The distinct nature of hematopoietic stem cell subpopulations studied in long-term stroma-associated culture in the mouse

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De verschillende eigenschappen van hemopoetische stamcel subpopulaties bestudeerd in lange-termijn stroma-geassocieerde culturen bij de muis

proefschrift

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Promotor:	Prof. Dr. O. Vos
Co-promotor:	Dr. R. E. Ploemacher
Overige leden:	Prof. Dr. B. Löwenberg Prof. Dr. R. Benner

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Later, later mag de zorg voor het begrijpen komen, - van de raadselen die de moeite waard zijn. Later, als de geest rijp genoeg is om onderscheid te zien tussen ernst en onernst.

(Theo Thijssen, De gelukkige klas)

Aan Monique en Matthijs

Aan mijn ouders

Contents

Chapter 1
Introduction to the thesis

The cells in the blood have a finite lifetime. To preserve their functions the cells have to be replaced by fresh blood cells. The processes that underlie the continuous production of blood cells take place in the hemato-lymphopoietic organs. These include the bone marrow, spleen, thymus, and lymph nodes. In the adult the bone marrow is the primary organ of hematopoiesis. It provides a proper micro-environment that allows hematopoietic progenitor cells to proliferate, differentiate and mature into new blood cells. Sufficient numbers of very primitive hematopoietic stem cells are maintained within the bone marrow to supply the body with fresh blood cells during the steady-state and hematologically stressed situations throughout life.

There is much discussion in the literature on the nature of hematopoietic stem cells. Stem cells are organized in a continuum of decreasing primitiveness according to mitotic history, proliferative ability, radiosensitivity, and sensitivity to cytotoxic drugs. Many *in vivo* and *in vitro* techniques are available to measure the different aspects of the subpopulations that can be found among the hematopoietic stem cells. The most primitive stem cells are recognized by their ability to reconstitute a recipient that received radiation or a treatment with cytostatic agents with all types of mature blood cells in the long run (long-term repopulating ability, LTRA). A major topic in stem cell research is the question whether or not the ability of a stem cell to form a macroscopic colony in the spleen upon transplantation in an irradiated recipient mouse (CFU-S) is a criterion of the most primitive hematopoietic stem cell. In other words, do CFU-S and LTRA cells necessarily represent two distinct subpopulations within the compartment of primitive hematopoietic stem cells?
In this thesis a number of experiments is described which give evidence for the notion of a distinct nature of CFU-S and cells with LTRA in the mouse. The distinct nature is illustrated by the difference in growth characteristics of CFU-S and LTRA cells on pre-established bone marrow-derived stromal cell layers. We have exploited the culture of stem cells on stromal layers for the development of an *in vitro* assay for the quantification of long-term repopulating stem cells, marrow repopulating stem cells, and spleen colony forming stem cells. The basic principle for this assay is the presence of a functional hematopoietic micro-environment *in vitro*, formed by the marrow-derived stromal layer. Stromal layers of long-term bone marrow cultures were studied on their ability to maintain stem cells with LTRA. For the measurement of LTRA we adapted a syngeneic sex-mismatched bone marrow transplantation model. In addition, the interaction of hematopoietic stem cells with fibronectin, a constituent of the

hematopoietic micro-environment, was studied.

The thesis starts with a description of the current definitions of hematopoietic stem cells (Chapter 2). This is followed by an outline of the *in vivo* and *in vitro* hematopoietic micro-environment of the bone marrow (Chapter 3). These two chapters precede the experimental work, which is described and discussed throughout the Chapters 4-8. An overview of the present state of research on the cell adhesion properties of fibronectin, focused on the hematopoietic cells, is part of Chapter 8. Finally, the most important results are discussed in Chapter 9.

Chapter 2
Hematopoietic stem cells

2.1 The hematopoietic compartment

Many researchers have been intrigued by the origin of the enormous numbers of mature red and white blood cells that are produced every day of a person's life. Two hundred billion (2×10^{11}) red blood cells, together with two billion (2×10^{9}) granulocytes, not to mention the lymphocytes, monocytes and platelets, is the sum of an everyday production. This production is necessary due to the limited life span of the mature blood cells. These cells die after days or weeks, and have to be replaced.
The hematopoietic system, consisting of a pool of progenitor cells that perform this replacement function, is embedded in a proper environment (Chapter 3), and functions as a highly organized cell renewal system. This cell renewal system consists of several stages of cells that precede the mature blood cells. All different lineages of mature blood cells, including erythrocytes, granulocytes, monocytes, lymphocytes and platelets, develop from a pool of so-called "stem cells". Mature cells develop through processes of cell division, differentiation and maturation. This process occurs in mammals predominantly in the bone marrow.
During this hematopoietic process of proliferation and differentiation several stages can be recognized. The major problem of determining the differentiation stage of the progenitor cells is their morphological likeness. Although late stages of development are morphologically recognizable, the early stages are not. Early progenitors appear as lymphocyte-like cells in routine May-Grünwald/Giemsa stained preparations, and cannot be recognized as progenitor cells because of their very low frequencies in the bone marrow.
The major property of hematopoietic progenitor cells by which they can be recognized and classified is their ability to form colonies in diverse *in vivo* and *in vitro* clonogenic assays. The consequence of the use of clonogenic assays for the determination of different subclasses of hematopoietic progenitor cells is that classification only takes place in retrospect. After the colony has been formed, one designates its origin a colony forming unit (CFU), i.e., the progenitor cell that has formed the colony.
A diversity of assays has been developed to characterize the various subclasses of progenitor cells. The definition of the progenitor cell that will proliferate and form a colony in an assay is determined by this particular assay (functional nomenclature). A progenitor cell that forms a colony in a semi-solid culture assay in the presence of a

cocktail of hematopoietic growth factors[¶] is called a CFU-C (colony forming unit in culture). Similarly, a progenitor cell that forms a colony in the spleen of an irradiated recipient animal upon bone marrow transplantation (BMT) is termed a CFU-S (colony forming unit in the spleen)[¶¶].

Often, the differentiation lineages that are present in a full grown colony are used to determine its origin. A CFU-C that gives rise to a colony containing granulocytes, erythroblasts, monocytes/macrophages, and megakaryocytes is termed a CFU-GEMM. Similarly, a colony containing only cells of the granulocytic and monocytic lineages originates from a CFU-GM. Again, the qualification of a progenitor cell depends on the conditions present in the assay. The multilineage expression of a CFU-GEMM takes only place in the presence of the right hematopoietic growth factors in proper concentrations. Otherwise, two differentiation lineages will be expressed when only G-CSF and M-CSF are present, and the progenitor cell is denoted CFU-GM.

The time needed for the appearance or full development of a colony is included in the schematic representation of the hematopoietic progenitor cells. This is especially evident in the CFU-S assay. A CFU-S that forms a macroscopic colony 8 days after injection is termed a CFU-S day-8 (CFU-S-8), while a CFU-S that forms a nodule on day-12 after transplantation is a CFU-S-12. The time needed for colony formation in this *in vivo* assay is a measure of the primitiveness of the progenitor cell. A CFU-S-12, therefore, is more primitive than a CFU-S-8.

Several stages of progenitor cells precede these *in vitro* and *in vivo* CFUs. The majority of the more primitive cells may not form colonies in the standard CFU-C and CFU-S assays just described. Other methods have been developed to make these progenitors recognizable. The first method is the radioprotection assay, in which a lethally irradiated recipient is rescued by a transplant of bone marrow cells (BMC). The reliability of this assay to measure only the more primitive stem cell subpopulations is questionable, and is discussed later in this chapter. The second method is the marrow repopulating ability (MRA)[46,89,90]. This is in essence a short-term repeated transplantation method. Upon transplantation in (lethally) irradiated animals a substantial part of the early progenitor

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[¶] The source of hematopoietic growth factors in standard semi-solid assays often is a culture medium conditioned by spleen cells treated with a mitogen. These conditioned media contain mostly IL-3, GM-CSF, G-CSF, and M-CSF.

[¶¶] When a mouse is irradiated with a lethal dose of radiation, it will die within 14 days after the irradiation due to lack of platelets, leading to massive internal bleeding. Depending on the dose and the dose rate, a large part of the precursor cells of the bone marrow will not survive the irradiation, which results in a stop in the formation of mature blood cells, and platelets, while the clearance of old mature blood cells and platelets continues. Such an animal can be rescued by transplanting a minimum of 5-10 × 10^3 of fresh bone marrow cells, injecting these into a lateral tail vein. Part of the precursor cells present in the bone marrow transplant will end up in the spleen to form spleen colonies.

cells end up in the bone marrow[¶], where they *generate* in 12-13 days new CFU-S or CFU-C. These secondary formed CFU-S and CFU-C can be assayed in standard spleen colony and semi-solid culture assays, respectively[¶¶]. Thus, progenitors of CFU-S and CFU-C form in the marrow new CFU-S and CFU-C that, in turn, form spleen colonies or *in vitro* colonies. A cell with the property to form new CFU-S, and that lacks the property to form a spleen colony itself, is termed "pre-CFU-S". It is more primitive than the CFU-S. These pre-CFU-S have marrow repopulating ability, because they repopulate the empty marrow cavities, once arrived, with new progenitor cells.

The third method to determine very primitive progenitor cells is to measure their long-term repopulating ability (LTRA). LTRA cells are the most primitive hematopoietic progenitors that can be assayed. The assay is based on the presumption that LTRA cells are responsible for the long-term reconstitution of (lethally) irradiated recipients upon BMT. Once transplanted, progenitor cells with *short*-term repopulating ability (STRA) immediately start to proliferate in bone marrow and spleen to supply the peripheral blood compartment with new, donor-derived blood cells and platelets. Cells with this quality are probably CFU-S-like progenitor cells[52,86,89,90,92,120]. This process will rescue the lethally irradiated recipient from a radiation-inflicted death. In the meantime the more primitive progenitor cells have developed to produce new CFU-S and CFU-C which, in turn, will produce new cohorts of mature peripheral red and white blood cells, and platelets. Some of the very primitive progenitor cells remain active in the production of new CFU-S, CFU-C, more differentiated progenitors, and new mature blood cells for a prolonged period of time. When donor-derived blood cells are detectable in the blood of an irradiated and transplanted recipient several months after BMT, they are derived from LTRA cells.

These very primitive progenitor cells also have the ability to replenish their own pool of very primitive cells by division, while retaining the same set of qualities, hereby creating a mechanism to protect the hematopoietic system against depletion, a property termed "self-renewal". From this pool of primitive progenitor cells a small number is activated, loosing their primitiveness in supplying the blood compartment with mature cells. The ability of self-renewal, together with the capacity to give rise to all hematopoietic lineages for a long period of time, is the definition we use of a primitive hematopoietic

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[¶] BMC also end up in other organs of the transplanted animal, like the spleen, liver, and the lungs.

[¶¶] MRA is expressed as the number of (progenitor) cells present in one femur and formed by the MRA cells of 10^5 fresh BMC in a period of 12-13 days. Thus, MRA[CFU-S-12] is the number of newly generated CFU-S-12 in a femur produced by 10^5 BMC, MRA[CFU-C] represents the number of newly generated CFU-C in a femur per 10^5 BMC injected, and MRA[cell] is the number of nucleated cells that have been generated in the femur 12-13 days after transplantation of 10^5 BMC. The determination of MRA is described in paragraph 4.2, and is compared with the erythroid repopulating ability (ERA) of a bone marrow graft, which measures the recovery of the red blood cell compartment of the blood after irradiation and transplantation.

stem cell (HSC).
The hematopoietic progenitors are arranged in a continuous hierarchic fashion. The most primitive cells, the HSC, form the source of all distal progenitor cells, that gradually loose the self-renewal and proliferating capacities upon differentiation. The stages of progenitor cells mentioned earlier and determined in various assays are recognizable hallmarks within the precursor cell continuum. Obviously, cells with a combination of the qualities of two consecutive stages occur. Some CFU-S-8 form colonies that are still visible on day-12, and therefore also will be assayed a CFU-S-12 (depending on the readout time of the spleen colony assay). Other CFU-S-8 colonies have vanished on day-12. Some MRA cells are able to form spleen colonies, while others are not. This has led to a lot of confusion with respect to the definition of the HSC. Especially the discussion whether HSC can form spleen colonies, and therefore may be termed CFU-S[¶], is a major topic in stem cell research. Therefore, separation technology should be improved to allow full resolution of tentative functional differences between the various HSC subsets. Pure preparations of stem cells with a restricted set of properties must be made, e.g., LTRA without spleen colony forming capacities, or CFU-S without long-term repopulating qualities. Doing this, we will be able to characterize accurately the phenotype, gene expression, cell biologic properties, and hematopoietic qualities of strictly defined stem cell populations, and the development of all intermediate stages.
A great deal of effort has been put in to characterize the HSC. However, the basic concept of the primitive HSC may not be similar to the concept mentioned above. Many conclusions concerning primitive HSC, therefore, may be subverted due to contamination of primitive HSC preparations with less primitive subpopulations. This is discussed in Paragraph 2.2.

2.2 Purification of hematopoietic progenitor cells

A variety of techniques has been developed to purify the most primitive precursor cell populations from fresh murine and human BMC. In some sorting protocols of murine bone marrow a distinction could be made between progenitor cells with spleen colony forming ability without marrow repopulating ability on the one hand, and MRA cells without the property to form spleen colonies on the other hand. This has successfully been undertaken in our laboratory, and precede the experiments described in this thesis, leading to the strict definition of HSC that forms the basis of the HSC concept used in this thesis. In the following section these purification methods are outlined, and compared with other purification protocols. In addition, differences between CFU-S and

[¶] The alternative formulation of the problem is whether a CFU-S is a LTRA cell, and therefore a HSC.

cells with MRA/LTRA are discussed, together with their role in radioprotection.

Rhodamine 123

The supravital fluorescent dye rhodamine-123 (Rh123) (Figure 2.1) allows a distinction between metabolically active and inactive cells. Rh123 is a probe for the localization of mitochondria in living cells[21,28,29,48,50]. Mitochondria play a crucial role in the energetic status of eukaryotic cells, and therefore are essential for survival and proliferation[33]. Although the exact nature of the specific binding of Rh123 to mitochondria is not known, it is very likely that there is no non-specific interaction with biological

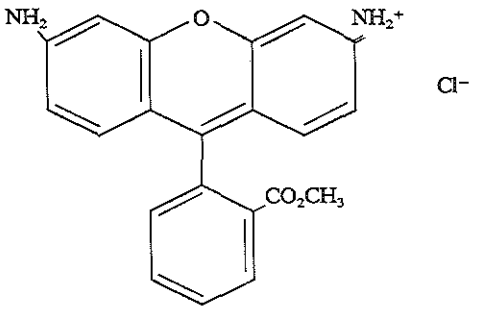

Figure 2.1. Schematic representation of rhodamine 123.

membranes. Rh123 is positively charged at physiological pH. Therefore, it has been suggested the positively charged Rh123 molecules are attracted by the relatively high electronegativity of the mitochondrial membrane. A relationship exists between Rh123 uptake by living cells and the Rh123 concentration in the incubation medium. Rh123 is not toxic to living (clonogenic) cells at concentrations up to 10 μg/mL during short incubation periods[28,29,50]. Continuous cell growth occurs in the presence of Rh123. Intracellular bound Rh123 remains detectable up to 48 hours after incubation, although the intensity slowly decreases, which makes it useful for supravital cell labeling. Rh123 is not suitable for cell tracing after 48 hours, or after more than two cell divisions. Cells show differences in their Rh123 uptake, and thus in their fluorescence characteristics, based on the energy state of their mitochondria and the number of these organelles. Cells incubated with Rh123 can be sorted by fluorescence-activated cell sorting[¶] based on their differences in fluorescence, i.e., their Rh123 uptake[28,29]. Sorting of cells that have taken up Rh123 using a laser beam may lead to cell death. Therefore, sorting is carried out at the lowest energetic (mW) settings. Maximal Rh123 uptake is seen at the time of DNA synthesis (S-phase) of cells in cycle. This coincides with an increase in the

[¶] When Rh is excited at a wavelength of 485 nm, it produces a green fluorescence (515-575 nm) in addition to the emission of red fluorescence (>600 nm) when excited at 546 nm.

number of mitochondria per cell, their sizes, and probably their energetic state (electronegativity). Increased Rh123 uptake is also seen in cells with a high RNA content, actively synthesizing proteins. Dead cells bind Rh123 for up to 8 hours after cell death, while dying cells are characterized by a bright fluorescence of both the cytoplasm and the nucleus. This is due to the release of the dye from the mitochondria. The fluorescence of such cells is often greater than of living cells.
In summary, Rh123 discriminates between cycling (high uptake) and protein synthesizing (high uptake) cells on the one hand, and non-cycling, quiescent (low uptake) cells on the other[¶]. This allows sorting of functionally distinct cell populations for further analysis.
Cells that have accumulated Rh123 may subsequently release the dye, especially when incubated in dye-free medium[¶¶17,67,78,79]. R123 is transported by the transmembrane efflux pump P-glycoprotein (P-gp), which is responsible for the phenomenon of multidrug resistance. P-gp is the product of the *MDR-1* gene. In general, normal peripheral blood and bone marrow cells express P-gp on a low level[27,80,85,107]. However, human lymphocytes differ in their Rh123 retention, as $CD4^+$ T-cells accumulate more Rh123 than $CD8^+$ T-cells[24]. P-gp is expressed in nearly all hematopoietic progenitor cells[20]. Staining of human $CD34^+$ hematopoietic progenitor cells with Rh123 is inversely correlated with P-gp expression. The highest level of P-gp expression is found among pluripotent stem cells, capable of initiating long-term *in vitro* hematopoiesis[20]. This indicates that the alternative explanation for the decreased accumulation of Rh123 in primitive HSC is found in the high expression of P-gp, resulting in an increased R123 efflux from these cells.

To make a distinction between metabolically active and inactive hematopoietic progenitor cells, Rh123 has been used in a number of studies. The sorting of murine BMC on the basis of Rh123 uptake resulted in the following findings. All nucleated BMC stain with Rh123[73]. The large majority of CFU-S-8 is present in the 50% most $Rh123^{bright}$ cells, while CFU-S-12 is more diffusely distributed over $Rh123^{bright}$ and $Rh123^{dull}$ cells[73], indicating that CFU-S-8 have more active mitochondria than have most CFU-S-12. Using a discontinuous BSA gradient for pre-enrichment, and sorting of Rh123 within a so-called "blast cell window", based on the forward and perpendicular light scatter characteristics of bone marrow clonogenic cells[119,124], Bertoncello and co-workers showed that the majority of MRA cells stained weakly with Rh123, while CFU-S were almost equally distributed among both the weak and bright fluorescent

[¶] It should be noted that peripheral blood lymphocytes are cells in a deep quiescent state, and therefore have a minimal Rh123 uptake. In contrast, plasma cells will stain extensively with Rh123.

[¶¶] In the R123 staining procedure of elutriated BMC described in Chapter 4 and 5, the Rh123 stained cells are incubated in Rh123-free medium for a period of 30-40 min.

BMC[15]. The cells were divided into a Rh123^{bright} and a Rh123^{dull} population, each population containing 50% of all nucleated cells present in the blast cell window.
It was the work of Ploemacher, who was technically assisted by Brons, that showed that MRA cells, CFU-S-12, and CFU-S-8 can be sequentially ordered on the basis of their Rh123 uptake, i.e., their mitochondrial activity[86,89,90]. The MRA could almost completely be separated from CFU-S-8/12 using the 15% most Rh123^{dull} cells vs. the 16% most Rh123^{bright} cells of all nucleated cells present in the light scatter blast cell window. Ninety-eight percent of all CFU-S-12 was not contained in the Rh123^{dull} fraction, while this fraction contained the majority (80%) of MRA. This indicates that, essentially, MRA cells and CFU-S-12 are two stem cell subpopulations with strictly distinct properties, MRA cells being the most primitive. This is not in agreement with the suggestion, done by others several years later, that HSC form a continuum of resting and activated CFU-S[102]. As a pre-enrichment step in the early studies on HSC of Ploemacher *et al.* counterflow elutriation, which sorts cells on the basis of size and density, was used. The enrichment of MRA in the Rh123^{dull} population, compared to fresh BMC, was 66.4 times for MRA[CFU-S-12], and 116.7 times for MRA[CFU-GM]. Interestingly, only 8% of all CFU-S-8 is in cell cycle[22,49,121,135], while 99% stain Rh123^{bright} [73,89]. This is probably due to differentiation processes within the CFU-S-8 which, as mentioned earlier, coincide with a high energetic state. Another feature that emerges from these studies is that MRA cells are pre-CFU-S. These are the cells that generate new CFU-S-12 without having the ability to form spleen colonies themselves, in contrast to the CFU-S-12 that hardly show any form of self-renewal, i.e., the generation of new CFU-S-12 by CFU-S-12. Therefore, earlier reports on the self-renewal ability of CFU-S[49,73,93,94,95,134] could, in retrospect, be explained by the assumption that pre-CFU-S were present in the precursor cell populations studied. A subpopulation of CFU-S-12, probably with marrow repopulating qualities, may also have contributed to observations of the self-renewal of CFU-S.
Both the Rh123^{bright} and Rh123^{dull} fractions contained progenitor cells able to generate cells of all differentiation lineages. However, Rh123^{bright} cells gave predominantly rise to macroscopic erythroid and megakaryocytic spleen colonies that disintegrated after day-12. The 15% most Rh123^{dull} cells, on the other hand, formed, in addition to new CFU-S, microscopic colonies in the spleen at day-12 after transplantation. These colonies, as the macroscopic spleen colonies found at day-16, were predominantly megakaryocytic and/or granulocytic[86]. The rapid generation of megakaryocytes and granulocytes by CFU-S is essential for the short-term survival of lethally irradiated animals. This is illustrated by the observation that the transplantation of only a few CFU-S-12 extend the survival time of these animals with several days[73,90]. By the *generation* of new CFU-S, the pre-CFU-S provide a prolonged survival, once the animals have overcome the first critical period. This indicates that radioprotection is a function of a combination of CFU-S and pre-CFU-S, and therefore is not a proper assay

for strictly distinct HSC subpopulations[¶].
The behavior *in vitro* of sorted pre-CFU-S and CFU-S on the basis of Rh123 is described in Chapter 4, and further extended in Chapter 5. It is concluded that the *in vitro* results perfectly resemble the *in vivo* observations, discriminating long-term *in vitro* repopulation by MRA/LTRA cells from the short-term *in vitro* growth by CFU-S. Rh123 has also been used in sorting protocols of human primitive hematopoietic progenitor cells[7,106,115]

Alternative purification protocols
Alternative protocols are being used for the purification of the distinct subclasses of HSC using fluorescence-activated cell sorting following one or more pre-enrichment steps[125]. Pre-enrichment is necessary to remove both the accessory cells and the mature hematopoietic cells. This can be achieved by (discontinuous) density gradients, counterflow elutriation, immunomagnetic beads sorting[13,14,26,97,98,101,104], and treatment of donor-mice with cytostatic agents several days before harvesting the BMC, especially with 5-fluorouracil (5-FU) and hydroxyurea[15,35,45,55,65,74,96,100,104,110,121,131,132].
One of the applications of immunomagnetic beads sorting is based on the specific binding of monoclonal antibodies to a series of surface markers specific for mature nucleated hematopoietic cells of all lineages. When the antibodies are first conjugated to paramagnetic beads, the mature cells bound to the antibodies will subsequently be removed by a strong magnetic field. This results in a so-called Lin^- fraction, depleted of differentiated hematopoietic cells.
The cytostatic agent 5-FU is frequently used in pre-enrichment protocols to obtain highly purified stem cell populations in the mouse. It causes inhibition of DNA synthesis through interference with the synthesis of thymine nucleotides. It acts, therefore, specifically during S-phase of the cell cycle. Rapidly cycling progenitor cells are killed by this antimetabolite. The compartment of cycling hematopoietic progenitors, i.e., CFU-S-12 and more mature cells, is severely depleted by a single intravenous injection of 150 μg/kg of body weight. The more primitive, quiescent progenitor cells are relatively enriched by this protocol, but the yield depends on the timepoint on which the BMC are harvested. When given intravenously, 5-FU has a short metabolic half-life. The depleted progenitor cell pools have to be endogenously reconstituted by the surviving stem cells. This results in the activation of the primitive HSC pool, which is reflected by a shift in the energetic state of these stem cells, which makes them unsuitable for sorting on the basis of Rh123 uptake (Ploemacher, unpublished

[¶] The radioprotective ability of a BMC sample is an essential parameter if it comes to survival studies after hematopoietic damage due to (therapeutic) irradiation. All progenitor subpopulations that are responsible for acute and prolonged hematopoietic recovery of the laboratory animal or patient must be included in the bone marrow transplant.

observations). Other sorting protocols, like those on the basis of the affinity to the lectin wheat-germ agglutinin (WGA), have successfully been carried out using BMC from 5-FU treated animals[92,120]. However, one never can be sure that the most primitive stem cells, obtained after 5-FU treatment, have not changed in their proliferative history, in the expression of surface markers, or in primitiveness. In fact, it has been demonstrated that at least part of the LTRA cells undergoes 1 or 2 mitoses in a period of 6 days following 5-FU treatment[62].

A fluorescence-activated cell sorter enables sorting on more than one parameter. It is based on forward light scatter of cells (a measure of size), perpendicular light scatter (a measure of the internal complexity of the cell), and the differential binding of one ore more fluorescent dyes. This dye may be Rh123, or a fluorescein conjugated to one or more cell surface markers[30]. These surface markers vary in the diverse purification protocols.
The binding of WGA appeared to be a powerful tool for the purification of HSC[4,8,71,82,126] and for the discrimination of various subtypes of HSC and CFU-S[87,88,91,92]. Also, the combination of WGA and Rh123 has been used[69,127,129].
A variety of monoclonal antibodies specifically directed against surface markers that are weakly or strongly expressed on CFU-S and more primitive progenitors have been developed. Although it is believed that some of these markers are indeed expressed on

Table 2.1 Cell surface antigens used for the purification of murine hematopoietic stem cells

marker	reference
Qa	11, 12, 40, 58
Thy-1	70, 82, 98, 100, 109, 112, 114, 132
Sca-1 (Ly-6)	44, 47, 63, 98, 100, 102, 114, 117
c-kit	47, 81, 83

cells with LTRA, it has not been unequivocally confirmed, due to co-enrichment of less primitive classes of progenitor cells, responsible for short-term repopulation. A list of these markers is given in Table 2.1. For the purification of human (primitive) progenitor cells monoclonal antibodies against the surface antigen CD34 are used for positive selection[1,2,7,23,61,105,111,123]. It has been reported that Thy-1 is also expressed on primitive human progenitor cells[7].

Alternative stem cell concepts

Different concepts for the definition of HSC result in a purification strategy that leads to the simultaneous purification of distinct stem cell activities, or vice versa. Sorting BMC on Thy-1^{lo}Lin$^-$Sca-1$^+$ characteristics generated a fraction that is nearly pure in CFU-S. This fraction contained LTRA cells[44,101,104,114], predominantly in the 25% Rh123^{lo} subpopulation of Thy-1^{lo}Lin$^-$Sca-1$^+$ [102]. CFU-S activity was distributed in both the Rh123^{lo} and Rh123^{hi} populations. This has resulted in the definition of HSC, which includes three activities. HSC must rescue lethally irradiated mice (radioprotection), they must self-renew, and they must restore all blood cell lineages permanently. It is beyond doubt that the Thy-1^{lo}Lin$^-$Sca-1$^+$ sorted BMC fraction contained LTRA cells. However, it also contained short-term repopulating cells, indicated by their radioprotective ability. The virtually complete separation of CFU-S and radioprotection away from the majority of MRA/LTRA, instead of using an almost pure CFU-S population, gives rise to a HSC definition that only includes long-term multilineage repopulation.

Ultimately, long-term reconstitution of all hemato-lymphopoietic cells in lethally irradiated mice could be obtained by transplanting a sorted BMC fraction by means of counterflow elutriation, that was entirely devoid of CFU-S[52]. Although this sorted fraction was certainly not pure, it contained cells with LTRA. Therefore, it is concluded that there consist two classes of engrafting cells: 1) progenitors that provide initial but transient engraftment, and 2) HSC, providing delayed but sustaining repopulation.

2.3 Detection of long-term repopulating activity of transplanted hematopoietic progenitor cells

A variety of transplantation models have been described for the detection of the progeny of long-term repopulating HSC.

Congenic mouse strains are frequently used for the identification of donor-type repopulation. In these models the donor and the recipient mice are identical except for polymorphism of a specific protein, e.g., an electrophoretically distinguishable allo-enzyme like phosphoglycerate kinase (PGK-A vs. PGK-B), or glucose phosphate isomerase (GPI-A vs. GPI-B)[6,43,75,77,122]. Other congenic transplantation models use allelic differences in surface markers which are present on all or part of the mature nucleated hematopoietic cells, e.g., Ly-5.1/Thy-1.2 vs. Ly-5.2/Thy-1.1[103,113]. These markers can be specifically detected by monoclonal antibodies.

The use of W/W^v mice to study long-term cure of a genetically anemic defect is well described[10,41]. The W/W^v mouse has a defect in the *W*-locus, which results in a macrocytic anemia in mice with this defect. The cure of the macrocytic anemia after

BMT is the readout system for donor-type hematopoietic repopulation. Recently, the use of α-thalassemic mice in studies on the long-term erythroid repopulation is described[5,118,130]. Homozygous α-thalassemic mice, suffering from a microcytic anemia, can be cured by transplantation of BMC of normal homozygous littermates after a sublethal dose of radiation. The different sizes of normal vs. thalassemic erythrocytes determine the amount of donor-derived erythroid repopulation. Mice that are homozygous for an autosomal recessive mutation that specifically impairs both T- and B-lymphoid differentiation, but not erythropoiesis and granulopoiesis (severe combined immuno-deficiency = SCID mice), can support lymphoid differentiation of donor bone marrow-derived HSC[37,38]. In this BMT model, therefore, all mature lymphoid cells are of donor origin, and can be assayed for many months after transplantation. Sublethally irradiated SCID mice have also been used as recipients for xenogeneic bone marrow grafts. It has been reported that a complete human hematopoietic system including all differentiation lineages can develop after transplantation of human BMC in these mice[7,59,60,66,84].

A number of papers report the successful insertion of foreign genetic material in hematopoietic progenitor cells. Upon transplantation these transfected cells may take part in a donor-derived reconstitution of all lineages of the hematopoietic system. Due to the random integration of the transfected construct in the genomic DNA of the hematopoietic target cells, the progeny of one progenitor cell can be detected using hybridization techniques on Southern blots of the DNA of nucleated peripheral blood cells, or cells from bone marrow, spleen and thymus. Usually, retroviral vectors carrying the genetic marker and a bacterial neomycin resistance gene to select the transfected cells from the non-transfected cells, are used to mark the progenitor cell. When the transfection has succeeded in a cell with LTRA, the foreign gene will be incorporated in an unique site in the DNA of the LTRA cell, and in all its clonogenic progeny of mature hematopoietic cells of the myeloid and lymphoid lineages in the blood, as well as in nucleated cells of bone marrow, spleen, thymus, and lymph nodes of transplanted animals several months after transplantation of the originally transfected cell[18,19,31,32,56,57,99,100,108,122]. Apart from the determination of donor-derived hematopoietic progeny, this technique can be used for gene therapy in a variety of genetic disorders by transferring a functional gene in hematopoietic target cells, that will synthesize a functional protein[3,9,16,25,32,54,64,116,133]. This applies only when a sufficient number of LTRA cells are transfected.

The repopulating capacity of a bone marrow transplant can also be monitored by using a sex-mismatched transplantation model. When transplanting male hematopoietic progenitor cells in a conditioned female recipient of the same inbred mouse strain, or vice versa, donor-derived nucleated blood cells can be distinguished from host-derived

cells using molecular hybridization of the Y-chromosome with a Y-chromosome-specific probe. This can either be performed by hybridization of DNA which is extracted from peripheral blood leukocytes and processed in a Southern blot[39,51,52,72,76,109,113], or by *in situ* hybridization on blood smears, and cytospin preparations of bone marrow, spleen and thymus[34,128]. The latter method is the subject of Chapter 6. As for the detection methods of genetically modified cells, filter hybridization of leukocyte DNA with a Y-probe can be made semi-quantitative using densitometry. In addition, the number of nucleated male cells minimally necessary to obtain a positive signal can be determined.

Several laboratories prefer a competitive repopulation assay, in which a mixture of donor-type and recipient-type BMC is transplanted after lethal irradiation of the recipient[36,42,68,109,110]. When HSC with only LTRA are transplanted, a co-transplant of syngeneic host-type short-term repopulating BMC may overcome the initial critical phase after irradiation by their immediate burst of proliferation, after which the donor long-term repopulating HSC will take over. These donor-type HSC encounter the competitive pressure of HSC of the syngeneic host-type. The authors concluded that this model is highly selective for a very primitive, totipotent, reconstituting HSC[109]. Essentially the same process, however, will occur after a sublethal dose of radiation, followed by only a donor-type transplant. In such a transplantation model the short-term repopulating cells of the irradiated host are not completely ablated, which is not relevant for the LTRA model, and immediately restore the damaged precursor cell pool. In addition, LTRA cells that have survived the irradiation will provide competitive pressure for the LTRA cells from the transplant.

Concluding remarks

Several important remarks need to be made with respect to the methods of determination of LTRA.

Many studies report the detection of very low levels of donor-type repopulation. This is especially the case in genetically marked donor-derived leukocytes, of which the marker DNA can be amplified by polymerase chain reaction to indicate blood leukocyte levels approaching 0.05% of donor-type origin. However, the physiological significance of very low fractions of donor-derived blood leukocytes is questionable. A few persisting long living lymphocytes of donor origin may result in such a weak donor-type signal, without the occurrence of an active donor-type stem cell clone at the very moment of assaying. Therefore, only considerable donor-type repopulation is suitable for a proper measurement of LTRA. In addition, high levels of donor-type repopulation (> 90%) may be a consequence of a reconstitution that has reached a plateau. The number of HSC that has been transplanted may have been higher than minimally required for 90%

donor-derived repopulation, without leading to higher repopulation levels. To encounter these pitfalls, a series of transplants, each containing a different number of LTRA cells, can be given, resulting in different values of donor-type engraftment. From these values the number of cells required for a specified percentage of repopulation (between 10 and 90) can be calculated. An example is given in Chapters 6 and 7.

The annotation "long-term" has, to date, not been defined properly. In many cases it is used as "no short-term", which is often past four weeks after BMT. A better definition emerges from a study of Jordan and Lemischka, who observed that the first four to six months after transplantation are characterized by fluctuation in stem cell proliferation and differentiation[53]. After this period a stable chimerism occurs. It is, therefore, convenient to define long-term repopulation by a certain level of detectable donor-type reconstitution beyond six months post-BMT.

The definition of the hematopoietic, long-term repopulating stem cell is certainly not equivocal. It depends on three fundamental principles, namely 1) a multilineage output, 2) the time of onset and the duration of the output, and 3) the extent of the output. The term "self-renewal" as such is not used in this description of HSC, because it is included in the duration and the extent of the output. By the amplification of the subpopulation of stem cells to which a HSC belongs, which essentially is self-renewal, a HSC enhances both the extent and the duration of the output.
A HSC is strictly defined by the assay in which it is determined. This definition depends on engraftment, detection level, and competition within the assay. This means that each assay creates its very own stem cell definition, which is not necessarily exchangeable with the definition of an other assay. Therefore, one should compare data from various stem cell assays on the repopulating ability of BMC samples with great care.

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Chapter 3
The hematopoietic micro-environment of the bone marrow

Preface

Hematopoietic stem cells (HSC) carry the entire genetic information which is required to amplify the stem cell subpopulation (self renewal), and to proliferate into differentiated progeny. These processes take place in a close association of progenitor cells and stromal cells of a hematopoietic micro-environment[1]. The hematopoietic micro-environment regulates the stem cell and progenitor cell lodgement, proliferation and differentiation and the release of mature blood cells into the circulation.
The hematopoietic micro-environment has been subject of extensive study. The vast amount of information on the regulation of hematopoiesis makes it hardly possible to give an integral view. Therefore, only a few aspects will be discussed in this thesis. In this chapter first a description is given of the composition of the bone marrow. Second, a bone marrow organ culture system, termed long-term bone marrow culture (LTBMC), is described with respect to the development of the system, and the morphologic and physiological characteristics of this type of *in vitro* hematopoiesis. This culture system plays a key role in the experimental work described in this thesis.

3.1 The *in vivo* hematopoietic micro-environment of the bone marrow

In the adult mouse the bone marrow is the physiologically most important site of hematopoiesis in normal situations. It is made up of vascular and non-vascular compartments. The bone marrow has a closed circulation, in which the arterioles flow out into venous sinuses, emptying into a large central sinus that is connected with the efferent venous system[56]. Between the sinuses lie the islets of hematopoietic tissue[261]. These hematopoietic bone marrow strands form the micro-environment in which the proliferation and differentiation of all hematopoietic cell lineages take place. These hematopoietic islets can be functionally subdivided into two compartments, 1) a stromal network, creating the environment in which 2) the hematopoietic cells can grow and differentiate optimally. Although these compartments can be studied separately, both morphologically and functionally, the cells and intercellular substances are intermixed in

[1] Stroma refers to the nonhematopoietic tissue cells in the medullary cavities (including the macrophages, which are of hematopoietic origin), that may take part in a functional hematopoietic micro-environment, in which hematopoietic cells develop. Other cell types, like T-lymphocytes, also may contribute to the micro-environment, without being a member of the bone marrow stromal compartment.

the hematopoietic strands, maintaining intimate physical relationships.
To enter the circulation, developing blood cells must pass through the sinus wall. Transplanted HSC have to cross this barrier in the opposite direction to enter the hematopoietic micro-environment.
In the following section a brief description is given of the cellular and non-cellular components of the bone marrow.

Endothelial cells. The endothelial cells form a complete lining of the luminal surface of a sinus. The cells are broad and flat, and their irregular cytoplasmic extensions overlap. In electron microscopic views, hematopoietic cells often can be seen migrating across

Table 3.1 Production of factors that influence hematopoiesis by endothelial cells

factor	reference
M-CSF	220, 222
G-CSF	220, 222, 286
GM-CSF	17, 37, 160, 220, 223, 286
IL-1	132, 222, 251, 254
IL-6	118, 143, 224
PDGF	49, 50, 66

the endothelium, forming temporary pores through the cytoplasm of the endothelial cells. Endothelial cells synthesize collagen type IV, laminin, and fibronectin[27]. In addition, endothelial cells synthesize hematopoietic growth factors and other cytokines[¶] (Table 3.1).
A very thin lamina basalis underlies the endothelial layer. It is not continuous, and consists of collagen types III and IV, laminin, fibronectin, and heparan sulphate proteoglycans.

[¶] Studies on factor production by endothelial cells as well as by other cell types that can be found in the hematopoietic micro-environments have for the greater part been done in cultures of cells of the same kind, not only from bone marrow, but also from other sources. Stromal cells from bone marrow were often obtained from the stromal layer of long-term bone marrow cultures. In several cases immortalized cell lines have been used. In addition, the presence of mRNA of a certain factor in a bone marrow preparation will not be a proof of actual production of the very cytokine in the bone marrow. Therefore, conclusions of such experiments with respect to the production of factors by bone marrow stromal cells must be carefully formulated.
The production of growth factors and cytokines in many experiments is triggered by stimulating agents like mitogens, other cytokines, etcetera. Much less is known about the production of such factors in the situation of normal hematopoiesis. However, one can argue whether normal hematopoiesis is a triggered situation.

Fibroblastic cells. Adventitial reticular cells[188] are located between the endothelium and the developing hematopoietic cells. They form the interrupted adventitial lining of the sinuses, while extensively branching cytoplasmic processes protrude into the stroma of the hematopoietic compartment. These branches form the cellular component of a spongy network, among which hematopoietic cells develop[258]. Reticular cells produce the so called reticular fibers[148], containing collagen type III (reticulin) that can be visualized by silver staining. Intimate physical associations between these cellular processes and the developing granulopoietic cells have been reported[227,259,263], while erythroid colonies have been observed in close contact with reticular cells after irradiation and transplantation of syngeneic marrow cells[22,130]. The reticular cells contain high concentrations of alkaline phosphatase in their membranes, suggesting that they are of fibroblastic nature[78,263]. In addition, they share a membrane antigen with cultured fibroblasts from hematopoietic organs[188]. The sinus adventitial reticular cell appeared to be the principal fibroblast-like cell in the bone marrow.
Other fibroblastoid cells from the marrow that form fibroblastic colonies *in vitro*

Table 3.2 Production of factors that influence hematopoiesis by fibroblasts

factor	reference
G-CSF	120, 126, 278
M-CSF	120, 203, 222
GM-CSF	38, 120, 126, 160, 278, 287
IL-1	131
IL-6	142, 246
IL-11	178
KL	8, 34, 82, 111, 167
LIF	230, 264
Activin A*	277

* Activin A is also termed Erythroid Differentiating Factor

(CFU-F)[43,84,182,183] may originate from the adventitia of marrow arteries, marrow nerve sheaths, or bone-associated cells. Fibroblasts are not derived from HSC[85]. Cultured marrow-derived adherent cells have shown to be largely fibroblasts that synthesize collagens types I, III, IV and V[28,43,134,197], laminin, and fibronectin[30,43,134,182]. Cultured marrow-derived fibroblastic stromal cells support hematopoiesis *in vitro*[114,162,191,281,283,284], and produce hematopoietic growth factors (Table 3.2). These properties are shared by subpopulations of fibroblasts of other organs[35]. An alternative fibroblastoid cell type that occurs in the bone marrow stroma resembles the myofibroblasts described in fibrotic tissues. These cells contain α-smooth muscle actin, as do myofibroblasts[179]. A recently

discovered fibroblastic cell type is the barrier cell[260,262]. Barrier cells are predominantly located in the distal metaphysis of the femur, and tend to lie in or near the bone-lining layer, which is composed of endosteum, osteoblasts, osteoclasts and so-called bone-lining cells. The femoral diaphysis and proximal metaphysis contain few barrier cells. The barrier cells extend from the bone-lining layer into the marrow, reaching the vascular sinuses. They tend to form syncytia and function as a structural unit of the hematopoietic stroma. These cells have been reported to branch among differentiated clusters of all types of developing hematopoietic cells. They control the release of hematopoietic cells into the circulation. Barrier cells are positive for fibronectin, and may be associated with collagen.
In rodents the adventitial reticular cell can function as a pre-adipocyte, capable of storing fat droplets[36,96,234,235,261]. Adipocytes in red bone marrow are resistant to starvation. The fat content of the bone marrow increases with age, although adipocytes remain relatively rare in mouse bone marrow.

Macrophages. Another type of stromal cell is the macrophage[205,207]. This is the only stromal cell type that is of hematopoietic origin, developing from monocytes[190]. A

Table 3.3 Production of factors that influence hematopoiesis by monocytes/macrophages

factor	reference
Epo	206 ,208, 250
G-CSF	176, 222, 247, 268
M-CSF	108, 175, 195, 222, 247, 268
GM-CSF	106, 222, 237, 271
IL-1	14, 74, 79, 129, 226
IL-6	19, 20, 241
PDGF	139, 221
TNF	174, 211, 252, 255
LIF	1, 9
TGF	12, 252

subpopulation of these cells is situated in the center of clusters of erythroblasts of all different maturation stages in synchronized development, enveloping the maturing red cells almost completely with their cytoplasmic processes, thus forming erythroblastic islets[32,57,113,154,186]. These erythroblastic islets are predominantly located near the sinus walls. Gap junctions have been observed between these central macrophages and their surrounding erythroblasts, allowing the passage of chemicals from cell to cell[23,42].

Central macrophages perform phagocytosis of the nuclei that are expelled during red cell differentiation. Macrophages are also found in close contact with eosinophilic granulocytes. Macrophages are a source of hematopoietic growth factors and other cytokines that have agonistic or antagonistic effects not only on erythropoiesis, but also on the development of monocytes, granulocytes, lymphocytes and platelets (Table 3.3). They also produce collagen type I. Thus, macrophages play a central role in hematopoietic micro-environmental regulation.

T-lymphocytes. T-lymphocytes, of hematopoietic origin and also present in the bone marrow, play a key role in the production of many hematopoietic growth factors and cytokines, as do B-lymphocytes[170] (Table 3.4), and therefore have micro-environmental functions[240]. The mouse bone marrow, however, contains few T-cells. The significance

Table 3.4 Production of factors that influence hematopoiesis by T- and B-lymphocytes and NK cells

factor	reference
G-CSF	161
M-CSF	184
GM-CSF	44, 107, 271
IL-1	215
IL-3	165, 271
IL-4	80, 110, 124
IL-5	77
IL-6	109, 225
IL-9	279
TNF	156, 161, 185, 232
LIF	90, 155, 264
TGF	121, 161

of T-cell-derived factors for normal hematopoiesis is still not clear. Additionally, hematopoietic cells themselves may produce hematopoiesis stimulating or inhibiting factors[12,13,16,72,89,177,217,238,239]. To what extent this is a physiological component of normal hematopoiesis in the bone marrow is, as yet, unclear.

Not discussed in this paragraph are the mast cells, the nervous structures and the cells involved in bone formation, of which the latter two are components of the non-hematopoietic part of the bone marrow.

Hematopoietic cells. The developing hematopoietic cells are grouped between the stromal elements of the hematopoietic micro-environment. The megakaryocytes are predominantly situated near the sinus wall, being in close physical relationship with endothelial and reticular cells[21,133,280]. This suggests that elements of the sinus wall provide a micro-environment for megakaryocyte growth and differentiation. Granulocytes develop near the center of the hematopoietic strands, while erythroblasts develop near the sinus wall. The bone marrow is also the site of the development of B-lymphocytes in mammals. Immature B-lineage cells are located in the subendostal regions of the bone marrow[18,105].
In addition to the localization of megakaryocytes and developing erythroblasts near the sinus wall and granulopoiesis in the center of the hematopoietic strands, it has been reported that CFU-S are predominantly distributed close to the bone surfaces of the murine femoral bone marrow[137]. The few CFU-S that are situated more centrally have a very low proliferative capacity and a high self-renewal property, thus seeming more primitive than the majority of the CFU-S located near the bony surfaces of the femoral shaft, which have high proliferative and low self-renewal properties[136]. Progenitor cells that form colonies *in vitro* (CFU-C) show a discontinuous radial distribution throughout the femoral marrow, with a peak concentration at a fixed distance from the inner bone surface[137]. CFU-F have their highest concentration in the center of the femoral marrow[276]. This spatial distribution of CFU-S and progenitor cells was later confirmed[91,140,187].

Extracellular matrix. The ground substance of the bone marrow contains fluid, which is bound by complexes of glycoproteins and proteoglycans. The most common extracellular matrix (ECM) component is collagen, of which several types have been described. In addition, the micro-environmental ECM contains fibronectin, laminin, and proteoglycans[168,169,291]. These proteoglycans contain core proteins to which various glycosaminoglycans (GAG) are covalently linked[213] (see also Chapter 8). The proteoglycans of the bone marrow contain chondroitin sulphate, heparan sulphate, and hyaluronic acid as GAG side chains, and are produced by both the stromal cells and all cell types of undifferentiated and mature hematopoietic cells[127,158,171,173]. The other components of the ECM are only produced by stromal cells[29,125]. Elevated levels of GAG, especially of hyaluronic acid and chondroitin sulphate, inhibit the erythroid differentiation, while granulopoiesis is unaffected or even increased[168,169,189,229]. One important feature, however, must be mentioned here. It has been demonstrated that proteoglycans containing heparan sulphate GAG bind GM-CSF[94,210]. This provides a mechanism to achieve very high local concentrations in distinct sites of the micro-environment. Another component of the ECM that is found in the bone marrow is hemonectin[10,40,41,180]. This protein has attachment qualities, restricted to granulocytic cells and their progenitors in hematopoietic organs.

The complexity of the hematopoietic micro-environment is illustrated by the large number of components and its large variety of functions. The lists of hematopoietic growth factors and cytokines that have their influences on hematopoiesis, given in Tables 3.1-3.4, are never meant to be comprehensive, but they give an impression of the versatility of factors that may be produced by one cell type in one organ-system. Certain factors released by cells of the bone marrow can stimulate marrow stromal cells, including the cells that produce these factors themselves, to produce hematopoiesis activating or inhibiting factors. Transmembrane forms of cytokines may provide a direct presentation of these factors to adjacent (hematopoietic) cells[95,129,203]. In addition, the stroma performs adhesive functions, mediated by components of the ECM, and by cell adhesion molecules on both stromal and hematopoietic cells. Adhesion of hematopoietic cells to the stroma is discussed in more detail in Chapter 8. Thus, the hematopoietic micro-environment forms an multicellular regulatory network which involves all cell types, including the hematopoietic cells, and the ECM.

In summary, the micro-environment of the bone marrow is not a loose association of mutually independent cells, fibers and amorphous ground substances, but a highly organized anatomical structure, that regulates the hematopoietic processes which are directed by triggers from the peripheral circulation. Many years ago this phenomenon has been recognized by Curry and Trentin[55,244], who used the term "hematopoietic inductive micro-environment" to denote the role bone marrow stroma plays in the determination of development and differentiation of hematopoietic stem cells.

It has been proposed that non-cycling HSC in the bone marrow micro-environment are lodged in specialized micro-areas, termed "niches"[218], that prevent the HSC from going into cycle. The exact nature of these niches is not known, and may even be only functional[¶]. The niche theory uses the idea of an inductive micro-environment as its basic principle. The route of development of a HSC is more likely to depend on micro-environmental signals, rather than on stochastic processes from within the HSC itself[60]. It has even been concluded that ".. the spatial positioning of stem cells and progenitors relative to stromal tissues determines their response to growth and differentiation signals"[282]. It should be possible to define the sites in the hematopoietic micro-environment of the bone marrow[136] that are responsible for the steady state maintenance of hematopoiesis.

[¶] The niches may not be morphologically distinguishable from other sites in the bone marrow stroma. However, cells in these niches may express different amounts of CAM or HGF (KL), compared to other stromal sites. In addition, the ECM may contain altered compositions of fibers (collagen, fibronectin, laminin) or ground substances (proteoglycans).

3.2 The bone marrow hematopoietic micro-environment *in vitro*

It is extremely difficult to conduct experiments to understand the *in vivo* interactions of cells and factors that take place in the hematopoietic micro-environment. The development of a three-dimensional bone marrow organ culture method has led to an opportunity to study hematopoiesis in a more integrated fashion than can be done in semi-solid or liquid suspension cultures.
Murine long-term bone marrow culture (LTBMC) has been developed in the mid seventies by Dexter and colleagues[61,275]. This culture system was later successfully adapted for human bone marrow[88]. It is based on the formation of an adherent multi-layered stromal cell layer in tissue culture flasks (or dishes) that forms a micro-environment for the clonal proliferation and differentiation of hematopoietic progenitor cells. This results in the release of nucleated cells in the supernatant culture medium. The culture medium consists basically of α-medium and 20% serum (a combination of selected batches of fetal bovine serum and horse serum in a ratio of 1:1 or 2:1), supplemented with hydrocortisone. The non-adherent cells are predominantly neutrophilic granulocytes and monocytes, but also CFU-C and CFU-S are found. The latter two cell types can be assayed by colony formation in a semi-solid culture assay *in vitro* and by spleen colony formation *in vivo*, respectively. LTBMC have been demonstrated to maintain the long-term growth of pluripotent HSC, CFU-S, CFU-GEMM, CFU-G/M, BFU-E, and CFU-meg. Although this culture system is primarily myeloid, modifications have been made to direct this culture system to erythropoietic differentiation or B-cell development.

The bone marrow stroma is an essential component for the maintenance of hematopoiesis in LTBMC. Most of the stromal cell types found in the bone marrow micro-environment *in vivo* are present in the stromal layer of these cultures. Knowledge of the nature of these cell types is important to understand the regulation of stromal cell-related hematopoiesis[3,5,6,7,26,48,64,71,101,115,136,194,200,231,236,288].

Endothelial cells. It is now evident that endothelial cells have an important contribution to the stromal cell layer of LTBMC[5,31,103,104]. In the past it has been difficult to demonstrate the presence of endothelial cells in these cultures due to the lack of specific markers. Recently, such markers have become available. Adjacent endothelial cells frequently show membrane contacts in the form of desmosomes. Endothelial cells in these cultures have the exact morphological characteristics of the so-called blanket cells (see below), have a relatively large size, and cover differentiating hematopoietic cells with a thin layer of cytoplasm. On the basis of these data blanket cells could be referred to as endothelial cells[103].

Fibroblastic cells. Reticular fibroblasts are identified by their large, oval nuclei with prominent nucleoli, by their alkaline phosphatase positivity, and the presence of reticular fibers[101,200]. Blanket cells are large cells with thin, well-spread cytoplasmic extensions, often showing gap junctions at sites of interaction with other blanket cells. They are also positive when stained for alkaline phosphatase. This indicates their fibroblastoid nature, an observation which is not in agreement with the assumption mentioned above, that blanket cells are endothelial cells. In addition, blanket cells contain α-actin, similar to cultured vascular smooth muscle cells[46], which may indicate that these cells are myo-fibroblasts[179]. Blanket cells are particularly present at sites of hematopoietic activity, the so-called "cobblestone areas" (CA). These CA occur beneath the cytoplasm of the blanket cells[64]. It is not unlikely that blanket cells form a niche for hematopoietic progenitor cells to develop in the myeloid direction. Whether blanket cells of fibroblastoid or endothelial origin form hematopoietic micro-environmental sites with distinct inductive properties (i.e., the ability to regulate or induce the commitment of stem cells towards a specific differentiation lineage), remains to be solved. Furthermore, adipocytes form a substantial component of a supportive stromal layer in LTBMC. Although it has previously been suggested that these adipocytes were of monocytic origin[235], adipocytes fail to stain with the macrophage-specific monoclonal antibody F4/80[15]. They are probably fat-storing fibroblasts, indicated by their alkaline phosphatase activity[64]. It has not been excluded that also endothelial cells accumulate lipids[179]. Adipocytes in LTBMC do not function in the support of hematopoiesis[242]. Storing lipid droplets seems a function of confluence of the stromal layer, and of the addition of corticosteroids to the culture medium[96]. Finally, in electron microscopic studies of the adherent stromal cell layer of LTBMC no evidence was obtained for the presence of barrier cells in these cultures[262].

Macrophages. Macrophages are also an important cell type in the stromal layer of LTBMC[4]. Like endothelial cells, fibroblasts, fat cells, and blanket cells, they are distributed through the entire culture. Macrophages are associated with granulopoiesis and, if the culture conditions are in favor of erythrocytic differentiation, with erythropoiesis. Erythropoietic progenitors are maintained in LTBMC, but maturation is blocked at the stage of BFU-E. Murine LTBMC can be directed towards erythroid differentiation beyond this stage by the addition of anemic mouse serum to the culture medium. Addition of erythropoietin alone often fails to exert this effect[65,75]. Red cell maturation is observed only on the surface of the adherent stromal layer[7]. In this case, erythroblastic islets are formed, consisting of a central macrophage with surrounding developing erythroblasts of all recognizable stages[4], resembling erythropoiesis *in vivo*. This includes close membrane contacts between the erythroblasts and the macrophage, and phagocytosis of expelled nuclei by macrophages prior to the release of mature erythrocytes into the culture medium[4,7]. There is no evidence of the occurrence of gap

junctions between macrophages and developing hematopoietic cells.

Hematopoietic cells. In LTBMC the site of active hematopoiesis is the adherent stromal layer itself, and not the supernatant. Although cell division may take place in the supernatant culture medium, as it does in suspension cultures, foci of hematopoietic activity are found on top of and within the stroma. The vast majority of the primitive hematopoietic progenitors is located within the stromal layer[53,70,141]. Here, these cells proliferate and form myeloid colonies. These colonies can be recognized using an inverted phase contrast microscope by the flattened appearance and polygonal shape of their tightly packed cells[101]. The clones are called cobblestone areas (CA). They are always covered by blanket cells, and one or more macrophages will be present. CA consist predominantly of myeloblasts and promyelocytes. Upon further differentiation these cells become more rounded, less densely packed, having the appearance of myelocytes and metamyelocytes, and migrate to the top of the adherent layer. Loosely adherent groups of maturated granuloid cells exist on top of the stromal layer[101,201]. Here they become spherical, detached from the stroma and are released in the supernatant culture medium[59]. However, a significant number of CFU-S and CFU-C can also be found in the supernatant. The measurement of these progenitor cells can be used to determine the hematopoietic activity of the culture. The release of progenitor cells into the culture medium may continue for many months after the culture was initiated.

Extracellular matrix. The ECM of the adherent layer of long-term marrow cultures consists of collagen types I, III and IV, laminin, fibronectin and proteoglycans[25,27,28,200,213,288,291]. The collagens are produced by all stromal cell types, fibronectin is produced by endothelial cells and fibroblasts, while laminin is produced only by endothelial cells. The importance of these molecules for the integrity of the adherent stromal cell layer is evident. Perturbation of collagen synthesis impaired the outgrowth of the adherent layer[290]. When an intact stromal cell layer is treated with an antibody against fibronectin, the adherent layer is completely destroyed. The GAG chains of the proteoglycans present in these cultures are hyaluronic acid, heparan sulphate, dermatan sulphate and chondroitin sulphate[269]. Heparan sulphate is the most abundant GAG of the adherent layer in murine cultures, while chondroitin sulphate dominates in the adherent layer of human LTBMC and in the supernatant of murine LTBMC[87,269]. Proteoglycans form an essential component of the adherent layer. This is indicated by the strong increase in hematopoietic activity when proteoglycan synthesis is enhanced in these cultures[64], and by the blocking of granulopoiesis in LTBMC and B-lymphopoiesis in Whitlock-Witte-type cultures due to the disturbance of the interaction of progenitor cell CD44 with hyaluronic acid[153]. It never has been reported that the distribution of different types of collagen have any specificity for the hematopoietic micro-environment. The same accounts for fibronectin, laminin, and the proteoglycans.

However, it has not been excluded that qualitative and/or quantitative differences exist in different sites in the hematopoietic stroma, thus contributing to a specific micro-environment. This speculation is further extended in Chapter 8 with respect to fibronectin. It is evident that ECM molecules are critical for the formation of an optimally supportive adherent layer. A recently discovered feature of the ECM of murine LTBMC is the induction of a functional hematopoietic micro-environment upon implantation of LTBMC-derived ECM under the murine kidney capsule, which gave rise to an ectopic site of hematopoiesis[214]. However, cell-free matrix alone cannot support hematopoiesis, although one report suggests a hematopoietic growth stimulating activity of fibronectin[257].

Although LTBMC are predominantly myeloid, immature progenitors of erythroid and megakaryoid cells, mature megakaryocytes, and progenitors of B- and T-lymphocytes are maintained in the adherent layer[70,119,166,194,219,243,270]. Stem cells from LTBMC are able to reconstitute the hematopoietic organs and the B- and T-lymphocyte compartments of (lethally) irradiated recipients upon transplantation (Chapter 7), but no pre-B- or B-cells, nor T-lymphocytes can be detected in LTBMC[63]. Long-term marrow cultures can be switched from primarily myeloid differentiation to stroma-dependent long-term B-lymphocytopoiesis[58,67,69,128,265,266,267,273,274]. These cultures maintain primitive marrow-derived B-cell progenitors, which proliferate and differentiate into pre-B and mature, immunoglobulin producing B-cells. Myeloid cells and their progenitors are absent. The culture medium contains a low concentration of serum, and it does not contain corticosteroids or other supplements. Cultures are maintained at 37°C instead of 33°C. The stromal layer consists predominantly of macrophages and "stromal cells", probably fibroblasts. At 3-5 weeks of culture the hematopoietic cells consist mainly of pre-B-cells and an increasing number of IgM expressing B-lymphocytes. The lymphoid cells are attached to the stromal cells or become enveloped by them, before they are released into the supernatant culture medium[123]. A substantial number of mature B-cells from these cultures is functionally defective[273].

For the *in vitro* differentiation of murine T-lymphocytes a thymus organ culture system has been developed[116,117,196]. This culture system is based on a fetal thymic lobule of which the T-cells and their progenitors have been removed by treatment with deoxyguanidine. The empty thymic micro-environment subsequently can be recharged with T-cell progenitors that will proliferate and differentiate into mature and functional T-lymphocytes.

The stromal cells of LTBMC have been reported to produce several hematopoietic factors and other cytokines during the culture period. A list of these factors is given in

Table 3.5[¶]. The production of IL-3 could not be detected in several studies[73,76], although it has been reported after PCR of the IL-3 mRNA[192]. The presence of *c-kit* ligand (KL) on stromal cells could be expected from experiments in which the LTBMC stroma of the genetically anemic Sl/Sl^d mice failed to support hematopoiesis[11,62,122], ever since the

Table 3.5 Production of factors that influence hematopoiesis in long-term bone marrow culture

factor	reference
G-CSF	73, 81, 138, 145
M-CSF	73, 81, 144, 145, 253
GM-CSF	47, 73, 76, 144, 145, 253
IL-1	73
IL-6	73, 112, 145, 147, 164
KL	73
TGF	73, 145

defect of these mice has been located in the *c-kit* ligand gene[51,82,111,159,272,285].

When the technique of LTBMC was first developed, a stromal cell layer was allowed to form that was recharged with a second inoculum of fresh syngeneic BMC. Later applications of this culture system used a one-phase method, by which the same BMC inoculum formed the stromal layer as well as the source of HSC. Stromal layers without hematopoietic activity could be established by irradiating a confluent adherent cell layer. Irradiation of more than 9 Gy abolishes all hematopoietic activity of the culture, leaving the stromal compartment intact. It has been reported that irradiated stromas support the growth and differentiation of the hematopoietic progenitor cells that were seeded onto these stromas[2,39,99,100,157,163,193,198,199,204,248,289]. Only a few papers mentioned negative effects of irradiation on the supportive capacity of stromal cell layers[97,98]. The use of irradiated stromal cell layers as the hematopoietic micro-environment for all subpopulations of hematopoietic stem and progenitor cells forms the basis of the experimental work described in this thesis.

[¶] Different methods for the detection of hematopoietic growth factors in LTBMC have been used. Culture medium, conditioned by the adherent stromal layer, has been tested on the presence of factors by using it, both plain and concentrated, in semi-solid colony assays with fresh BMC or factor-dependent cell lines. The LTBMC-conditioned medium has also been tested in various RIA and ELISA methods for the occurrence of growth factors and cytokines. Colony formation in agar overlaid on the stroma has been done, and several reports show the detection of growth factor mRNA in cells of the adherent layer.

One alternative bone marrow culture system needs to be mentioned here. This concerns a functional stromal layer that can be grown from murine and human bone marrow and fetal liver cells. These stromal layers have another composition than their LTBMC counterparts, due to different preparation and culture conditions, and consist predominantly of macrophages and fibroblasts. Such stromal layers provide feeder layers for so-called "blast colony-forming cells", that adhere to these layers. This adherence is one of the criteria to call a progenitor cell a blast colony forming cell. These progenitor cells are believed to be more primitive than CFU-GEMM[92,93,209].

Many attempts have been made and are being made to obtain cell lines, often originating from LTBMC, that are able to support hematopoietic growth. Although some success was achieved, none of the reported "supportive" cell lines is widely used for this purpose in a variety of laboratories. The supportive capacity of irradiated stroma shows its own kinetics with respect to maintenance, growth and development of HSC (this thesis). Therefore, all future attempts to mimic this supportive activity with cloned stromal cell lines (originating from bone marrow or fetal liver) to define their functional properties have to be related to the original bone marrow stroma. This includes the support of primitive LTRA cells vs. less primitive CFU-S, the appearance and disappearance of hematopoietic clones, selection of supporting primitive and more mature progenitors on an equal basis, and the maintenance of LTRA cells. These criteria also apply for stromal cell lines that have been genetically engineered to produce growth factors[52,212,233].

Numerous factors have been listed (Tables 3.1-3.4) that influence the maintenance, proliferation and differentiation of primitive HSC and hematopoietic progenitor cells *in vivo*. A delicate balance of inducing, synergizing and inhibiting factors, together with other micro-environmental conditions, regulate the formation of mature blood cells, the ultimate task of the bone marrow. Some of these factors have been identified in LTBMC (Table 3.5), where they probably perform identical functions as in the marrow tissue *in situ*. It is therefore likely that the control of stem cell and progenitor cell activation in these cultures involve a similar balance between inducing and inhibiting regulatory factors as *in vivo*, which may differ in the various sites in the *in vitro* micro-environment, by which an inductive niche may be created.

The applications of LTBMC are versatile. Long-term marrow cultures have been used as a source of cultured HSC that repopulate (lethally) irradiated recipients of syngeneic, semi-allogeneic or allogeneic BMC. Especially the cells from the adherent layers have been found to have the ability to reconstitute the hemato-lymphopoietic compartments upon transplantation[63,86,102,146,181,219,228]. LTBMC have been shown to be devoid of mature T- and B-lymphocytes[63], which diminishes the development of a graft-versus-host

reaction upon transplantation with LTBMC-passed (semi-)allogeneic BMC. In addition, it has been reported that LTBMC of leukemic BMC support the development of normal hematopoietic cells, while leukemic growth is gradually disappearing[45,54,216]. Therefore, LTBMC have been successfully used in purging protocols for autologous bone marrow transplantation[45,245].
LTBMC have been shown to expand a subpopulation of stem cells with LTRA at a low level[33,83,172]. This was a promising result with respect to the use of LTBMC fostering stem cells for gene transfer, which could be employed to cure a variety of genetic disorders. The overall maintenance of HSC in these cultures, however, has not been determined properly, and is the subject of Chapter 7.
Attempts to determine the number of HSC in a given BMC sample using LTBMC have been made using a limiting dilution technique in which LTBMC stromal layers function as feeders for stem cells[202]. These experiments were very laborious because they were carried out in flask cultures. LTBMC have been miniaturized[39,135,149,150,151]. These two applications form the basis for the use of micro-LTBMC in a limiting-dilution set-up (Chapter 5)[152,256].

Recently, it has been reported that human hematopoietic progenitor cells do not require direct contact with the bone marrow stroma for long-term *in vitro* hematopoiesis[249]. Hematopoietic progenitors are conserved to a greater extent when only in contact with the culture medium, separated from the stroma by an ultra-thin membrane that was impermeable for cells, and produce more CFU-GM than did progenitors that had physical contact with stromal cells. This is in sharp contrast with earlier observations, done with diffusion chambers in murine LTBMC, that show the essential requirement of physical stromal contact for optimal maintenance and proliferation of primitive hematopoietic cells[24]. The biological mechanisms behind these different observations are, as yet, not clear, and require further study.

The fact that progenitor cell production in LTBMC may last for a prolonged period of time indicates that these cultures can function as an *in vitro* model for hematopoietic cell proliferation, regulated by close-range interactions between the hematopoietic cells, the various types of stromal cells, the ECM, and diffusible regulatory molecules, in brief, the hematopoietic micro-environment. LTBMC techniques have significantly contributed to the understanding of the interrelationships between stroma and developing cells during the process of *in vitro* hematopoiesis. They have further defined and extended the original hypothesis of the inductive hematopoietic micro-environment[55] and the "niche" theory[218]. However, one must realize that this culture system never completely establishes the complexity of intact bone marrow *in situ*[68].

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Chapter 4
Growth kinetics of distinct murine stem cell subpopulations in hematopoietic repopulation assays *in vivo* and *in vitro*

Preface

This chapter describes a study on the ability of subpopulations of hematopoietic stem cells, obtained from murine bone marrow using elutriation and multiparameter sorting, to establish and maintain hematopoiesis following their deposition on irradiated stromal layers of long-term bone marrow cultures. Two fractions were obtained which differed in their mitochondrial activity as indicated by the retention of rhodamine-123 (Rh123). The $Rh123^{bright}$ cell fraction, containing the majority of day-8 and day-12 CFU-S and *in vitro* clonable progenitors, showed hematopoiesis only in the first weeks. In contrast, the $Rh123^{dull}$ fraction, which was depleted for day-8 CFU-S and which contained the majority of cells with marrow repopulating ability, maintained hematopoiesis for a prolonged time after an initial week of delay. These data fully support and extend previously published *in vivo* data, indicating that CFU-S have low capability to generate new CFU-S and CFU-C (i.e., CFU-G, CFU-M, CFU-GM), and that cells responsible for long-term generation of hematopoietic progenitor cell and for the maintenance of hematopoiesis both *in vivo* and *in vitro* are the precursors of CFU-S and of *in vitro* clonable progenitor cells. Furthermore, the present findings form the basis for an *in vitro* assay for primitive precursors of CFU-S, namely cells with marrow repopulating ability (MRA) and long-term repopulating ability (LTRA) (Chapter 5).
In addition, we evaluate two assays used for the determination of the repopulation potential of stem cells in murine bone marrow grafts. Marrow repopulating ability and erythroid repopulating ability (ERA) have been analyzed on their reliability with respect to the ranges in graft size. Also, stroma-dependent hematopoiesis *in vitro* used to measure short-term and long-term hematopoietic growth is evaluated with reference to the number of bone marrow cells (BMC) seeded onto the stroma.

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4.1 Marrow repopulating cells, but not CFU-S, establish long-term *in vitro* hematopoiesis on marrow-derived stromal layers

Introduction

The population of hematopoietic progenitor cells forms a continuum in which the various cell types are arranged in a hierarchic model of self-renewal, pluripotency and proliferative capacity.
The CFU-S has been generally regarded as the most primitive hematopoietic stem cell (HSC). However, measurements of CFU-S do not necessarily correlate with life-sparing and long-term repopulating ability[2,18]. In the last 10 years evidence accumulated that the CFU-S may not be the pluripotent self-renewing stem cell[4,7,10,23].
The most primitive cell types appear to be extremely resistant to the cytostatic effects of 5-fluorouracil[10], and contain very few and/or relatively inactive mitochondria, as indicated by the low retention of the supravital fluorochrome rhodamine-123[1,20]. These cells have high MRA, as evidenced by their ability to generate high numbers of day-12 CFU-S, CFU-C *in vivo*[23]. In contrast, CFU-S have been demonstrated to have high affinity for Rh123 and to generate very few secondary CFU-S and CFU-C *in vivo*, indicating their low MRA.
The aim of the study described in this paragraph was to investigate whether the large differences in repopulating ability between MRA cells and CFU-S measured *in vivo* could be mimicked in an *in vitro* model of stroma-dependent hematopoiesis. This model provides an essential prerequisite for the development of an *in vitro* assay of a long-term repopulating cell *in vivo*.

Materials and methods

Cell purification. Bone marrow of both tibiae and femora of 15-20 male (CBA × C57Bl)F_1 mice, aged 20-40 weeks, purchased from the Radiobiological Institute TNO, Rijswijk, The Netherlands, was elutriated as described elsewhere[22]. Cells which had left the separation chamber at a flow rate of between 11 and 13 mL/min at constant rotation of a JE-6 Beckman rotor at 1200 rpm (E(11-13) fraction) were washed, and 10^6 elutriated cells per mL were incubated for 45 minutes at 37°C in RPMI with 0.4%

bovine serum albumin (BSA, fraction V; Sigma), containing 0.1 μg/mL Rh123 (Eastman Kodak). Subsequently the cells were washed twice and incubated for 30 minutes in RPMI with 0.4% BSA to remove the unbound Rh123, and resuspended (3×10^6 cells/mL) in the same medium. The Rh123-labeled cells from the E(11-13) elutriation preparation were analyzed and sorted by a FACS II (Becton Dickenson) with an argon laser set at 488 nm and a sorting speed of 2500 cells per second.
Elutriated cells in the forward and perpendicular light scatter (FLS/PLS) blast window were divided into four fractions (windows I-IV) of increasing fluorescence, of which window I contained an average of 20% and window IV 7% of the nucleated cells present in the blast window. Cells from window I were called $Rh123^{dull}$, and from window IV $Rh123^{bright}$ [23].

Determination of CFU-S and MRA. Unfractionated and sorted cells were injected into the lateral tail veins of 8-10 lethally irradiated male mice per experimental group. Irradiation was carried out by two opposing ^{137}Cs sources (Gammacell 40, Atomic Energy of Canada). The total dose of 10.5 Gy per mouse was given at a dose rate of 1.13 Gy/min. Macroscopic spleen colonies were counted on day-8 (CFU-S-8) and day-12 (CFU-S-12) after treatment. To measure MRA, unfractionated or sorted cells were injected into 4-5 lethally irradiated mice per experimental group. After 13 days aliquots of their marrow were assayed for the presence of CFU-S-8[9,10]. MRA was expressed as the number of newly generated CFU-S-8 per femur equivalent per 10^5 cells injected, denoted as MRA[CFU-S-8]. Control irradiated mice never showed endogenous reconstitution as determined by CFU-S and MRA[CFU-S-8].

Long-term bone marrow cultures. The marrow content of one femur was flushed into a 25 cm^2 tissue culture flask (Costar) using 3 mL α-medium (α-modification of Dulbecco's Modified Eagle's Medium), containing 10% horse serum (HS; Boehringer), 10% fetal bovine serum (FBS), 0.5 mg/mL Fe-saturated human transferrin (Hoechst-Behring), 10^{-5}M hydrocortisone-21-hemisuccinate (Sigma) and 10^{-4}M β-mercaptoethanol (Merck). The cultures were maintained at 33°C, 10% CO_2 and 100% humidity, and were fed weekly by removing all the growth medium and adding an equal volume of fresh medium. After 4 weeks of culture the flasks, at that time containing a confluent hematopoietically active stromal layer, were irradiated with a total dose of 20 Gy at a dose rate of 1.13 Gy/min in order to remove all endogenous hematopoietic activity.

Culture of sorted hematopoietic cells on irradiated stromal layers. Unfractionated and elutriated cells and the sorted $Rh123^{dull}$ and $Rh123^{bright}$ fractions were overlaid on stromal layers which had been irradiated 1-4 days previously. Cultures were maintained at 33°C and 10% CO_2. With weekly intervals the supernatants of the cultures were

removed completely and replaced by fresh medium. The harvested supernatant cells were assayed for total nucleated cell count and for CFU-C using semi-solid culture.

Semi-solid culture. CFU-C of LTBMC supernatants were determined by culturing 5×10^4 nucleated supernatant cells in a semi-solid culture system, containing 0.8% methylcellulose (Methocel AP4 Premium; Dow Chemicals), 10% FBS, 1% BSA, 10% concanavalin-A stimulated mouse spleen conditioned medium (Con-A MSCM) as the source of hematopoietic growth factors, 3.3×10^{-3}M L-glutamine (Merck), 80 U/mL penicillin (Gibco), and 80 μg/mL streptomycin (Gibco) (final concentrations). These cultures were kept at 37°C and 5% CO_2. Colonies consisting of 50 or more cells were counted on day-7 of culture.

Results

CFU-S and MRA cells. The contents of CFU-S-8, CFU-S-12, and MRA cells of normal bone marrow, $Rh123^{bright}$ and $Rh123^{dull}$ cells are listed in Table 4.1. These data are

Table 4.1 Contents of CFU-S and MRA of $Rh123^{dull}$, $Rh123^{bright}$, and unsorted BMC

Cell fraction	CFU-S-8*	CFU-S-12*	MRA[CFU-C]†
$Rh123^{dull}$	<4.5‡	38.2 (18.5)	5368.5 (49.9)
$Rh123^{bright}$	1469.0 (103.7)	1125.0 (94.6)	<130.8
fresh BMC	13.6 (2.0)	25.3 (3.7)	61.9 (8.4)

* Number of CFU-S per 10^5 BMC injected
† MRA expressed as the number of newly generated CFU-S-8 per femur equivalent per 10^5 BMC injected
‡ Data are from a representative of 4 experiments

similar to those in an earlier study[23], showing that the $Rh123^{dull}$ fraction contained the cells responsible for long-term repopulation *in vivo*, while the $Rh123^{bright}$ fraction contained CFU-S, but no MRA cells.

Growth kinetics of Rh123-sorted stem cells on irradiated stromal layers. When irradiated stroma was seeded with 2.5×10^4 $Rh123^{bright}$ cells, an immediate but short-lived burst of hematopoiesis was seen (Figure 4.1[b] and 4.1[d]). About 250 CFU-C per flask were produced during the first week. This number then fell to a low level at week 2,

and CFU-C production faded out after the fourth week. Nucleated cell production was very high at week 2, but declined hereafter. After week 5 no nucleated cell production could be detected.

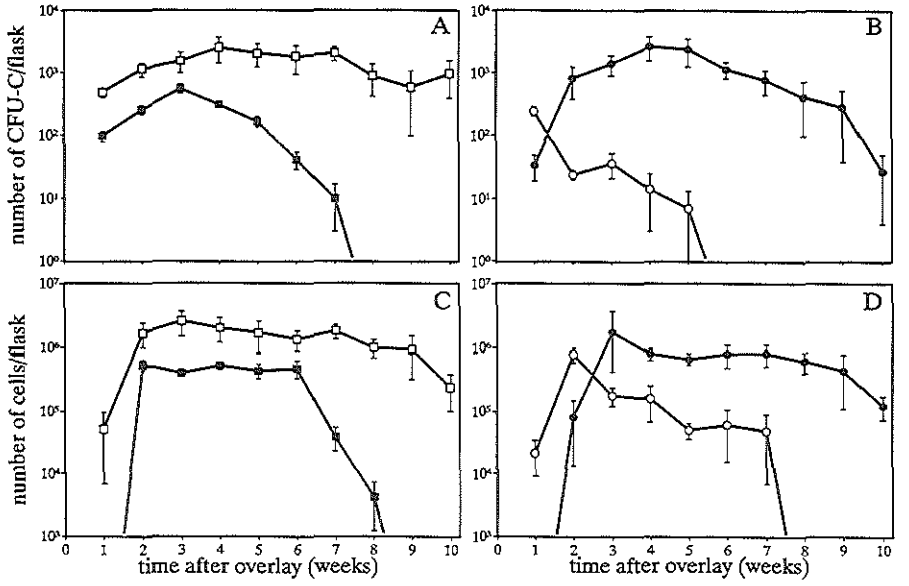

Figure 4.1. Numbers of CFU-C (A, B) and cells (C, D) per flask. Fresh BMC (■), E(11-13) cells (□), $Rh123^{bright}$ (○), and $Rh123^{dull}$ cells (●) (4 experiments (± 1 SEM).

In contrast, when irradiated stromal layers were overlaid with equal numbers of $Rh123^{dull}$ cells, hematopoiesis could hardly be detected in the first week. From the second week on hematopoiesis was established and peaked around the fourth and fifth week (Figure 4.1[b] and 4.1[d]). The levels of CFU-C and cell production could be maintained from the second to the nineth week. This led to a cumulative production of 10,000 CFU-C in 9 weeks per flask, while in the same period only 325 CFU-C were harvested from flasks containing $Rh123^{bright}$ cells.

In vitro hematopoiesis of an inoculum of 10^6 E(11-13) elutriated cells followed kinetics that was a composite of the $Rh123^{bright}$ and $Rh123^{dull}$ fractions (Figure 4.1[a] and 4.1[c]). Release of CFU-C in the supernatant showed a relatively constant level from the first week on. Production of CFU-C could be maintained for at least 10 weeks. The numbers of nucleated cells in the culture medium followed similar kinetics.

When 10^5 fresh BMC were seeded on irradiated stromal layers, production of CFU-C showed an increase in the first 3 weeks followed by a gradual decrease in the 4 weeks thereafter (Figure 4.1[a] and 4.1[c]).

In none of the experiments CFU-C production was observed on irradiated stromal layers which had not been overlaid with cells, nor did we encounter hematopoietic foci or any other kind of hematopoietic activity.

In order to compare the ability of the various cell suspensions to generate CFU-S *in vivo*

Table 4.2 Production of CFU-C by $Rh123^{dull}$, $Rh123^{bright}$, and unsorted BMC

Cell fraction	week 1*	week 4
$Rh123^{dull}$	136 (61)†	10,788 (4,577)
$Rh123^{bright}$	974 (167)	54 (42)
fresh BMC‡	98 (18)	307 (35)

* Time after overlay

† Number of CFU-C per flask, expressed per 10^5 BMC inoculated. The original inocula per flask were: $Rh123^{dull}$: 2.5×10^4; $Rh123^{bright}$: 2.5×10^4; fresh BMC: 1.0×10^5. Data represent the arithmetic means of 4 experiments (1 SEM)

‡ Data for fresh BMC represent the arithmetic means of 2 experiments (1 SEM)

MRA[CFU-S-8] (Table 4.1), and to produce CFU-C *in vitro*, we have calculated the production of CFU-C per flask on week 1 and week 4 on the basis of 10^5 cells originally inoculated (Table 4.2). It is clearly shown that CFU-C production in the first week largely descends from cells bearing the characteristics of $Rh123^{bright}$ cells (high CFU-S incidence), while the majority of CFU-C production on week 4 is caused by $Rh123^{dull}$ cells (high MRA[CFU-S-8] incidence).

Discussion

The data presented here show that a suspension containing MRA cells, which is depleted of CFU-S-8 ($Rh123^{dull}$)[23], has the ability to generate secondary CFU-C for many weeks on a pre-established stromal layer. This observation leads to the tentative identification of the long-term repopulating cell in stroma-dependent hematopoiesis *in vitro* as a cell that ensures long-term maintenance of hematopoiesis *in vivo*, by virtue of its ability to generate new CFU-S-12 *in vivo*.

In addition, we show that cell suspensions enriched for CFU-S-8 and CFU-S-12, but depleted for MRA cells ($Rh123^{bright}$), can sustain hematopoiesis and CFU-C production on an adherent stromal layer only for a limited period of time. This notion is in complete agreement with previous findings, that such $Rh123^{bright}$ cells lack the capacity to generate *in vivo* new CFU-S-12, CFU-C, and cells that ensure survival of lethally irradiated recipients[23,24]. The data also support and extend earlier observations indicating

that CFU-S cannot initiate long-term *in vitro*[4,27,30]. Taken together, the evidence strongly suggests that the majority of CFU-S-8 and CFU-S-12 lack the ability to self-renew, and have limited capacity to generate new CFU-C both *in vivo* and *in vitro*.

Sorting of elutriated BMC based on Rh123-retention represents a powerful method to separate CFU-S and CFU-C away from pre-CFU-S[23]. $Rh123^{bright}$ cells, which have high mitochondrial activity, are highly enriched for CFU-S-8 and CFU-S-12, but depleted for MRA cells, i.e., cells that generate new CFU-S-12 in the irradiated marrow cavities. Most of the CFU-S in this $Rh123^{bright}$ fraction form transient erythroid nodules in the irradiated spleen and are deficient in generating megakaryocytic and granulocytic spleen colonies[21]. In contrast, $Rh123^{dull}$ cells, showing low mitochondrial activity, are depleted of CFU-S, but contain cells associated with MRA. The differentiation potential of such cells in the spleen is mainly megakaryocytic at 8 days after infusion, while later on they form large megakaryocytic and erythrocytic spleen colonies[21].

The observation that $Rh123^{bright}$ cells form many secondary CFU-C in the first week but hardly detectable numbers on week 4, while $Rh123^{dull}$ cells appear to do the opposite, can be taken as characteristic feature of short- and long-term repopulating cells *in vitro*, respectively. This implies that the production of CFU-C in the first weeks descends from short-term repopulating cells, CFU-S, whereas hematopoietic activity in the fourth week is due to HSC with long-term repopulating ability, namely MRA cells. Thus, production of CFU-C on week 4, when related to the number of cells overlaid on the stroma, gives quantitative information on the long-term repopulating ability of a given cell suspension, which is well demonstrated in Table 4.2. If these findings apply to human bone marrow as well, such calculations may form the essence of an *in vitro* assay for the quantification of MRA of a cell suspension for use in bone marrow transplantation, e.g., following purging regiment.

The use of established non-hematopoietic stromal layers is essential in the present study, since the sorted cell fractions are depleted for stromal cells. Previous studies report an equality of both irradiated and non-irradiated stromal layers in supporting prolonged hematopoiesis with respect to maintenance of the quality of CFU-S[3,28,36]. This indicates that the results obtained by inoculating HSC on irradiated stromal layers were not negatively influenced by stromal condition, and that the hematopoiesis sustaining potential of these cells remain fully active in this culture system. Therefore, our results must be due to the characteristic capacities of both $Rh123^{bright}$ and $Rh123^{dull}$ cells.

For human hematopoietic progenitor cells a similar hierarchic division in primitiveness can be made as for their murine counterparts. Human LTBMC in 35 mm culture dishes have been modified into a quantitative assay for so-called "long-term culture-initiating cells (LTC-IC)[31]. Clonogenic cells present after 5-8 weeks of culture of human BMC under limiting dilution conditions on pre-established human bone marrow-derived irradiated stromal layers formed the basis of the quantitative determination of LTC-IC[32]. In these studies it is also demonstrated that the production of clonogenic cells is totally

dependent on the presence of an intact stromal cell layer.
These studies represent the human part in the search of *in vitro* assays for primitive HSC. Urged by these papers and the data described in this chapter, we proceeded to develop a quantitative *in vitro* assay for marrow repopulating and long-term repopulating HSC on the basis of time-dependent clone formation in pre-established stromal layers in the mouse. This assay is described in detail in Chapter 5.

4.2 Evaluation of *in vivo* and *in vitro* repopulation assays in the mouse

Introduction

In recent years the definition of the spleen colony forming cell (CFU-S) being the most primitive and long-term engrafting HSC[33] has been challenged. Since the proposal of Magli *et al.*[17] that cells forming spleen colonies on day-10 after transplantation or later represent the pluripotent HSC rather than day-8 CFU-S, evidence accumulated that cells forming early spleen colonies (day-8) differ from cells forming late spleen colonies (day-11/14) in various respects, including lectin-binding properties[14,22], antigenic load on their surface membrane[6], growth- and self-renewal characteristics[16,19,35], and sensitivity to cytostatic drugs[34]. Some authors even doubted the self-renewal properties of cells forming late spleen colonies (day-12 CFU-S) (Paragraph 4.1)[27,30], although others showed data on retransplantation studies of day-12 colonies, that were able to form new colonies in a second recipient[28,29].

Treatment of mice with the cytostatic agent 5-fluorouracil, which eliminates proliferating cells and spares non-cycling (stem) cells, led to the suggestion that the day-13 CFU-S assay is not a proper indicator of the property of a graft to repopulate the bone marrow of a lethally irradiated host, and gave evidence for a pre-CFU-S cell[10]. This class of primitive HSC does not form macroscopic splenic nodules within a 13-day period, but is capable of producing new day-13 CFU-S as their progeny[10,34].
Ploemacher and Brons[23,24] were able to separate pre-CFU-S almost completely from CFU-S on the basis of differential retention of the supravital fluorochrome Rh123. The separate identity of pre-CFU-S and CFU-S was confirmed by Jones *et al.*[15], who separated cells with long-term repopulating ability from spleen colony-forming cells using counterflow centrifugation. It now becomes increasingly accepted that the majority of CFU-S is distinct from the stem cell subset responsible for long-term maintenance of hematopoiesis[13].

The assay for the pre-CFU-S activity of a bone marrow sample as described by Hodgson and colleagues[11] and adapted by others[23,24] is the MRA. MRA denotes the ability of a

graft to repopulate the marrow cavities of an irradiated recipient either with nucleated cells or new hematopoietic precursor cells in a period of 12-13 days. In addition to the MRA assay, the ability of the transplant to repopulate the erythroid blood compartment (erythroid repopulating ability, ERA) has been used as an indicator for primitive stem cell activity[1,8,12]. In support of the *in vivo* evidence, it has been shown that MRA cells, rather than CFU-S, are responsible for long-term hematopoiesis *in vitro* (Paragraph 4.1)[27,30]. Using long-term cultures of an inoculum of BMC on pre-established irradiated stromal layers, we have shown that the generation of hematopoietic precursors (CFU-C) in the fourth week of culture correlates well with the MRA of the cell suspensions determined *in vivo* (Paragraph 4.1). In contrast, we have found that CFU-S activity is reflected by the progenitor cell generation during the first week of culture only, which indicates the transient repopulating ability of these stem cells.

In the present study we have carried out a series of experiments in order to reveal possible limitations of the *in vivo* and *in vitro* assays mentioned above with respect to the range of BMC numbers that can be used in a graft or an inoculum. We considered an assay suitable for measurements of repopulation if a constant value of a chosen endpoint is obtained for a large range of BMC input. The rationale comes from the practical problem of determining MRA, ERA, or long-term repopulating ability *in vitro* using long-term bone marrow cultures, when transplants or inocula of (highly) enriched primitive stem cell populations of an unknown purity are tested in these assays.

Material and methods

Animals. Male (CBA × C57Bl)F_1 mice were bred and maintained at the Laboratory Animal Center under clean conventional conditions, and were used for the experiments at 10-20 weeks of age. Recipients for the *in vivo* assays were given a lethal dose of 9.2 Gy γ-irradiation from two opposing ^{137}Cs sources at a dose rate of 1.08 Gy/min. Donor mice were killed, the marrow contents of both femora and tibiae were harvested and a single cell suspension was made in Dutton's balanced salt solution (Gibco). BMC were infused 2-4 hours after irradiation of the recipient mice.

Marrow repopulating ability (MRA). MRA measures the ability of the transplant to repopulate the irradiated marrow cavities of the recipient either with nucleated cells (MRA[cell]), or with hematopoietic progenitors (MRA[CFU-C]), in a period of twelve days. This period is determined by the survival time of the recipient mice for the irradiation dose used, with 3% of the mice dying before or at day-12, and a 50% survival of 14-15 days. Per experiment, six groups of five irradiated mice were injected intravenously with increasing doses of BMC, ranging from 3×10^4 to 10^7 cells per mouse,

three-fold apart. Five irradiated mice that received no cells served as control group. Twelve days after transplantation the bone marrow of the recipient's femora in each group was harvested, pooled and assayed for cellularity and the presence of *in vitro* clonable hematopoietic progenitors (CFU-C) in a semi-solid culture system. MRA was expressed as the number of nucleated cells contained in one femur of a recipient, generated by 10^5 transplanted cells (MRA[cell]), or as the number of CFU-C per femur per 10^5 BMC injected (MRA[CFU-C]).

Erythroid repopulating ability (ERA). The ERA measures the capacity of a graft to repopulate the erythroid blood compartment of an irradiated host. From the same mice as used in the MRA assays, a blood sample of each individual animal was taken twelve days after transplantation. Erythrocytes were counted on a Coulter Counter (Coulter Electronics), and the hematocrit (Ht) was determined using a micro-hematocrit centrifuge and reader (Hawksley). The percentage of reticulocytes was determined by FACScan (Becton Dickenson) using the RetiCount software program, after staining 1 mL whole blood with 0.1 μg thiazole orange in 1 mL of 2 mM EDTA/PBS for 60 min, and measuring the fluorescence at 530 nm. ERA was expressed, A) as the absolute number of erythrocytes per mL recipient blood on the basis of 10^5 BMC injected (ERA[ery]), B) as the hematocrit of the recipient blood per 10^5 cells injected (ERA[Ht]), and C) as the absolute number of reticulocytes per mL blood per 10^5 BMC injected (ERA[reti]).

CFU-C assay. To determine the number of femoral CFU-C in recipient mice, generated by the graft in 12 days, fractions of the pooled bone marrow samples of each group were put in a semi-solid culture assay in a series of dilutions ranging from 0.5 femur per dish for the irradiation control bone marrow to 0.005 femur per dish for mice, that had received a transplant of 10^7 cells. The culture medium consisted of α-medium at 280 mOsm, 1.2% methylcellulose (Fluka), 20% HS (Gibco), 1% BSA, 10% pokeweed mitogen (Gibco) mouse spleen conditioned medium as the source of hematopoietic growth factors, 80 U/mL penicillin, 80 μg/mL streptomycin, 8×10^{-5}M β-mercaptoethanol, 3.3×10^{-3}M L-glutamine, and 8×10^{-5}M sodium selenite (Merck). One mL of culture medium was put in each of two duplicate 35 mm culture dishes per group. The cultures were kept at 37°C, 5% CO_2 and 100% humidity. Colonies consisting of more than 50 cells were counted on day-7 of culture.

Long-term bone marrow culture (LTBMC). In this culture technique BMC are grown on pre-established bone marrow-derived stromal cell layers. The weekly production of non-adherent nucleated cells and CFU-C forms an indication of *in vitro* repopulating activity.

Both femora and tibiae of 10 mice were crunched using an aseptic mortar and pestle.

The bone marrow was harvested in LTBMC medium. The LTBMC medium consisted of α-medium, 10% FBS (HyClone), 5% HS, 0.41 mg/mL Fe-saturated human transferrin, 8×10^{-5}M β-mercaptoethanol, 10^{-5}M hydrocortisone-21-hemisuccinate, 80 U/mL penicillin and 80 μg/mL streptomycin, 3.3×10^{-3}M L-glutamine, and 8×10^{-5}M sodium selenite. No attempts were made to prepare a single cell suspension. The BMC were then put into thirty 25 cm^2 culture flasks. One flask contained about 3×10^7 BMC in 3 mL LTBMC medium. The cultures were kept at 33°C, 10% CO_2, 100% humidity, and were fed weekly by replacing all of the growth medium. After 3 weeks the cultures consisted of a confluent and hematopoietically active stromal cell layer. The stromas subsequently received 20 Gy γ-irradiation at a dose rate of 1.08 Gy/min, which removed all hematopoietic activity. One day after irradiation the culture medium was completely changed for LTBMC medium with 20% HS. Three days after irradiation the stromas were overlaid with fresh BMC in a series of dilutions ranging from 3×10^4 to 10^7 cells per flask. Each dilution group consisted of 5 flasks. The supernatants of the cultures were harvested weekly from 1 to 4 weeks after overlay, and individually assayed for the number of nucleated cells and CFU-C. After 4 weeks of culture the adherent layers were trypsinized, single cell suspensions were prepared of each individual flask, and the number of adherent CFU-C per flask was estimated.

Statistical analysis. The data were analyzed using the Student-Newman-Keuls test for multiple comparison.

Table 4.3 Numbers of nucleated cells and CFU-C, measured 12 days after transplantation

Number of cells injected per mouse	Number of cells per femur*	Number of CFU-C per femur*
3.0×10^4	1.08×10^6 (3.56×10^5)	466 (357)
1.0×10^5	2.05×10^6 (5.45×10^5)	1,490 (670)
3.0×10^5	5.60×10^6 (1.07×10^6)	5,480 (1,630)
1.0×10^6	1.07×10^7 (6.53×10^5)	14,190 (2,525)
3.0×10^6	1.59×10^7 (9.08×10^5)	28,074 (3,824)
1.0×10^7	2.13×10^7 (3.69×10^6)	39,323 (5,815)
0	6.24×10^5 (3.98×10^5)	94 (80)

Results

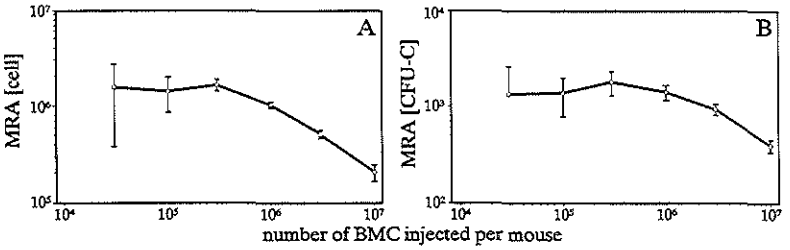

Figure 4.2. MRA, measured as the femoral content of nucleated cells (A), and CFU-C (B). Data represent the arithmetic means of 3 experiments (± 1 SEM).

Marrow repopulating ability (MRA). Twelve days after transplantation the mice were assayed for the number of nucleated cells and CFU-C per femur (Table 4.3). Both these numbers increased with increasing numbers of marrow cells used for transplantation. The irradiation control values were subtracted from the transplantation values, and these were normalized per 10^5 cells injected in order to calculate MRA (Figure 4.2). MRA[CFU-C] gave statistically identical values over a large input range (3×10^4 to 3×10^6

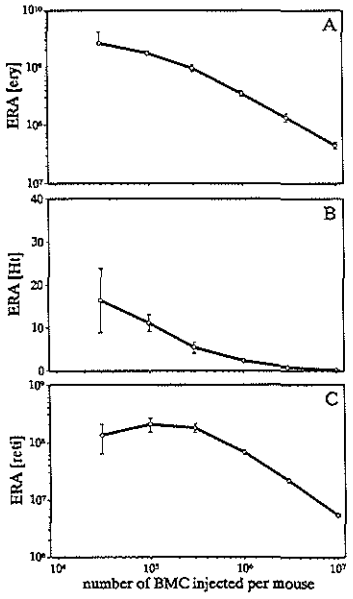

Figure 4.3. Erythroid repopulating ability, measured as (A) ERA[ery], (B) ERA[Ht], and (C) ERA[reti]. Data represent the arithmetic means of 2 (C) or 3 (A, B) experiments (± 1 SEM).

Table 4.4 Erythroid blood parameters, 12 days after transplantation

Number of cells injected per mouse	Number of erythrocytes per mL blood*	Ht*	Number of reticulocytes per mL blood†
3.0×10^4	5.03×10^9 (1.11×10^9)	26.28 (4.24)	5.44×10^7 (2.29×10^7)
1.0×10^5	6.23×10^9 (2.93×10^8)	32.05 (5.18)	2.18×10^8 (7.34×10^7)
3.0×10^5	7.38×10^9 (7.17×10^8)	37.20 (9.65)	5.48×10^8 (1.28×10^8)
1.0×10^6	8.00×10^9 (6.50×10^8)	45.98 (2.06)	6.84×10^8 (1.15×10^8)
3.0×10^6	8.35×10^9 (1.16×10^9)	48.08 (1.42)	6.48×10^8 (7.89×10^7)
1.0×10^7	8.73×10^9 (9.33×10^8)	48.65 (2.40)	5.38×10^8 (2.11×10^7)
0	4.45×10^9 (1.48×10^9)	21.62 (4.72)	1.44×10^7 (6.16×10^6)

* Data represent the arithmetic means of 3 experiments (1 SEM)

† Data represent the arithmetic means of 2 experiments (1 SEM)

BMC), but the MRA[cell] values were stable over a smaller range (3×10^4 to 3×10^5 BMC). Although the Student-Newman-Keuls test for multiple comparison did not reveal statistical significant differences ($\alpha_T < 0.05$) between MRA[CFU-C] as well as MRA[cell] values measured at different graft sizes, it is evident from the curves, that the MRA[cell] assay consistently underestimated the ability of a graft when more than 3×10^5 cells were infused.

Erythroid repopulating ability (ERA). Table 4.4 shows the values of the erythroid blood parameters that were used to measure the repopulating capacity of the grafts, 12 days after transplantation. Following subtraction of the irradiation control values and normalization of the data to 10^5 cells injected per animal, we obtained the values for ERA[ery], ERA[Ht] and ERA[reti] as they are presented in Figure 4.3. From these curves it is clear, that all ERA parameters tested decreased with increasing cell numbers injected and thus underestimated the repopulating activity of a graft when more BMC were infused. The ERA[ery] parameter even showed a 45.1 times difference between the values obtained after injection of 3×10^4 and 10^7 BMC. The values of ERA[ery] and ERA[Ht] after infusion of 3×10^4 and 10^5 BMC were statistically different from values obtained from 3×10^5 to 10^7 BMC. In the two experiments in which the absolute number of reticulocytes in the blood was determined, it was found that the ERA[reti] values obtained from the 3×10^4, 10^5 and 3×10^5 BMC groups were statistically not different, but they differed from the values from 10^6 to 10^7 BMC injected. All data were processed at $\alpha_T < 0.05$.

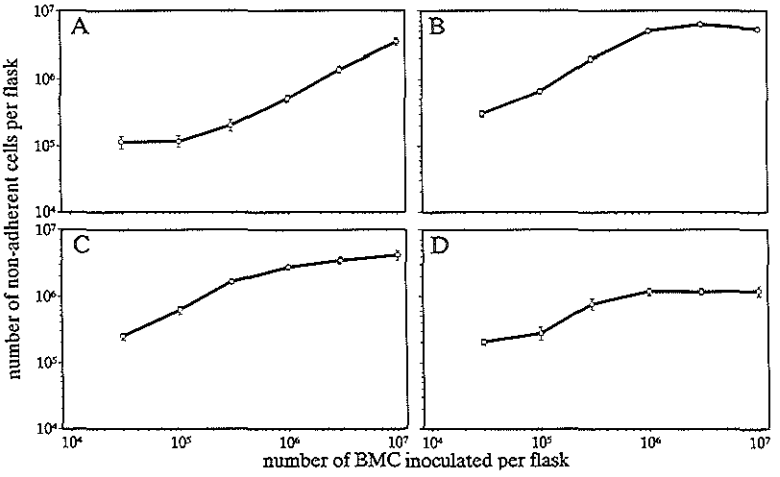

Figure 4.4. Release of nucleated cells into the supernatant of LTBMC culture flasks at (A) week 1, (B) week 2, (C) week 3, and (D) week 4 (arithmetic means of 3 experiments ± 1 SEM).

Long-term bone marrow culture. Over a period of 4 weeks the supernatants of each individual flask were assayed for the number of nucleated cells and CFU-C generated per week. The results are shown in Figures 4.4 and 4.5. In general, an increase in the production of both non-adherent cells and CFU-C was observed with increasing numbers of inoculated BMC. Except for week 1, however, culturing more than 10^6 BMC on an irradiated stromal layer did not further increase the production of CFU-C and nucleated cells. Following normalization of the data to the number of non-adherent cells and CFU-C produced per 10^5 BMC inoculated per flask, we obtained the curves for week 1 and 4 as depicted in Figure 4.6. These data clearly show that the use of larger BMC inocula increasingly underestimated the production of non-adherent cells and CFU-C. The only exception was the production of non-adherent CFU-C in the first week of culture, which showed statistically identical values over the range of BMC input tested ($\alpha_T < 0.01$). The data for weeks 2 and 3 mimicked those for week 4.

In Table 4.5 the cumulative production of non-adherent cells and CFU-C of the flasks is shown, together with their values normalized for 10^5 BMC seeded per flask. This again illustrates that the larger inocula seeded per flask produce new nucleated cells and CFU-C ineffectively.

In contrast to the release of non-adherent CFU-C into the flask supernatants at 4 weeks of culture (Figure 4.6), the *content* of CFU-C of the adherent layer at the same time appeared to correlate with the number of BMC inoculated over a long range (3×10^4 to 3×10^6 BMC per flask) (Figure 4.7[a]). This is especially evident after normalization of

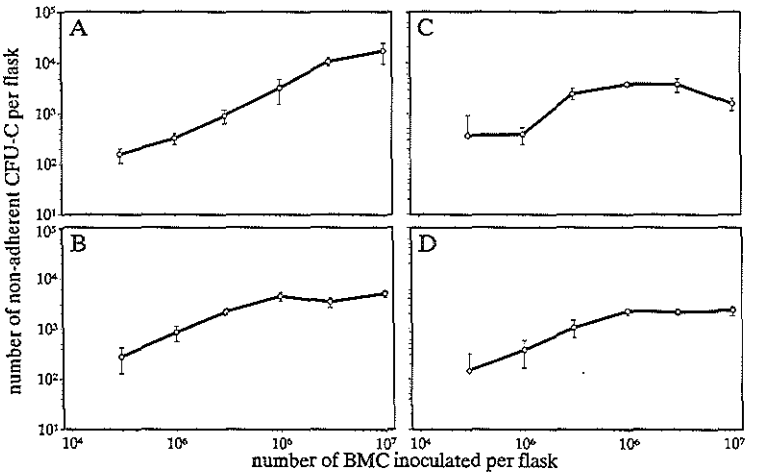

Figure 4.5. Release of CFU-C into the supernatants of LTBMC culture flasks. (A) week 1, (B) week 2, (C) week 3, and (D) week 4 (3 experiments ± 1 SEM).

these data on the basis of a 10^5 BMC inoculum (Figure 4.7[b]). Measurements of the

Table 4.5 **Cumulative production of non-adherent cells and CFU-C over a period of 4 weeks**

Number of cells inoculated per flask	Number of cells per flask*	Number of cells per flask per 10^5 cells inoculated	Number of CFU-C per flask	Number of CFU-C per flask per 10^5 cells inoculated
3.0×10^4	8.75×10^5	2.92×10^6	940	3135
1.0×10^5	1.66×10^6	1.66×10^6	1910	1910
3.0×10^5	4.59×10^6	1.53×10^6	6612	2204
1.0×10^6	9.51×10^6	9.51×10^5	13525	1352
3.0×10^6	1.23×10^7	4.13×10^5	19730	658
1.0×10^7	1.41×10^7	1.41×10^5	26026	260

* Data represent the arithmetic means of 3 experiments (1 SEM)

long-term *in vitro* repopulating ability therefore are not limited by variations in cell input in this range. No statistical difference between all these data points could be found. This indicates, that cell numbers up to 10^7 BMC seeded onto stroma in this culture system do not underestimate the week 4 CFU-C production within the adherent layer.

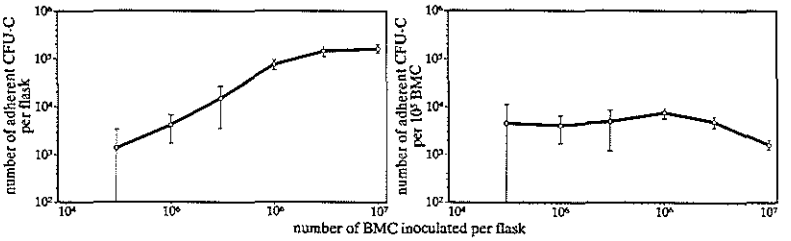

Figure 4.7. Number of CFU-C present in the adherent layer of a LTBMC flask at 4 weeks of culture. Data represent the arithmetic means of 2 experiments (± 1 SEM).

Discussion

In the present paper we report on the applicability of the MRA, ERA, and LTBMC assays as tools for the measurement of hematopoietic repopulation potential with respect to the range of graft sizes.

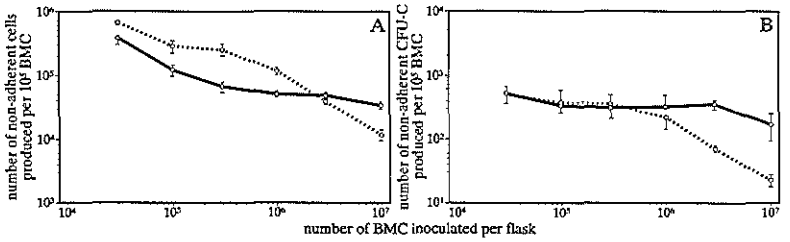

Figure 4.6. Weekly harvest of nucleated cells (A) and CFU-C (B) from supernatants of flasks at 1 week (—) and week 4 (▪▪▪) after seeding.

Of these assays, the ERA[ery], ERA[Ht], and ERA[reti] assays appear to underestimate dramatically the potential of the graft to repopulate the erythroid system of an irradiated recipient. Similarly, measurements of femoral cellularity in the MRA assay tend to underestimate the proliferative potential of the grafts at higher (3×10^5 to 10^7) BMC inputs, which limits the use of this particular assay. The MRA[CFU-C] assay gave estimations on the ability of a graft to generate new CFU-C which were quite independent of graft size. If we assess the frequency of stem cells with MRA in normal

bone marrow at about 5 per 10^5 cells (Chapter 5), one can give as much as 500 stem cells in a transplant to obtain reliable estimates of MRA[CFU-C], which is equivalent to the number of stem cells contained in 10^7 fresh BMC. The large range of MRA values in transplants of 3×10^4 BMC per mouse (Figure 4.2) is due to the fact that these values in individual experiments approximated the values obtained in irradiation control mice. The recommended input dose for MRA[CFU-C] measurements therefore ranges from 10^5 to 10^7 fresh BMC, or an equivalent number of stem cells.

Measurements of early progenitors by assaying end-stage cells (ERA, MRA[cell]) failed, probably because these functional cell compartments are being restored rapidly after transplantation by relatively mature progenitors, and are completely repopulated at the time the precursor cell compartment is still being built. Therefore, it is preferable to measure the repopulating ability of bone marrow samples using assays in which repopulation with precursor cells is determined (MRA[CFU-C]) rather than measuring a completely repopulated compartment of end-stage cells.

In addition to the *in vivo* methods to monitor the activity of various types of stem and precursor cells in a sample of BMC, the long-term bone marrow culture system, introduced by Dexter *et al.*[5] and further characterized by many researchers, gives the opportunity to study long-term maintenance of hematopoiesis *in vitro*. By virtue of the formation of a stromal compartment in which hematopoietic progenitor cells and stem cells lodge to retain their properties of establishing and maintaining hematopoietic activity, ongoing *in vitro* hematopoiesis can be longitudinally followed by measuring the release of newly generated nucleated cells into the supernatants of these cultures. Part of these cells are precursors, capable of forming colonies in various clonal assays.
In order to create a stromal cell layer, 10^7 fresh BMC are put into a 25 cm^2 culture flask. In 3 weeks a confluent stromal cell layer will have developed, containing many hematopoietic foci. Irradiation of these cultures at this stage with a total dose of 9 Gy or more completely abolishes the hematopoietic activity. The confluent stromal layer, however, remains intact and is able to function as a micro-environment for hematopoietic stem and precursor cells that are subsequently inoculated (Paragraph 4.1, Chapter 5)[3,25,26,28,36]. This technique suits the requirements for *in vitro* studies on highly purified stem cell fractions, which are not able to form their own micro-environment (Paragraph 4.1, Chapter 5)[30].
Because of the importance of the use of this culture system in the search for *in vitro* methods equivalent to the *in vivo* assays, the behavior of various stem cell subsets *in vitro*, and the interactions of stem cells with the micro-environment, we attempted to define its validity over a wide range of cell inputs. The production of non-adherent CFU-C in the first week of culture was found directly to relate to the graft size. Since CFU-C production in the first LTBMC week has been shown to correlate with the

CFU-S incidence of a graft (Paragraph 4.1), this observation indicates that CFU-S activity can be reliably measured *in vitro* by this parameter over a wide range of BMC inputs.
The CFU-C content of the adherent layers at 4 weeks of culture showed a good correlation with the input BMC number throughout the entire range of inoculum sizes tested, in contrast to the production of non-adherent CFU-C at the same time. As with the MRA[CFU-C] assay, small inocula (3×10^4 BMC per flask) gave large errors, due to the fact, that some flasks did not contain detectable CFU-C. We have previously shown that the non-adherent CFU-C production in the fourth week yields quantitative information on the MRA of the graft. The present data lead us to conclude, that the MRA of a graft can be reliably determined *in vitro* by measuring the adherent CFU-C content of a flask at 4 weeks of culture, using inocula ranging from 10^5 to 10^7 fresh BMC, or an equivalent stem cell number, per flask.

In summary, the use of the ERA technique underestimated considerably the graft potentials, and should therefore be omitted. Both MRA[CFU-C] and long-term production of adherent CFU-C in LTBMC can be applied as quantitative estimates of the repopulation potential of primitive stem cells in a graft. The application over a wide range of stem cell inputs is essential when bone marrow samples of unknown stem cell frequencies are tested, especially in samples of physically purified BMC subsets.

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Chapter 5
An *in vitro* limiting dilution assay for long-term repopulating hematopoietic stem cells in the mouse

Preface

This chapter describes an *in vitro* assay that allows the quantification of the various subpopulations of the entire range of hematopoietic stem cell subsets, from the relative mature CFU-S-8 to cells with long-term repopulating ability. The assay is based on the time-dependence of clone formation by stem cells on a pre-established bone marrow-derived stromal cell layer, using a limiting dilution set-up. This technique is an immediate continuation of the study described in Paragraph 4.1, in which primitive and less primitive stem cells show different growth kinetics after inoculation on stromal layers in tissue culture flasks.
In the first paragraph the methodology, the choice of the endpoint of the assay, and the behavior of various stem cell subsets in this assay are described. The second paragraph deals with the correlation between the various stem cell subsets and time-dependent clone formation in more detail. In addition, the nature of the clones themselves is described. The third paragraph gives some comments on the statistical background of limiting dilution assays.
The data contained in this chapter have already been published. Paragraph 5.1 has been published in:

- Ploemacher RE, Van der Sluijs JP, Voerman JSA and Brons NHC (1989). An in vitro limiting-dilution assay of long-term repopulating hematopoietic stem cells in the mouse. Blood 74: 2755-2763.
- Ploemacher RE and Van der Sluijs JP (1991). In vitro frequency analysis of spleen colony-forming and marrow-repopulating hemopoietic stem cells in the mouse. J Tiss Cult Meth 13: 63-68.

Paragraph 5.2 has been published as:

- Ploemacher RE, Van der Sluijs JP, Van Beurden CAJ, Baert MRM and Chan PL (1991). Use of limiting-dilution type long-term bone marrow cultures in frequency analysis of marrow repopulating and spleen colony-forming hematopoietic stem cells in the mouse. Blood 78: 2527-2533.

Paragraph 5.3 has been written in cooperation with J.C.M. van der Loo, after helpful

advise of professor R. van Strik, from the Department of Epidemiology and Biostatistics of this University.

5.1 An *in vitro* limiting dilution assay for long-term repopulating hematopoietic stem cells in the mouse

Introduction

Evidence is increasing that the hematopoietic stem cell compartment is extremely heterogenous. It is organized in a hierarchy of primitive cells, showing a decreasing ability to generate new stem cells, decreasing proliferative potential and pluripotentiality, and increasing turnover rate on maturation. This includes that the most primitive stem cells have the ability to generate many spleen colony-forming units (CFU-S), and cells that ensure survival of fatally irradiated recipients in the irradiated marrow *in vivo*[41]. This marrow repopulating ability (MRA) is largely associated with resting cells, as evidenced by the increased MRA of cells surviving the cytostatic agents 5-fluorouracil (5-FU)[15], bromodeoxyuridine[16], or hydroxyurea[17,47]. Furthermore, in contrast to CFU-S and *in vitro* clonable progenitor cells, these MRA cells, also called pre-CFU-S, have low mitochondrial activity per cell, as suggested by the minimal retention of the supravital fluorochrome rhodamine-123 (Rh123)[1,36].

It has not been possible to determine the frequency of MRA cells in suspensions, because the *in vivo* assay for MRA measures the total number of CFU-S generated on a basis of the number of bone marrow cells (BMC) injected without yielding information about the number of progenitors contributing to the total number of CFU-S formed. Unfortunately, any attempt to perform limiting dilution assays *in vivo* regularly is prohibited by the large numbers of animals required.

Recently, the notion emerged that CFU-S give only short-lived, stroma-associated hematopoiesis *in vitro*[3,6,52]. We have demonstrated that the ability of a stem cell for long-term engraftment of an irradiated stromal cell layer in flask cultures is inversely related to its mitochondrial activity, which corresponds well with its MRA *in vivo* (Chapter 4). In addition, we have proposed that CFU-C production in long-term bone marrow culture (LTBMC) at 1 or 4 weeks after overlay of BMC can be taken as a semi-quantitative measure of inoculated CFU-S or MRA, respectively (Chapter 4).

In view of these observations we have set out to develop a quantitative *in vitro* assay that permits direct measurement of the frequencies of hematopoietic stem cells responsible for short-term or long-term engraftment both *in vivo* and *in vitro*. It has been demonstrated earlier that clonal analysis using a limiting dilution assay in Dexter-type cultures is feasible, but limited by the large numbers of flasks that have to be analyzed[45]. The present assay has been designed to meet requirements for a simple and unequivocal

endpoint, routine use, and a sufficient number of cultures to permit reliable Poisson statistics.

Materials and methods

Mice. Male (CBA × C57Bl)F_1 mice, 12 to 30 weeks old, were purchased from the Medical Biological Laboratory TNO (Rijwijk, The Netherlands) and maintained at the Laboratory Animals Center under clean conventional conditions. The drinking water was acidified to pH 2.8. In specific experiments, mice were injected with either 150 or 75 mg 5-FU (Sigma) in phosphate buffered saline (PBS)/kg of body weight in the lateral tail vein. Three days later they were killed and single cell suspensions were prepared. This BMC suspension is denoted 5-FU_{3d} BMC.

Sorting of bone marrow cells. Bone marrow cells (BMC) were prepared by cleaning femora from muscles and tendons, and grinding them in a mortar using PBS. The cell suspensions were sieved over a nylon filter (mesh size, 100 μm). In order to prepare cells that were either enriched for MRA cells and depleted for CFU-S, or vice versa[36], we used fluorescence-activated cell sorting of BMC following a pre-enrichment step that included centrifugal elutriation[36], or paramagnetic bead-mediated negative selection of cells lacking the expression of lineage-specific markers[51]. RPMI medium with 0.4% bovine serum albumin (BSA), penicillin (10 IU/mL), and streptomycin (10 mg/mL) was used as standard counter current centrifugal elutriation (CCE) medium. For paramagnetic bead sorting BMC were washed twice in Dulbecco's Modified Eagle's Medium (DMEM), and incubated for 1 hour on ice with the pooled supernatants of five hybridoma cell lines producing rat immunoglobulin G (IgG) antibodies to CD4 and CD8 determinants on T lymphocytes (antibodies H129.19 and 53.6.72, respectively), Mac-1 (on monocytes, macrophages, granulocytic cells; antibody M1/70), B220 on B lymphocytes (antibody RA3.6B2), and GR-1 on granulocytes (antibody RB6.8C5). After washing twice in DMEM containing 0.02% gelatin, the cells were incubated with goat-anti-rat IgG-coated paramagnetic beads (Advanced Magnetics) in DMEM and 2% mouse serum, and incubated at 0°C for 30 minutes. The labeled cells were then withdrawn against an inner tube wall using a strong magnet, and the nonbound cells were collected. These cells will hereafter be called Lin^- cells to designate the absence of expression of a series of lineage markers. Following CCE or paramagnetic bead sorting, cells were incubated for 45 minutes at 37°C in DMEM containing 2 μg/mL Rh123 (Eastman Kodak). In order to remove the excess of Rh123, the cells were incubated for 30 minutes in DMEM at 37°C and were washed twice.

Analysis and sorting of the Rh123-labeled cells was performed by a FACS II (Becton Dickinson) with an argon laser set at 488 nm. The cells from the E(11-12 mL/min)

elutriation preparation, or the Lin$^-$ cells, were sorted within a light scatter blast cell window. The lower limit of the forward scatter was set to include approximately half of the lymphocytic cells, while most of the granulocytes were excluded on the basis of perpendicular light scatter ("blast cell window"), as previously described[41]. This light scatter window was set using Rh123-labeled unfractionated BMC. Two fractions differing in Rh123-retention (Rh123^{dull} containing 10% of cells present in the blast window with little uptake, and Rh123^{bright} cells, which included 10% of the most brightly fluorescent cells of the blast cell window) were obtained.

Colony assays. The day-8 and day-12 CFU-S content (CFU-S-8 and CFU-S-12, respectively) of cell suspensions were determined by injecting the appropriate BMC dilutions into a lateral tail vein of lethally irradiated mice. The dilutions used were estimated from previous experiments to give between one and five surface nodules on day-8 and between zero and six on day-12 to avoid confluence. Two opposing ^{137}Cs sources (Gammacell 40, Atomic Energy of Canada) were used to irradiate the recipient mice at a dose rate of 1.15 Gy/min with a total dose of 9.9 to 10.5 Gy. Control irradiated mice that did not receive cells were included for all observation days in each experiment. Eight or 12 days later their spleens were excised, fixed in Telleyesniczky's solution, and the macroscopic surface colonies were counted.
Quantification of CFU-C (CFU-G, CFU-M and CFU-GM) was performed using a semisolid (0.8% methylcellulose, Methocel AP4 Premium; Dow Chemical) culture medium. The culture medium consisted of α-medium, 10% fetal bovine serum (FBS), 1% BSA (fraction V; Sigma), 20% Con-A-stimulated mouse spleen conditioned medium (Con-A MSCM) as the source of hematopoietic growth factors, 80 U/mL penicillin (Gibco), 80 μg/mL streptomycin (Gibco), 3.3×10^{-3}M L-glutamine (Merck). One mL of culture medium was put in each of two duplicate 35 mm culture dishes (Costar) per group. The cultures were kept at 37°C, 5% CO_2 and 100% humidity.
Granulocyte/macrophage colonies consisting of more than 50 cells were counted on day-7 of culture using an inverted microscope. In the study of the *in vitro* clonogenic properties of Rh123^{dull} and Rh123^{bright} cells, Con-A MSCM was replaced by 10% pokeweed mitogen (PWM; Gibco) MSCM, 1 IU sheep erythropoietin/mL (Connaught Laboratories), and 2×10^4 U of human recombinant interleukin-6/mL (IL-6; purified from *Escherichia Coli*; (Dr. L. Aarden, CLB Amsterdam). These growth factor concentrations were found to be optimal for colony formation in the system used.

Marrow repopulating ability (MRA). The MRA describes the ability of a cell suspension to generate new CFU-C or CFU-S in the bone marrow of a lethally irradiated recipient mouse over a period of about 12-13 days[36]. This period is determined by the survival of fatally irradiated mice that have not been grafted with BMC. Since we required a survival of at least 4 of 5 mice per group, the regeneration period was set to

13 days in this study. Thirteen days after injection of unfractionated or sorted BMC into five lethally irradiated mice, aliquots of their marrow cells were assayed for the presence of CFU-S-12 and CFU-C. MRA was expressed as the number of CFU-S-12 or CFU-C per femur equivalent per 10^5 BMC injected[36]. Control irradiated mice were included in each experiment and were never found to have endogenous regeneration as determined by the CFU-S-12 and CFU-C content of their femora on day-13.

Methodology of the micro-LTBMC technique. This section describes the essential methodology of a limiting dilution-type miniaturized long-term bone marrow culture. The method is based on the long-term bone marrow culture (LTBMC) in 25cm^2 flasks[4], and is done by overlaying cells on pre-established, irradiated stromal cell layers in microtiter wells instead of flasks[45]. These layers are hematopoietically inactive but still support clonal growth of HSC and their descendants (Chapter 4).
For the preparation of marrow-derived stromal cell layers (CBA × C57Bl)F_1 mice, 10-30 weeks of age, were killed by gassing with CO_2. One mouse is required for setting up 4 flat bottomed microtiter plates (Costar or Falcon), of which the outer wells are not used (240 inner wells). Both femora and tibiae are taken out and placed in ice-cold Dutton's balanced salt solution (Gibco). The muscles and tendons are removed and the bones are placed in a sterile mortar (up to 100 femora and tibiae can easily be processed at a time) in a few milliliters of Dutton with 5% FBS. The bony shafts are opened by grinding them with a pestle, and the cell clumps are removed from the bones by repeated flushing using a wide-bore pipette (5 mL). The cell-rich medium is collected in a centrifuge tube, and the bony spicules are allowed to settle down. The supernatant is collected. It is essential not to make a single cell suspension. Violent stirring of the cell suspension is avoided at all times to preserve sufficient cell clumps required for optimal outgrowth of stromal elements. The marrow equivalent of one mouse (two hind legs) is diluted in 52 mL of LTBMC medium. The LTBMC medium consists of α-medium at 280 mOsm, 10% of FBS (HyClone), 5-10% of horse serum (HS; Gibco)[¶], 8×10^{-5}M ß-mercaptoethanol (Merck), 10^{-5}M hydrocortisone-21-hemisuccinate (Sigma), 80 U/mL penicillin, 80 μg/mL streptomycin, 3.3×10^{-3}M L-glutamine, and 8×10^{-5}M sodium selenite (Merck) (final concentrations). This volume is sufficient for filling the inner 60 wells of each of 4 microtiter plates with 0.2 mL (about 5×10^5 BMC) per well. The outer rim of wells may be filled with sterile water. The cultures are put in a fully humidified incubator at 33°C and 10% CO_2. The outgrowth of adherent clones is checked from day-7 of culture. An optimal hematopoietically active stromal layer will develop in 10-14 days. If a confluent adherent layer develops within less than 10 days, this is

[¶] Both FBS and HS are from selected batches. The FBS is selected on its performance in a semi-solid CFU-F assay, while the HS is selected on the growth of myeloid progenitors in a semi-solid CFU-C assay.

generally a sign of excessive macrophage outgrowth at the expense of fibroblastic and endothelial cells due to the HS batch. Inasmuch as this circumstance is associated with unstable and poorly supportive stromal layers, these cultures should be terminated and a new attempt should be made with a lower percentage of the HS batch used. Optimal layer development is associated with the appearance of phase-contrast non-refractile clones of hematopoietic cells (cobblestone areas; CA) under the stromal cells.
As soon as all well bottoms in a plate are covered by an adherent layer, the plate is exposed to 20 Gy of γ-radiation using two opposing ^{137}Cs sources (Gammacell 40, Atomic Energy of Canada) at a dose rate of 1.08 Gy/min, to completely eradicate the endogenous hematopoietic activity from the adherent layers. This treatment does not critically affect the ability of the LTBMC stroma to support hematopoiesis[9,11,52,67]. Leaving the plates unirradiated after reaching confluence may lead to retraction of stromal layers at the well borders and subsequent increasing loss of layer integrity.
One day after irradiation, the culture medium in the wells is completely removed and fresh LTBMC medium is added, now containing 20% HS instead of 10% FBS and 5-10% HS. Although the plates can be used as of this moment for overlay of fresh

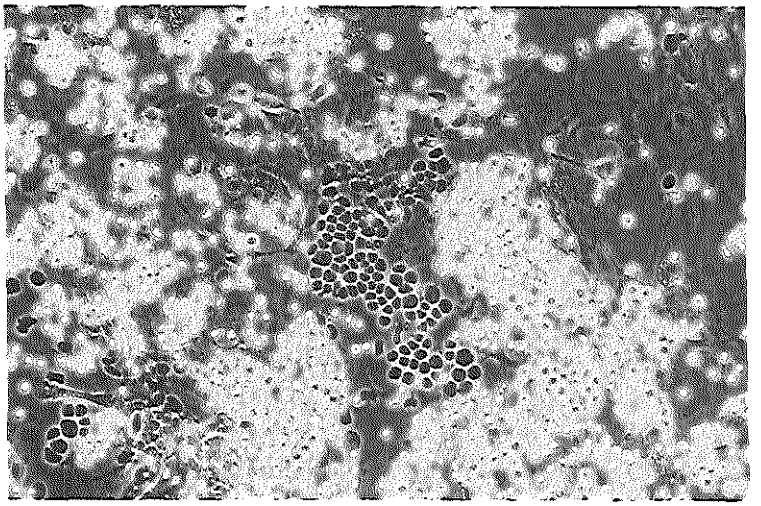

Figure 5.1. Phase-contrast micrograph of differentiating clones in LTBMC. The dark-appearing cells form the CA, which are covered by the adherent layer (original magnification 250×).

BMC, disappearing endogenous CA can be traced up to 3 days after irradiation.
Irradiated layers can be maintained for reinoculation for 8 weeks, and require a half medium change any 2 to 3 weeks.
Single cell suspensions of fresh BMC are prepared as described above. The cells are subsequently sieved through a 100 μm and 30 μm gauze sterile nylon sieve. Fresh BMC are diluted as follows: 27,000 → 9,000 → 3000 → 1000 → 333 → 111 cells per well in

0.2 mL of LTBMC medium. Each group of dilutions consists of 20 wells, distributed over 2 plates. An alternative is: 50,000 → 25,000 → 12,500 → 6,250 → 3,125 → 1,042 → 347 → 116 cells per well (0.2 mL). In the latter case, each dilution consists of 15 wells. The wells are half or completely refed weekly. It is recommended not to use H-2^k histocompatibility type BMC as a second inoculum, since these cells may have a decreased long-term maintenance[5,44].
As of 3 days after inoculation the wells can be inspected for the presence of CA that contain at least 5 cells, but may contain up to many thousands of non-refractile cells (see Figure 5.1). Inspection is done at 100× total magnification using an inverted phase-contrast microscope (Olympus). It is recommended not to count within 24 hours after a medium change due to transient release of CA cells to the stromal surface, resulting in a underestimation of CA frequencies. A well is regarded positive if at least **one** CA is found.

Comments on the statistic background together with the experimental setup of limiting dilution assays are given in Paragraph 5.3.

Replating of CA. In replating studies cultures were terminated to be able to determine the number of CFU-C contained in the adherent layer of a single well. To this purpose, the medium was removed from a well and replaced by 0.1 mL of 0.5% trypsin/PBS for one to two minutes. The digestion process was stopped by adding 0.1 mL of ice-cold HS. A single-cell suspension was made by repeated passage of the well content through an 18-gauge needle. The well content was then taken up in 5 mL of α-medium, washed, diluted in LTBMC medium, and 1/3 to 1/30 of the well content per dish was plated in the CFU-C assay.

Results

***In vitro* colony formation of Rh123-sorted stem cells.** It is shown in Table 5.1 that the presence of optimal concentrations of pokeweed mitogen stimulated mouse spleen conditioned medium (PWM-MSCM) and rIL-6 supported a high cloning efficiency of $Rh123^{bright}$ cells, which was in agreement with their 10-40-fold enrichment of CFU-S-8 as compared with unfractionated BMC[36]. In contrast, $Rh123^{dull}$ cells formed only few small macrophage and blast cell colonies after day-7 of culture. Although their number increased significantly up to 21 days, the size and number of these colonies did not reflect the presence of cells that have high proliferation potential, i.e., cells associated with MRA *in vivo*[36] and with long-term generation of CFU-C in LTBMC (Chapter 4). However, it should be noted that culture conditions as described for expression of high proliferative potential colony-forming cell (HPP-CFC)[1] were not exactly met in our

studies.

Micro-LTBMC. Following overlay of a sufficient number of hematopoietic cells on pre-irradiated stromal layers, hematopoiesis developed in a way characteristic of the cell fraction tested. When using unfractionated BMC, some progenitors adhered to the

Table 5.1 Colony-forming ability in methylcellulose of cells separated on the basis of differences in Rh123 retention

Cell sources	Colonies counted on day 7	12	21
Elutriated BMC	75 - 210	57 - 230	150 - 285
CCE/Rh123^{dull*}	0	0 - 13	26 - 59
CCE/Rh123^{bright*}	85 - 21,000	672 - 19,000	843 - 19,000

Data represent the range of the colony numbers per 10^5 cells plated in 3 separate experiments. Cultures were stimulated with PWM-MSCM, erythropoietin and rIL-6
* Sorted in the light scatter blast cell window from elutriated BMC

stroma and formed clusters or small colonies on top of the stroma within the first 3 days. As of day-2 single cells were observed in between or fully beneath the stromal cells as judged from their lack of refractiveness in phase-contrast and their large diameter, which was indicative of flattening. As of day-3 such stroma-covered cells were observed to have proliferated and have formed clusters of often tightly packed cells that are referred to as cobblestone areas (CA)[5]. Some CA kept on growing for a few weeks and then contained many thousands of cells, and no cells in that area were observed to have migrated up to the surface to become differentiated granulocytes and monocytes/macrophages. Light microscopy of the cells collected from such wells by trypsinization indicated the presence of many blast cells. Other CA disappeared after a few days or weeks by migrating to the upper surface of the stromal layer while loosing their phase density. In a few days these cells differentiated, acquired the light halo-effect of spherical cells and then migrated away over the stromal surface (Figure 5.1). Remarkably, no cells were found in the culture medium throughout the culture period.

Choice of endpoint in micro-LTBMC using limiting dilution strategy. From Table 5.2 it appears that no nonadherent CFU-C were found in single wells with extensive hematopoiesis including the presence of CA. The demonstration of replatable CFU-C was strictly associated with the presence of CA in any well tested, but not with stroma-adherent hematopoietic cells on the interface with the medium. On the basis of these data, and the consistent observation that cells contained within the stromal layer are associated with actively proliferating and more primitive hematopoietic cells[5], we

Table 5.2. Localization of replatable* CFU-C in micro-LTBMC

component tested	number of wells tested	CA	hematopoietic cells on stroma	number of wells containing CFU-C
supernatant	80	+	+	0
adherent layer	35	-	-	0
adherent layer	46	-	+	1
adherent layer	39	+	-	18
adherent layer	81	+	+	69

* replated between 7-28 days after overlay
abbreviations: +, present; -, absent

decided to use the presence of CA in a well as an endpoint for limiting dilution-type assays. Subsequently, we tested whether the number of BMC overlaid on pre-irradiated layers in micro-LTBMC correlated with the occurrence of CA as an endpoint. To this purpose we overlaid the wells with a series of dilutions ranging from 1 cell to 80,000 BMC per well, and using 20 to 30 wells per dilution. Microtiter plates were repeatedly screened over a period of 32 days under phase-contrast, and wells were scored positive when at least one CA containing five or more cells was encountered. A typical experiment is presented in Figure 5.2. It is clear that the percentage of negative wells in any dilution series is strictly determined by the number of cells inoculated per well. In conjunction with the association of replatable CFU-C, this observation provides graphically the validity of the CA as endpoint for limiting dilution assays of primitive hematopoietic stem cells.

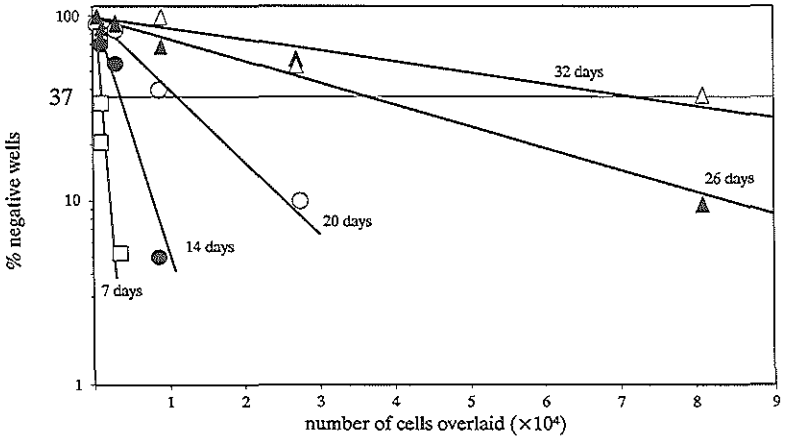

Figure 5.2. Scoring of CA as endpoint in limiting dilution analysis of stem cells is strictly determined by the number of BMC overlaid. Here, 6 dilutions of 20 wells per aliquot were included.

Table 5.3 Comparative precursor cell frequencies in bone marrow

progenitor type	progenitor frequency (range)	number of progenitors per 10^5 nucleated cells mean (1 SEM)	range
CFU-C*	1/385 - 1/710	205 (20)	140 - 260
CFU-S-7†	1/100 - 1/265	675 (73)	375 - 1,000
CFU-S-12†	1/100 - 1/265	725 (110)	375 - 1,000
CAFC-3	1/990 - 1/3,300‡	59 (17)	30 - 101
CAFC-5	1/170 - 1/1,150	259 (67)	87 - 588
CAFC-7	1/205 - 1/500	339 (30)	200 - 488
CAFC-14	1/1,110 - 1/2,500	68 (8)	40 - 90
CAFC-21	1/3,200 - 1/15,000	17 (3)	7 - 31
CAFC-28	1/9,000 - 1/50,000	6 (1)	2 - 11

* Stimulated by 10% PWM-MSCM.
† Corrected for seeding efficiency ($f_{24\ hours} \approx 4\%$).
‡ CAFC frequency data from 12 separate experiments.

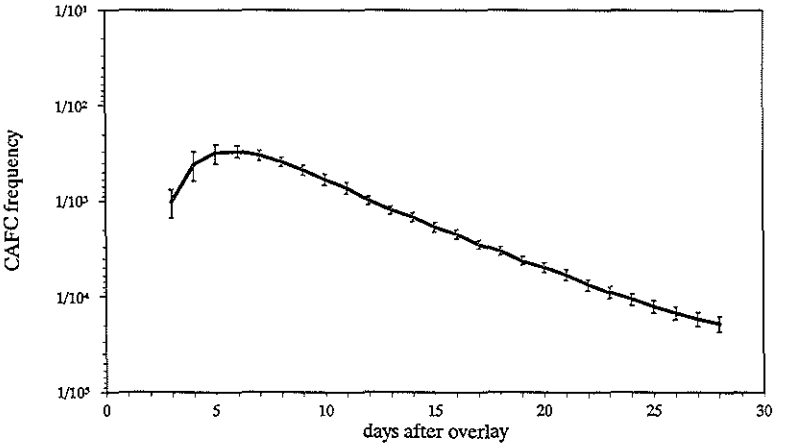

Figure 5.3. Distribution of day-3 to day-28 CAFC frequencies in normal bone marrow. Data are the arithmetic means of 12 individual experiments (± 1 SEM).

Frequency of cobblestone area-forming cells in normal marrow. The number of cells in each aliquot was chosen such that a fraction of cultures at a certain observation time would not contain any cobblestone area-forming cells (CAFC). From the fraction of nonresponding cultures, using the Poisson equation, it was then possible to calculate the CAFC frequency[10]. From experiments presented in Figure 5.2 we have calculated average CAFC frequencies in normal murine bone marrow. As is clear from Table 5.3

Table 5.4 Frequencies of short- and long-term repopulating stem cell subsets *in vivo* and *in vitro*

	CAFC per 10^5 nucleated cells			CFU-S per 10^5 nucleated cells*		
cell source	day-5	day-7	day-28	day-7	day-12	MRA†
unfractionated BMC	248 (62)	245 (53)	3.3 (2.0)	17 (4)	27 (6)	240 (59)
CCE/Rh123^{dull}‡	47 (12)	189 (31)	127 (28)	2 (2)	72 (24)	8,640 (2,419)
CCE/Rh123^{bright}‡	4,044 (1,469)	7,800 (1,848)	0.6 (0.6)	1,423 (189)	1,017 (91)	0 (0)

Numbers are the arithmetic means of 4 experiments (1 SD)
* not corrected for seeding efficiency
† number of CFU-S-12 contained in a femur of an irradiated recipient on a basis of 10^5 BMC injected, determined at day-13
‡ sorted from elutriated BMC within the light scatter blast cell window.

and Figure 5.3, progenitors forming CA between day-5 and day-7 after overlay (CAFC-5 and CAFC-7) occur with highest frequencies in the marrow, while progenitors for later CA formation occurred with decreasing frequencies.

Distribution of CAFC frequencies of Rh123-sorted stem cells. In order to investigate

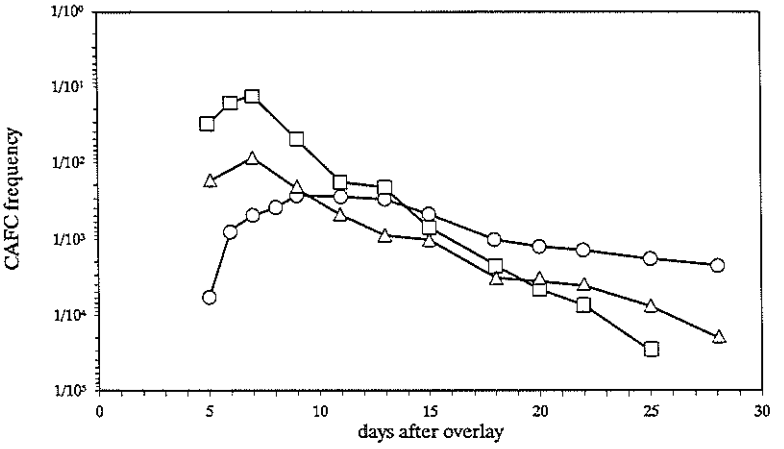

Figure 5.4. Distribution of CAFC frequencies of elutriatiated BMC (△), further enriched by Rh123^{bright} (□), or Rh123^{dull} (○) sorting.

any heterogeneity in CAFC scored after different periods of culture, we overlaid stromal layers in microcultures with BMC sorted on the basis of differences in cellular mitochondrial activity as indicated by their Rh123 retention. As reported earlier[36], $Rh123^{bright}$ cells contain many CFU-S-7 and CFU-S-12, but do not contain cells that generate new day-12 CFU-S and CFU-C in vivo, i.e., cells with MRA. Conversely, $Rh123^{dull}$ cells are depleted for CFU-S, but contain most of the MRA. By limiting dilution (Figure 5.4), $Rh123^{bright}$ cells appeared to have high frequencies of CAFC-7, but low frequencies of BMC forming late appearing CA (e.g., CAFC-28). In contrast, $Rh123^{dull}$ cells formed far more late CA and fewer early CA than unfractionated and $Rh123^{bright}$ BMC. These data clearly indicate that early and late CAFC differ with respect to their mitochondrial activity. Table 5.4 shows the agreement in frequency ratios between the various cell suspensions as measured by the *in vitro* CAFC assay and the *in vivo* acquired data for CFU-S and MRA cells. Apparently, the differences between frequencies for CAFC-5 and CAFC-7 in the various cell suspensions are comparable with the CFU-S numbers, whereas CAFC-28 frequencies relate to the data for MRA.

Distribution of CAFC frequencies in bone marrow from 5-FU treated mice. The hematopoietic stem cell compartment in the mouse is structured as a concatenated series of stem cells with progressively limited proliferative potential, and increasing turnover rate and maturity[16,18,47]. Thus, treatment of mice with 5-FU reduces CFU-C, and cells forming spleen colonies in irradiated mice on day-8 and day-10 (CFU-S-8 and CFU-S-10) considerably more than CFU-S-13, which in turn are affected far more than MRA cells[15,18].

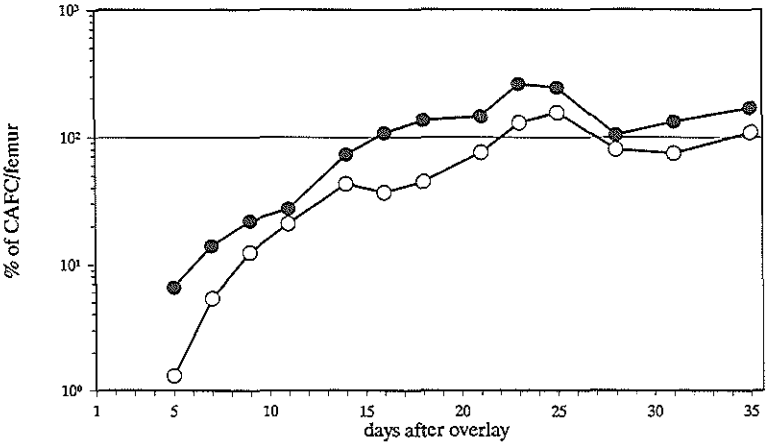

Figure 5.5. Femoral content of CAFC of mice, infected with 75 mg/kg BW (●), or 150 mg/kg BW (○) of 5-FU, 3 days before harvest of the bone marrow (arithmetic means of 3 experiments).

We have measured the CAFC frequency in the bone marrow of mice injected 3 days previously with either 150 or 75 mg 5-FU/kg body weight (BW). Figure 5.5 shows that

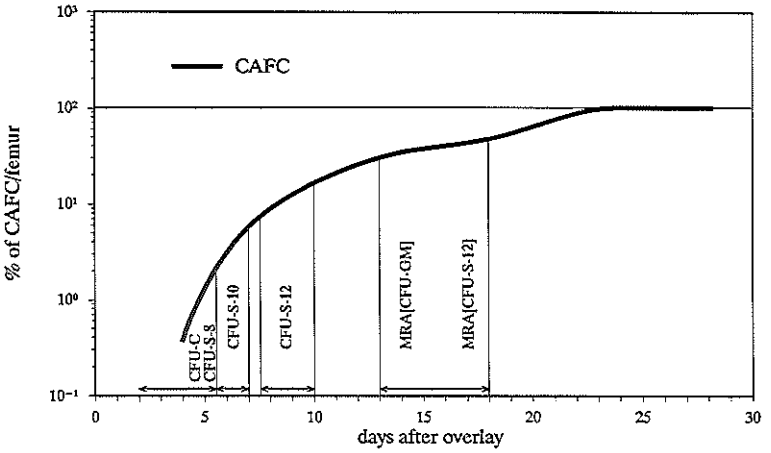

Figure 5.6. Femoral content of diverse progenitor cells of mice, treated with 150 mg/kg BW of 5-FU. Arrows indicate the ranges of depletion of the progenitor cell pools.

treatment with 150 mg 5-FU depleted the femoral content of early appearing CAFC (CAFC-5 to CAFC-9), but increasingly spared the progenitors of later appearing CA. Progenitor cells responsible for CA formation after day-21 were not reduced by the high dose 5-FU, indicating their essentially noncycling status. As compared with control mice, femora of mice injected with 75 mg/kg BW three days before contained even more progenitors that formed CA after 3 weeks of culture. It appears, therefore, that the cohort of CAFC differing in time-dependent CA formation reflects the basis of turnover rate and ability to generate new stem cells. Figure 5.6 comprises the effect of 150 mg 5-FU/kg BW on the femoral content of CAFC, CFU-C, CFU-S-8, and CFU-S-12, and MRA cells. It can be seen that both CFU-C and CFU-S-8 are depleted to less than 1% following this 5-FU protocol, whereas only approximately 4% to 7% of CFU-S-12 and 30% to 60% of MRA cells survive. These data confirm that frequencies of early appearing CAFC reflect those of CFU-C and CFU-S, whereas late appearing CAFC frequencies relate to those of MRA cells. The observation that the CAFC-28 represents a hematopoietic progenitor cell type which is not affected by 5-FU treatment suggests that this progenitor is the most primitive subset able to repopulate the irradiated bone marrow.

Half-life of CA. During the first days after overlay of BMC on the stroma, developing CA outnumber disappearing hematopoietic clones, leading to a maximum CA frequency. Hereafter, a process of clonal extinction can be observed in time that is characteristic for the cell type studied. Table 5.5 shows the half-life for CA observed in cultures of $Rh123^{bright}$ and $Rh123^{dull}$ BMC, 5-FU-resistant BMC, and normal BMC during this extinction process. Short half-lives for CA evoked by $Rh123^{bright}$ cells, and long values for $Rh123^{dull}$ BMC, and BMC from mice 3 days after 5-FU injection, are evident.

Table 5.5 Average half-life of CA in microcultures of various BMC fractions

cell origin	number of experiments	half-life of CA (days) (1 SD)	observation period (days)
unfractionated BMC	7	3.6 (0.1)	7 - 28
CCE/Rh123^{bright*}	3	1.7 (0.1)	7 - 28
CCE/Rh123^{dull*}	3	6.0 (0.4)	9 - 28
Lin$^-$/Rh123^{bright*}	3	1.5 (0.2)	7 - 28
Lin$^-$/Rh123^{dull*}	3	17.2 (0.6)	9 - 28
5-FU$_{3d}$ (150 mg/kg)	3	5.0 (0.1)	9 - 32
5-FU$_{3d}$ (75 mg/kg)	3	4.0 (0.2)	9 - 32

* sorted in the light scatter blast cell window from elutriated (CCE) or magnetic bead-treated (Lin$^-$) BMC.

Discussion

This study describes an *in vitro* limiting dilution assay for estimating the frequency of a series of hematopoietic stem cells that give rise to time-dependent clonal amplification under the stromal layer in LTBMC. We propose the term cobblestone area-forming cells, or CAFC, for these primitive cells. An important implication of the presented data is that the time stem cells form CA directly relates to (1) their turnover time as indicated by their resistance to 5-FU[16], (2) their ability to generate secondary CFU-S and CFU-C both *in vitro* and *in vivo*, and (3) inversely relates to their mitochondrial activity. This concept is compatible with a previously proposed model considering bone marrow functionally organized as a concatenated series of stem cell compartments in which turnover time decreases as maturity increases[16,47]. Time course studies of colony formation by stem cells proliferating either *in vivo*[25] or *in vitro*[55] have revealed a positive correlation between the onset of growth and extent of self-renewal subsequently observed. We propose, therefore, that the frequency of the various subsets belonging to this stem cell hierarchy can be quantified by scoring CAFC frequencies as a function of time after overlay on stromal layers *in vitro*.

The present report introduces a frequency analysis assay *in vitro* of stem cells associated with marrow repopulating ability. MRA cells have been shown to have a high capacity for the generation of secondary CFU-S-12, of cells with radioprotective ability[41], and CFU-C both *in vitro* (Chapter 4) and *in vivo*[15,36]. As demonstrated in Table 5.1, colony formation of Rh123^{dull} cells in semisolid medium was poor, and contrasted with their ability for long-term repopulation of irradiated mice and stroma layers *in vitro*. It should be noted that Rh123^{dull} cells did only give rise to small colonies, even in the presence of

rIL-6 and IL-3 (contained in PWM-MSCM). IL-6 and IL-3 have been described to act synergistically to hasten the appearance of multilineage blast cell colonies[37]. Up to the present the MRA of a cell suspension could only be determined *in vivo*, but frequency analyses were impracticable due to the large number of animals required for the limiting dilution technique. In addition to the use of this micro-LTBMC for MRA cell frequency analysis, Table 5.4 and Figure 5.4 clearly show that a good indication of the CFU-S-12 frequency can be obtained from limiting dilution analysis of CAFC-7. The extinction of the majority of normal BMC-derived clones (see Table 5.5) in our miniaturized LTBMC assay is fully compatible with the observation that the total life of individual clones in flask cultures is between 3 and 15 days[45]. The rapid decay of hematopoietic clones generated by $Rh123^{bright}$ cells also firmly supports our earlier observation[37] that such cells form a majority of transient erythrocytic spleen nodules in an irradiated recipient. Although scoring of CA as an endpoint in the presently described limiting dilution analysis is time-consuming, it presents as an unambiguous measure of proliferative activity. It is superior to the scoring of hematopoietic cell clusters on top of the layer, because such cells are more mature and may have lost their proliferative activity days before the time of observation. It has been consistently observed that the actively proliferating and more primitive hematopoietic cells are preferentially located within the adherent layer, whereas with increasing maturity the cells migrate to the surface of the layer and into the culture medium[60,64]. Moreover, CFU-S in the adherent layer have been reported to have a significantly higher self-renewal than have the non-adherent CFU-S[27]. In support of these data, we have observed replatable CFU-C only in wells containing CA, and never in CA-negative wells that contained hematopoietic cells adherent to the surface of the stromal layer. Remarkably, in our micro-LTBMC all cells and CFU-C were associated with the adherent layer. This contrasts with ample reports on the shed of CFU-C to the medium in flask cultures, and with an earlier study on the culture of human BMC in microtiter wells[30]. Our observations, therefore, indicate that the measurement of CFU-C associated with the adherent layer gives information of the total well production of CFU-C.

The miniaturized LTBMC technique used here differs from previous studies with respect to the eradication of endogenous hematopoietic activity. Micro-LTBMC have been reported earlier using 1.67 cm^2 surface area wells[24,61], but the use of 96-wells plates has only once been reported for murine LTBMC[30]. To ensure optimal survival and clonal amplification of any progenitor cell inoculated, a pre-existing and non-hematopoietic stromal layer is required. This certainly does apply to fractionated BMC, in which the essential stromal cells are selectively depleted, but also to anti-neoplastic agents-treated BMC which might be inhibited in the establishment of the stroma, leading to loss of clonogenic hematopoietic cells. In our hands, eradication of endogenous hematopoiesis by radiation of the established layer in the flasks (Chapter 4)[3,9,52] was highly reproducible. In contrast, we experienced either loss of stromal support for

hematopoiesis or recurrent hematopoietic activity by lowering the horse serum and hydrocortisone concentrations in the culture media to free stromal layers of hematopoiesis, according to the method of Reincke *et al.*[45].

It has been reported that 5-FU treatment of mice *in vivo* leads to dramatic reduction of the number of erythroblasts, CFU-C, CFU-S-7, and CFU-S-13, but far less of MRA, in the bone marrow[15,16]. Similarly, it appears from Figure 5.4 that the frequencies of cells forming CA between day-5 and day-21 after overlay are decreasingly reduced in the marrow of 5-FU (150 mg/kg BW) treated mice, as compared with untreated mice. The micro-LTBMC assay using the limiting dilution strategy, therefore, seems to offer an insight into the frequency distribution of the total stem cell hierarchy, ranging from the most mature progenitor cells as measured by the CAFC-3 frequencies to the most primitive hematopoietic stem cells (i.e., MRA cells), detected by CA formation after day-21. By virtue of their ability to generate new stem cells, the cells with MRA have long-term repopulating ability *in vivo*, and are of particular relevance to successful marrow transplantation. It appears from our study that the frequency of such cells in normal bone marrow, as determined by the presence of CA at day-28 or later, averages 6 per 10^5 fresh BMC (see Table 5.3), or less. This is similar to the number of primitive stem cells in normal bone marrow estimated by limiting dilution calculating using (temporary) cure of the anemic W/W^v mice as an endpoint[2], and agrees reasonably well with the frequency of cells producing bone marrow clones in previously irradiated and subsequently repopulated mice[31].

A one-phase micro-LTBMC assay has recently been described for human cells[29]. Provided sufficiently large human marrow biopsies, a two-phase assay can be developed using pre-established stromal layers. There exists no assay that quantifies human hematopoietic progenitor cells equivalent to the murine stem cell assays for CFU-S and MRA. Since the quantification of CFU-C or CFU-Mix in a bone marrow transplant does not consistently relate to the extent and success of bone marrow transplantation (BMT)[64], it is evident that a human assay for the enumeration of the frequency of long-term marrow repopulating cells would be a unique tool in the qualification of an *in vitro* purged graft for autologous BMT purposes. It would also add a new dimension to the delineation of the defective stem cell pool in aplastic anemia or leukemia, and to the quantification of hematopoietic toxicity accompanying cytoreductive therapy and BMT.

5.2 The use of limiting dilution-type long-term bone marrow culture in frequency analysis of marrow repopulating and spleen colony forming hematopoietic stem cells in the mouse

Introduction

In recent years the concept of the HSC has dramatically changed. The established assumption of a single homogeneous stem cell group, the members of which would be assayable in well-defined *in vitro* and *in vivo* assays, has been complicated by a series of reports on extensive heterogeneity in the progenitor cell compartment. The population of HSC probably represents a hierarchy of more or less primitive cells on the basis of pluripotentiality, and proliferative potential, and turnover rate[36,47,65]. Variation in length of interval required for their clonal expansion has been recognized as a further aspect of stem cell heterogeneity (Chapter 4, Paragraph 5.1)[14,19,25,48]. The accessibility of the murine system has permitted intensive studies aimed at the improvement of bone marrow transplantation and at the treatment of immunological and hematological diseases. HSC subsets have been characterized that can be identified by their colony formation *in vitro* or *in vivo*, allow survival of lethally irradiated mice, or give long-term engraftment in a depleted hematopoietic system. In this respect, it has become gradually apparent that both in man and mouse the conventional colony assays are not predictive of the MRA or LTRA of a graft[12,13,20,28,49,62,66]. In the past few years it has even been shown that the majority of murine CFU-S could be physically separated from the more primitive stem cells which repopulate the bone marrow, thymus, or spleen, and from cells providing radioprotection[1,33,34,38,39,62]. In contrast to CFU-S, cells with MRA are largely associated with resting cells as inferred by their resistance to the cytostatic effect of 5-FU[15], bromodeoxyuridine[16], or hydroxyurea[47], and by their low mitochondrial mass as suggested by the low retention of Rh123[1]. These MRA cells, but not the majority of CFU-S-12[52], have been shown to be associated with cells initiating long-term engraftment of an irradiated stromal layer in flask cultures (Chapter 4). In the previous paragraph we described a miniaturized long-term bone marrow culture, allowing the frequency analysis of cells with LTRA *in vitro* via limiting dilution analysis (Paragraph 5.1). A similar technique has been developed recently for human marrow[56]. However, it has yet to be demonstrated that these *in vitro* limiting dilution assays in mice and men can be employed for quantitative measurement of primitive MRA and LTRA *in vivo*. In order to carry out a statistical evaluation of the validity of this *in vitro* stem cell assay, we have physically sorted murine bone marrow cells with the aim to prepare cell suspensions in which the various *in vivo* defined HSC subsets occurred in different frequencies. These cell populations were simultaneously tested in the CAFC assay and *in vivo*, and frequencies of the different HSC subsets were compared using linear regression analysis. The absence of human analogues for the *in vivo* stem cell assays

prohibits a similar correlation study in man.

Materials and methods

Mice. Male (CBA × C57Bl)F_1 mice, 12-30 weeks old, were either bred in the Central Animal Department of the Erasmus University Rotterdam, or purchased from the Medical Biological Laboratory (Rijswijk, The Netherlands), and maintained under clean conventional conditions. The drinking water was acidified to pH 2.8. In specific experiments mice were injected with 150 mg 5-FU in phosphate buffered saline (PBS)/kg BW in a lateral tail vein. Three or six days later they were killed and single cell suspensions prepared from their femora and tibiae ($5\text{-}FU_{3d}$ and $5\text{-}FU_{6d}$, respectively).

Sorting of bone marrow cells. Bone marrow cells were prepared by cleaning femora and tibiae from muscles and tendons and grinding them in a mortar using PBS. The cell suspensions were sieved over a nylon filter (mesh size 100 μm and 30 μm). In order to prepare cells that differed in their relative frequencies (either enrichment or depletion) of CFU-S-7/8, CFU-S-12, and MRA cells[36], we used fluorescence-activated cell sorting (FACS) following a pre-enrichment step that included either immunomagnetic bead-mediated negative selection of BMC lacking the expression of certain lineage-specific epitopes[51], or buoyant density centrifugation. The immunomagnetic bead-mediated selection is described in Paragraph 5.1. For buyoant density centrifugation a modification of a discontinuous Ficoll-400 (Pharmacia) gradient designed to isolate thymocyte and bone marrow subpopulations[8,35] was used. The gradient was prepared at 4°C in 10 mL polyallomer centrifuge tubes (DuPont) with 3.6 mL of a bottom layer (1.078 g/mL), 2.4 mL of 1.069 g/mL, and 1.2 mL of 1.065 g/mL, on top of which 1 mL of a BMC suspension was layered containing $2.5\text{-}3.0\times10^8$ BMC/mL. The gradients were spun at 23,500 g for 30 minutes at 4°C using a Sorvall centrifuge (DuPont), mounted with a HB-04 fixed-angle rotor. Cells from the interface 1.069-1.078 g/mL were then collected, washed twice in PBS and 5% FBS, and properly diluted. Previous to sorting, the cells were either incubated with 0.1 μg/mL Rh123 for 30 minutes at 37°C, or with 0.5 μg/mL fluorescinated wheat germ agglutinin (WGA-FITC; Polysciences), as previously described[36,38]. Analysis and sorting of the cells was performed by the FACS II using a single argon laser at 488 nm. FITC and Rh123 fluorescence were measured using a 510-515 LP filter. After sorting the WGA-labeled cell suspensions were added to equal volumes of 0.4M of the competitive sugar *N*-acetyl-D-glucosamine (Polysciences) in distilled water. The cells were not washed before injection in order to prevent loss of cells.

Colony assays. The CFU-S-7 and CFU-S-12 content of cell suspensions was determined

by injecting the appropriate dilutions into a lateral tail vein of lethally irradiated mice. Two opposing ^{137}Cs sources were used to irradiate the recipient mice at a dose rate of 1.05-1.15 Gy/min with a total dose of 9.3-10.5 Gy. Control irradiated mice that did not receive cells were included for all observation days in each experiment. No endogeneous spleen colonies were found in the latter mice. Seven and 12 days later their spleens were excised, fixed in Telleyesniczky's solution, and the macroscopic surface colonies counted.

Quantification of CFU-C was performed using the semisolid culture technique described in Paragraph 5.1. The cultures contained 10% PWM-MSCM as the source of hematopoietic growth factors, and 20% HS. Colonies were counted on day-7 of culture with an inverted microscope.

Marrow repopulating ability (MRA). The MRA describes the ability of a cell suspension to generate new CFU-C or CFU-S-12 in the bone marrow of a lethally irradiated recipient mouse over a period of 12-13 days[36]. This period is determined by the survival of fatally irradiated mice that have not been grafted with BMC. Since we required a survival of at least 4 of 5 mice per group, the regeneration period was set to 12 days in this study. To measure MRA 12 days after injection of sorted or unsorted BMC into 5 lethally irradiated mice per group, different aliquots of their femoral marrow content were assayed for the presence of CFU-C. MRA was expressed as the number of CFU-C or nucleated cells per femur equivalent per 10^5 BMC injected. Control irradiated mice were included in each experiment and their endogeneous CFU-C (1-200 per femur) and nucleated cell number ($2\text{-}4\times10^5$ per femur) on day-12 was used to correct experimental data.

Long-term repopulating ability (LTRA). LTRA of day-28 CA was estimated using a sex-mismatched syngeneic chimeric model. The percentage of donor (male) type contribution to the peripheral blood leukocytes at 4 months post-transplantation into sublethally (8.25 Gy) γ-irradiated syngeneic female hosts was measured with a fluorescent *in situ* hybridization technique using an Y-chromosome-specific probe[62]. This technique is described in detail in Chapter 6.

Micro-LTBMC technique and replating of CA. The CAFC assay was performed as described in Paragraph 5.1. Unfractionated BMC from normal mice, or from 5-FU treated mice at 3 days after injection, were overlaid in 5 parallel series on irradiated stromal layers in microtiter plates in 6 concentrations, starting with 81,000 cells per well down to 333 cells per well, the concentrations being 3-fold apart and using 20 wells per dilution. Cultures were fed weekly by a complete medium change, and CAFC frequencies were determined over a period of 4 weeks. On day-7, -14, -21, and -28, one of the parallel series was used for replating studies. The culture medium was removed

from the wells and the adherent layers trypsinized by adding 0.05 mL of 0.5% trypsin for 1-3 minutes at room temperature. The digestion process was stopped by adding 0.1 mL of ice-cold FBS. The cells from 20 wells for each concentration were pooled, and single-cell suspensions made by repeated passage through an 23-gauge needle. Cells were then centrifuged, resuspended in 5 mL of α-medium, and aliquots of the well contents were plated in the CFU-C assay. Recombinant human erythropoietin (2 U/mL; CILAG) was added as additional growth factor, and the number of colonies was determined on day-7. In some cases single colonies were picked on day-11, cytospinned and stained with May-Grünwald/Giemsa to allow the determination of differentiation lineages in each colony with light microscopy.

Results

Cobblestone-area formation by various BMC populations. The kinetics of CA formation could be varied dramatically by sorting BMC on the basis of different criteria as previously shown (Paragraph 5.1)[62]. This is exemplified in Figure 5.7, that contains data from an experiment in which the light density fraction of BMC from 5-FU treated donor mice were subjected to FACS on the basis of Rh123 retention. The cells were sorted using forward and perpendicular light scatter thresholds that excluded most of the remaining granulocytes and part of the lymphocytes (blast window)[36]. It appears that 5-FU_{6d} bone marrow was far more enriched for CAFC-28 (32×) than for CAFC-6 (1.5×) as compared to their frequencies in normal BMC. The light density fraction also showed an increasing enrichment for CA-formation with increasing culture time. In these light

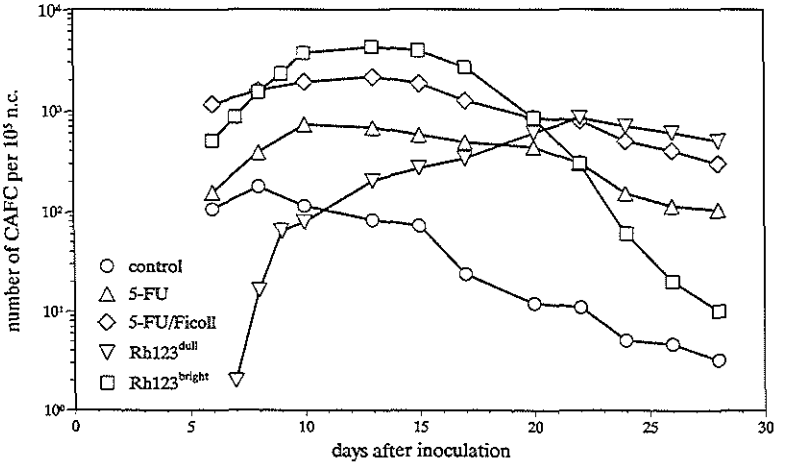

Figure 5.7. CAFC frequencies of fresh and enriched BMC, using 5-FU_{6d} bone marrow. Data are from a representative experiment.

density fractions the frequency of CAFC-6 was 10× higher than in unfractionated marrow, while CAFC-28 were even 93× enriched over control BMC. Sorting out 10% of the most $Rh123^{dull}$ cells of this low density preparation of $5\text{-}FU_{3d}$ BMC in the "blast window" (this window contained 30% of all nucleated cells sorted) gave a large depletion of CAFC-5 (less than 1% of control BMC), however, CAFC-28 were further enriched (154× over control BMC). This sorting protocol thus led to a CAFC-28/CAFC-6 enrichment ratio that exceeded 15,000 in one cell suspension. In a less extreme way, 10% of the most $Rh123^{bright}$ cells in the lightscatter blast window were 113-fold enriched for CAFC-17, while containing far lower numbers of progenitors forming CA on day-28 (3-fold enrichment). These observations clearly indicate that early and late CAFC differ with respect to their sensitivity for the cytostatic agent 5-FU, and their affinity for Rh123. It is evident that the low Rh123 retention and a relative insensitivity for 5-FU of CAFC-28 are features shared with MRA cells *in vivo*, while opposite properties characterize the CFU-S-8 and CFU-S-12[15,36].

Correlation studies of *in vivo* and *in vitro* defined stem cell subsets. In a series of similar sorting experiments, data were collected that allowed the comparison of *in vivo* stem cell assays (CFU-S and MRA) with the *in vitro* CAFC assay. The methods used for physical separation of the BMC have been described in Paragraph 5.1.

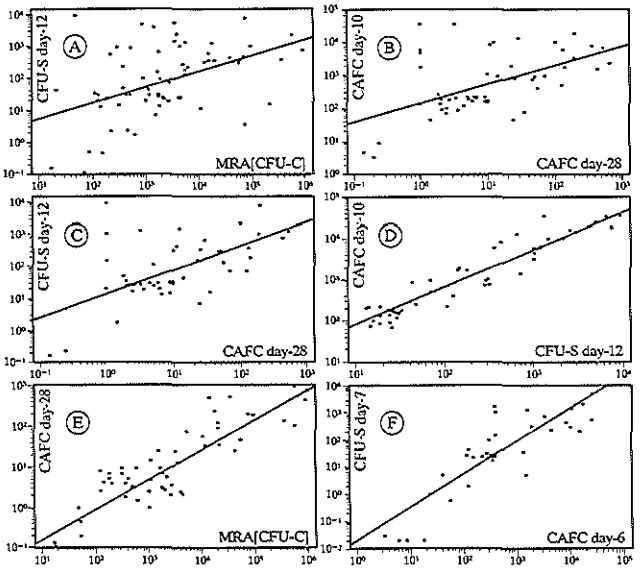

Figure 5.8. Linear regression analysis for the correlation between CAFC and in vivo stem cell parameters.

Figure 5.8[a] confirms and extends earlier observations on a lack of correlation between the incidences of CFU-S-12 and MRA[CFU-C] activity in a linear regression analysis

(coefficient of determination r^2=0.236; n=69), indicating that colony formation in the spleen and generation of new stem cells in the bone marrow are properties of physically largely separable stem cell subsets. It is evident that neither the frequencies of CAFC-10 (Figure 5.8[b]; r^2=0.334; n=53) nor those of CFU-S-12 (Figure 5.8[c]; r^2=0.387; n=51) show good correlation with the CAFC-28 incidence, and that the average early and late appearing CA significantly differ in a series of physical parameters. Comparison of the frequencies of all CAFC-types with those of CFU-S-7 and CFU-S-12 and the activity of MRA cells indicated three significant correlations. First, a high coefficient of determination (r^2=0.924; n=55) in a linear regression analysis was observed between frequencies of CFU-S-12 and CAFC-10 (Figure 5.8[d]). When assaying low numbers of these stem cells (lower left hand side of Figure 5.8[d]), 6.7 CAFC-10 were measured on a single CFU-S-12, indicating that the *in vivo* "seeding efficiency" of CFU-S-12 would be about 15% as approximated by this limiting dilution assay *in vitro*. In the BMC fractions that were highly enriched for these stem cell types 4.7 CAFC-10 was scored on any CFU-S-12, representing an *in vivo* seeding efficiency for CFU-S-12 of about 21%.
A second remarkable high coefficient of determination (r^2=0.788; n=53) was observed for the number of CFU-C generated in the marrow by stem cells with MRA

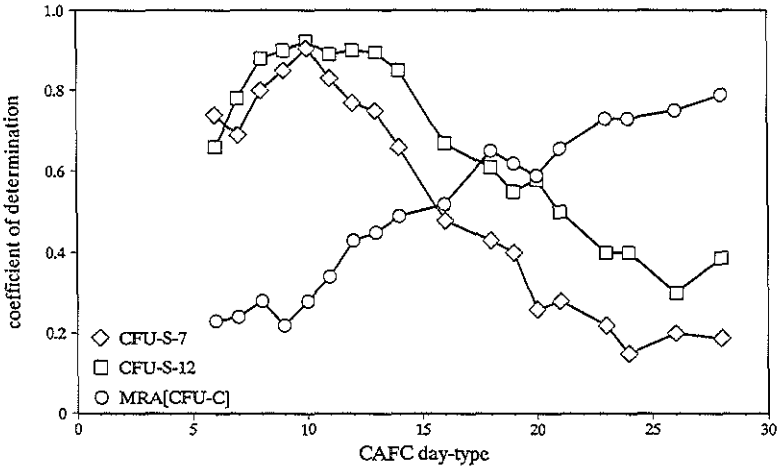

Figure 5.9. Compilation of coefficients of determination (r^2) of CAFC values and values of CFU-S-7, CFU-S-12, and MRA[CFU-C].

(MRA[CFU-C]), and the frequency of CAFC-28 in a variety of cell suspensions tested (Figure 5.8[e]). When the CAFC-28 frequency was low, the data indicate that 0.18 CAFC-28 equalled 10 CFU-C produced in one femur in a period of 12 days, i.e., in this period the presence of 1 CAFC-28 compared to a production of about 1,300 secondary CFU-C in the total marrow space of an irradiated mouse, accepting that one femur represents 4.5% of the whole marrow mass in these rodents. In difference with this was

the calculated potency of highly enriched CAFC-28 (right hand side of the fitted curve in Figure 5.8[c]) where 1 CAFC-28 equalled the generation of about 21,000 CFU-C in the total marrow in a 12-day period, whereas in unsorted marrow 1 CAFC-28 was observed to correspond with 500-4,000 newly formed CFU-C. This lack of congruency in Figure 5.8[c], in combination with good linear fit, may have been caused by the circumstance that a clonal assay (CAFC) is compared with the ability of a cell suspension to generate new CFU-C, irrespectively of the number of progenitors contributing to such a progenitor cell production. Apparently, the enrichment protocols used for MRA cells selected for a relative small population of potent CFU-C producers while excluding a majority of less capable MRA[CFU-C].

A third linear correlation (r^2=0.743; n=36) was observed between the numbers of CAFC-6 and those of CFU-S-7 in the cell suspensions (Figure 5.8[f]). However, the limited number of data do not allow conclusive statistics on the validity of the CAFC assay as replacement for the *in vivo* CFU-S-7 assay.

A full summary of the correlation coefficients calculated by linear regression analysis of the CAFC day-5/28 frequencies and those of CFU-S-7, CFU-S-12 and MRA activity is presented in Figure 5.9. The data indicate that the best approximation of actual CFU-S-12 frequencies is done by counting CA between day-8 and day-13 of culture. In addition, in the same cultures the frequency of CAFC-28 showed a best linear fit with the *in vivo* activity of MRA cells. It appears furthermore, that the CAFC assay is not fit to measure the CFU-S-7 population as accurately as it does CFU-S-12.

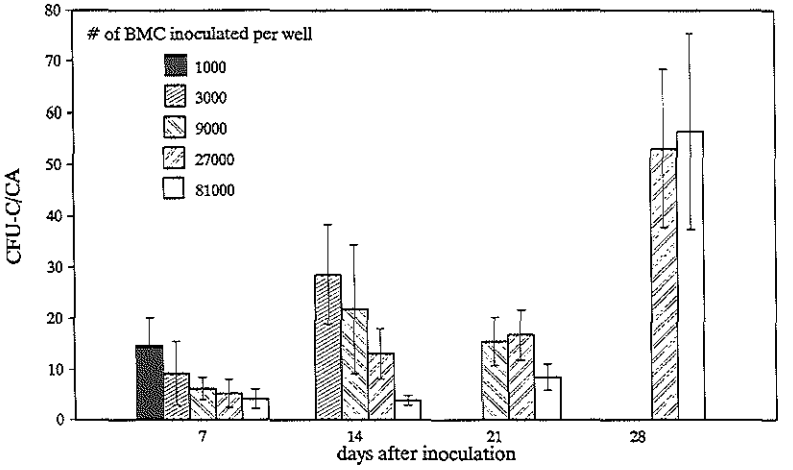

Figure 5.10. Effect of inoculum size (1-81×10^3 of fresh BMC) on the CFU-C content of CA, as determined in the first 4 weeks of micro-LTBMC.

Presence of CFU-C in cobblestone areas. In order to investigate whether the potency of CAFC subtypes to generate secondary progenitor cells differed with the length of the

interval required for their clonal expression, micro-LTBMC cultures of different ages were investigated for their content of CFU-C. Since we have previously shown that the

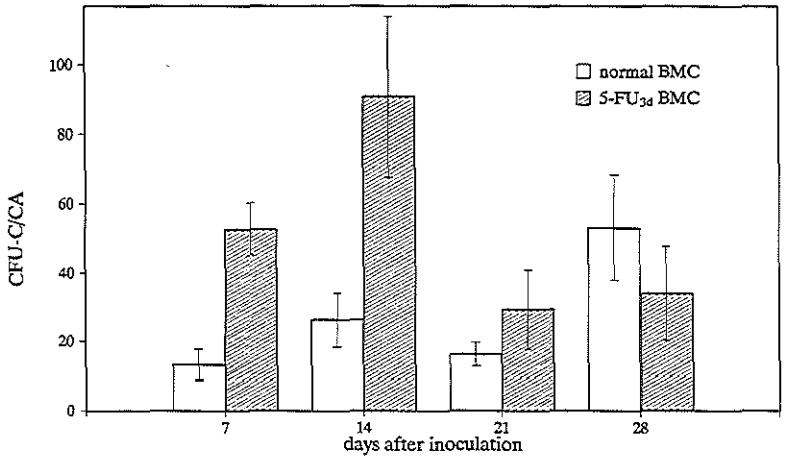

Figure 5.11. CFU-C content of CA developing in micro-LTBMC from normal and 5-FU_{3d}) bone marrow.

presence of replatable CFU-C in micro-LTBMC is strictly associated with the presence of CA, the CFU-C content of a CAFC was estimated by dividing the number of replatable CFU-C per well by the number of CAFC per well as determined by limiting dilution analysis in the same cultures. From Figure 5.10 it appears that increasing numbers of normal BMC inoculated per well consistently decreased the number of secondary CFU-C detected per cobblestone area. Since large variability in replating data was observed when less than an average of 1 CA was detected per well, such data were excluded from this study. For these reasons, only data from series containing between 1 and 10 CA per well were included in the comparative replating study presented in Figure 5.11. It is evident that normal bone marrow-derived CA present between week 1 and 3 only contained 10-25 CFU-C, while 50 CFU-C were detected in the few CA present on week 4. These data suggest that the average CAFC-28 has a higher ability than earlier detectable CAFC to produce *in vitro* clonable progenitors. Except for week 4, CAFC in bone marrow from 5-FU treated mice contained consistently more CFU-C than CAFC in control marrow, suggesting that 5-FU is selectively toxic for less potent CAFC.

In normal bone marrow, CFU-C replated from day-28 CA formed a higher percentage of mixed colonies (GEM, GEMM) than CFU-C from day-7 CA (Table 5.6). In bone marrow from 5-FU treated mice both CAFC-7 and CAFC-28 generated a relatively high percentage of CFU-Mix. These data again support the assumption that CAFC-28 in normal marrow, and most CAFC in 5-FU marrow, are more primitive than CAFC giving

Table 5.6 **Differential capacity of CFU-C replated from CA on day-7 or day-28 of culture**

source of BMC	day of replating	% M/GM colonies	% GEM/GEMM colonies	number of colonies
control	7	95	5	200
5-FU	7	78	22	156
control	28	83	17	276
5-FU	28	74	26	188

Data represent percentages of CFU-C-derived colonies containing combinations of granulocyte (G), monocyte/macrophage (M), erythrocytic (E) or megakaryocytic (M) differentiation lineages.

rise to early CA formation in normal marrow.

Presence of stem cells in day-28 cobblestone areas. From preliminary experiments it was clear that the content of *in vivo* LTRA cells, CFU-S-12, and CAFC-10/28 in single day-28 CA varies widely. In order to obtain a representative estimate of the content of the more primitive stem cells in day-28 CA, each well in fourty 96-well plates, containing irradiated marrow-derived stroma, was seeded with 3000 normal BMC, and the medium was changed weekly. Nine percent of the wells contained CA at day-28, indicating that the probability of finding a single clone per CA-containing well was higher than 95%, according to Poisson statistics. Hundred CA-containing wells were than pooled and aliquots equalling either 1, 2, or 4 CA were injected into lethally irradiated recipient mice, and macroscopic spleen nodules were counted on day-12. Part of the pooled CA-containing wells was overlaid in the micro-LTBMC assay (8 dilutions of 15 wells each, two-fold apart, starting with an aliquot of 4 CA-containing wells per well). These experiments indicated that an average day-28 CA contained 2.2 CFU-S-12, 15.6 CAFC-10, and 0.29 CAFC-28. In similar tests using identical numbers of empty wells we did not detect replatable CFU-S-12 or CAFC.
In vivo LTRA was estimated by pooling 200 CA-containing wells on day-28 and injecting 1/5 of this suspension intravenously into each of 5 syngeneic female mice per group that had been conditioned with a sublethal irradiation dose of 8.25 Gy. Four months later the male donor cells constituted 0, 2, 9, 15, and 16 percent, respectively, of all nucleated cells in the peripheral blood, i.e., 3 out of 5 mice showed a substantial donor-type signal. We did not find donor-type repopulation in female mice that received an equivalent of 40 empty wells on day-28.

Discussion

In order to obtain statistical support for our earlier suggestion, mentioned in the previous paragraph, that the time-dependent CA formation in stroma-dependent liquid cultures reflects the turnover time and primitiveness of CAFC, we have done a comprehensive study on the qualitative and quantitative relation between *in vivo* and *in vitro* stem cell assays. This study was facilitated by the application of purification protocols that give extreme variations in the relative frequencies of the various HSC subsets. The combined use of 5-FU and FACS on the basis of light scatter, affinity for WGA and Rh123 indeed allowed the acquisition of subset ratios exceeding several hundred-fold (Paragraph 5.1)[18,62].

The data present evidence that the CAFC-10 assay accurately measures CFU-S-12, irrespectively of the presence or absence of cells with MRA activity. The regression analysis also showed that 4.7-6.7 CAFC-10 are detected on every CFU-S-12, suggesting an *in vivo* spleen seeding efficiency for CFU-S-12 of 15-20%. This high figure can be explained by assuming that CFU-S-12 have either a higher seeding in the irradiated spleen than the 5-12%, as previously established for CFU-S-7[7,21,32,59] and CFU-S-12[40] using *in vivo* retransplantation studies, or the cloning efficiency of the CAFC assay was lower than 100%.

A fairly good linear fit was observed for the CAFC-28 and the MRA of the cell suspensions. The suggestion of Reincke and colleagues[46], that murine stem cells giving rise to day-28 clones in LTBMC are indicative of CFU-S activity, is thus not supported by our data. It should be noted that the present data have been plotted in an absolute way, which leads to significantly lower correlation coefficients than observed when comparing enrichments for these HSC subsets in single experiments. Although the linear regression analysis showed excellent fit, the ratio of CFU-C generated per femur and the frequency of CAFC-28 increased with increasing presence of these cells in the cell suspensions tested, leading to a deviation of the congruency line. In order to comprehend this it is recalled that the MRA assay is a non-clonal *in vivo* regeneration assay, and that a selection for more potent MRA cells using the protocols applied may thus give higher enrichments for *in vivo* activity than for clonogenic capacity in the CAFC test. This notion is supported by our observation that *in vitro* the number of CFU-C produced per CAFC may vary considerably with the protocol used to obtain the cells (Figure 5.11).

In conjuction with the regression data, the primitive characteristics of CAFC-28 is also apparent from the observation (Figure 5.11, Table 5.6) that these stem cell subsets produce more CFU-C than cells forming early detectable CA, and that relatively more CA-derived CFU-C colonies containing 3 or more differentiation lineages. The absolute frequency of CAFC-28, i.e., 1-4 per 10^5 cells in normal marrow, is in line with earlier reports on the incidence of extensively proliferating, or long-term repopulating HSC in

mice[2,31] and humans[56], and demonstrates that the CFU-S and CFU-C assays significantly overestimate the incidence of stem cells with MRA and LTRA.

Although good linear fit is demonstrated between frequencies of hematopoietic clones *in vivo* and *in vitro* on particular days, and the clone-initiating cell may have quite primitive characteristics, our correlation data do not imply that a single colony (either in the spleen or in a culture well) consequently still contains primitive HSC after weeks of culture. Conditions for obtaining single clones in liquid LTBMC can only be created in a statistical manner, i.e., the percentage of positive wells should ideally be kept lower than 10 percent to give a higher than 95 percent probability that a positive well contains not more than 1 clone. Although preliminary experiments have shown that not every single clone includes secondary CFU-S and CAFC, pooled single clones at day-28 of culture appear to contain *in vivo* LTRA cells, CFU-S-12, CAFC-10, and CAFC-28, suggesting that at least part of CAFC-28 should be classified to the most primitive HSC subset identified today. Since CAFC-28 have gone through 3 to over 20 divisions in order to generate a day-28 CA, such individual colonies are unlikely to all contain detectable primitive stem cells.

The murine CAFC assay offers an attractive alternative for the costly and animal requiring *in vivo* HSC assays. Secondly, when the assay is read out during 4 weeks, it supplies the observer with a full cross section of HSC subset frequencies in a particular cell suspension, ranging from transient spleen colony-forming cells to the primitive MRA cells. This is of particular importance when quantitative information is required on the life-sparing capacity of a graft, which should contain both a sufficiently large number of rapidly proliferating CFU-S responsible for short term survival, and stem cell-generating MRA cells, that may have a delayed onset in proliferative activity and therefore have low radioprotective ability[38]. Such a delayed initiation of clonal expansion *in vitro* is characteristic of cell suspensions enriched in MRA, or depleted for CFU-S, as is the case in BMC from 5-FU treated mice and in $Rh123^{dull}$ BMC. Finally, the availability of the CAFC system should facilitate studies of molecular events associated with the regulation of proliferation and differentiation of single LTRA cells.

The present data encourage further study on the validity of a limiting dilution-type LTBMC, both in man and mouse, as a clonal assay for enumeration of cells, that determine the succes of bone marrow transplantation. In mice, such long-term engraftment studies using sex-mismatched donor-host combinations or polymorphism for Ly-5/Thy-1 are currently carried out in our laboratory.

5.3 Comments on the statistics of the *in vitro* limiting dilution assay for the quantification of primitive murine hematopoietic stem cells.

A limiting dilution assay (LDA) to quantify the number of hematopoietic stem cells in a sample of unsorted or sorted bone marrow cells is a major tool in describing the composition of stem cell subsets in a cell suspension *in vitro*. Statistical evaluation of data from experiments with a limiting dilution setup has been a subject of discussion, as has been the proper way of performing limiting dilution-type studies. Therefore, it is useful to give a brief description of the principles of limiting dilution analysis.

Identical samples of the same cell suspension do not contain the same number of cells, and thus vary in the number of stem cells. When such a suspension is properly made, the number of cells per sample are randomly and independently distributed. A sample can contain 1, 2, 3, ..., i cells. The probability with which a sample contains a certain number of cells is described by the Poisson probability distribution:

$$P_i = e^{-m} \cdot \frac{m^i}{i!} \qquad (1)$$

(P_i is the probability of the presence of a sample containing i cells)
(m is the mean number of cells per sample)
(i is the real number of cells per sample)

This equation applies for the number of hematopoietic stem cells in a given sample, where P_i is the probability of the presence of a sample containing i stem cells, m is the mean number of stem cells per sample, and i is the real number of stem cells per sample.

To determine the number of stem cells in a sample, we decided to use the presence of a cobblestone area (CA) within the stromal layer of a microculture as the (visually) perceptible entity of one stem cell.

In limiting dilution assays there are 4 possible models for the presence of a positive response[57]:

1. only 1 cell of only 1 cell type is necessary to obtain a positive response (single-hit).
2. a number of cells (≥2) of 1 cell type is necessary for a positive response (multi-hit).
3. only 1 cell of more than one cell type gives a positive response (multi-target).
4. combination of the multi-hit and multi-target models.

For the present, we assume our culture system follows single-hit kinetics. Later in this paragraph we shall show this a proper assumption.
Definitions for this single-hit model are:

1. the progenitor cells are diluted to limiting doses.
2. every progenitor cell generates a detectable response, in our case a CA.
3. every other circumstance present in the culture is non-limiting.

If we do not know the exact number of (responding) stem cells in a sample, we only can obtain information about the presence of one or more stem cells in a sample by observing the presence or absence of a response in a series of identical samples. If there is a response we call the sample positive, if there is not, the sample is called negative. With this we obtain a quantal dilution assay (i.e., yes/no responses), and are allowed to use the Poisson equation in the zero term:

$$p_0 = e^{-m} \cdot \frac{m^0}{0!} \tag{2}$$

$$p_0 = e^{-m} \tag{3}$$

(p_0 is the probability of the presence of a sample containing zero progenitor cells)

A logarithmic transformation of this equation is:

$$-\ln p_0 = m \tag{4}$$

For **one** dilution (i.e., a number of replicate wells containing i cells), the negative logarithm of the fraction of non-responding cultures ($\ln p_0$) is proportional to the mean number of stem cells per culture (m). We do, however, not observe p_0, but we observe the fraction of nonresponders of a series of replicate cultures. The fraction of negative responders can be described as:

$$p_0 = \frac{r_i}{n_i} \tag{5}$$

(r_i is the number of negative cultures of dilution i)
(n_i is the total number of cultures with i responder cells)

Therefore, m is an estimate of the true number of progenitors per culture[10].

The method that can be used to determine the frequency of responder cells from **more**

than one dilution in a limiting dilution assay has been subject of an extensive discussion. Taswell[57] briefly reviewed and compared four possible methods, namely least square fitting, weighed averaging, likelihood maximization and chi-square minimization. In his paper he concluded, among others, that Minimal Chi-Square (MC) is superior to the other methods, including Maximum Likelihood. This has been challenged by Fazekas de St.Groth[10], Smith *et al.*[50], Mantel[26], and Strijbosch *et al.*[54]. These authors concluded that, using MC, test group sizes have to be indefinitely large for proper estimation of the frequency, however numerous the number of dilutions. Maximum Likelihood estimates (ML) do not show this inconsistent behavior, and therefore ML is the method of choice in bioassays with necessarily limited test-group sizes. Strijbosch proposed the "jackknife" version of the Maximum Likelihood method as the statistical procedure of choice[53,54]. This method can, however, only be applied if all the dilution groups contain an equal number of replicate cultures. In long-term cultures like our *in vitro* assay for primitive hematopoietic stem cells, a risk of infection of individual wells is present, and thus an equal number of wells in all dilutions cannot be guaranteed during the entire culture period. Therefore, Maximum Likelihood is the alternative.

Performing linear regression analysis (least square fitting)[23] still is a widespread used method, because it is easy to do (see also Figure 5.2). Therefore it is discussed in this paragraph, together with ML estimation.

Using Least Square fitting (LS) one estimates the stem cell frequency observing the fractions of negative responding cultures in several series of dilutions. Every series of replicate cultures of 1 dilution generates a datapoint, since $m=-\ln p_0$. Several datapoints provide a straight line through the origin, best done by using LS fitting. This is represented graphically in a semi-logarithmic plot with on the y-axis the negative logarithm of the fraction of non-responding cultures (p_0) and on the x-axis the linear scale of cell input. This line allows the estimation of the frequency of progenitor cells by interpolating at the level of 37% non-responding cultures (note that $-\ln 0{,}37 = 1$). The size of the sample containing an average of 1 progenitor can thus be estimated. Furthermore, a straight line indicates the LDA fits in the single-hit model. Any deviation in linearity means the assay follows other than single-hit kinetics[22,23].
This method, however, has several drawbacks. In the linear regression graph the cell input has been given as an independent variable, i.e., free from error. Consequently, all variation is attributed to r/n, and the variation of the cell input is denied, which cannot be right[10]. In comparing this method with other methods, Taswell concluded the LS fitting to be inferior[57,58].

The Maximum Likelihood estimation (ML) is basically a maximization of the *binominal term*, applied to the zero term of the Poisson probability distribution. So we obtain for

the likelihood (l):

$$l=\frac{n!}{n!\,(n-r)\,!}\cdot(p_0)^r\cdot(1-p_0)^{n-r} \tag{6}$$

or:

$$l=\frac{n!}{n!\,(n-r)\,!}\cdot(e^{-m})^r\cdot(1-e^{-m})^{n-r} \tag{7}$$

Maximization (determining the maximum likelihood) is obtained by differentiating with respect to m, after logarithmic transformation (L is the logarithm of likelihood; M is the logarithm of m), and equating the differential to zero:

$$\frac{dL}{dM}=-r+(n-r)\cdot\frac{e^{-m}}{1-e^{-m}}=0 \tag{8}$$

Solving, we obtain:

$$m=-\ln\left(\frac{r}{n}\right) \tag{9}$$

For a single dilution, this is exactly the same as the zero-term of the Poisson equation, used as data points for linear regression in the LS method. When the number of stem cells has to be determined from a series of cell concentrations, the outcomes of the individual dilutions have to be combined. From this point LS and ML diverge. The ML solution does not give the same outcome as does fitting a linear regression.
To combine the outcomes of the various dilutions in determining the maximum likelihood of the progenitor cell frequency, we have:

$$L=\sum_{i=1}^{i=k}\left[\ln\left(\frac{n_i!}{r_i!\,(n_i-r_i)\,!}\right)-r_ic_if+(n_i-r_i)\cdot\ln(1-e^{-c_if})\right] \tag{10}$$

(L is the logarithm of the Maximum Likelihood)
(k is the number of dilutions)
(c_i is the concentration of cells for dilution i)
(n_i is the number of replicate cultures for dilution i)
(r_i is the number of negative cultures for dilution i)
(f is the frequency of stem cells)
($c_if = m_i$)

This equation can only be solved by an iterative procedure. First, a provisional value of m is generated by calculating the arithmetic mean of all values of m obtained from the

individual dilutions (m_0). Next, the first cycle of iteration provides an adjustment to the provisional m_0. The next cycles of iteration each gives an adjustment of m, that was obtained by the previous cycle. This procedure continues, until the approvement of the last estimate of m is negligible.
After the last cycle the variance and the 95% confident limits are calculated. The procedures of iterations and the calculations of variance and confident limits are described in more detail by Taswell[57], Fazekas de St.Groth[10], and Strijbosch[53].
The variance and confidence limits tell us nothing about the compatibility of the observations with the hypothesis that our data behave within the single-hit Poisson model. Decisions concerning the validity of the assay have to be made, based on the results of test statistics[58], rather than on the shape of a line in a graph[23]. Therefore, we test for the goodness of fit of the estimate of m using a chi-square (χ^2-) test[10,57,58] for which we use the value of m of the last cycle of iterations. The χ^2-test detects the presence of every phenomenon, that is not in agreement with the single-hit Poisson model: multi-hit, multi-target, variable number of false negatives (dose-dependent) or constant number of false positives (dose-independent)[58].

The p-value, corresponding to the calculated value of χ^2 and the degrees of freedom, should not be less then 0,05 (p<0,05). If it does, the data do not fit in a single-hit Poisson model. In almost all our experiments, most data points fit in the single-hit Poisson model.

In order to present data and conclusions from limiting dilution assays in a convenient way, one has to report the *real* frequencies (not the reciprokes), in combination with the test statistics, the corresponding p-values, and the confident limits[58].

To get an idea of an unknown stem cell frequency, an experimental design with a large number of dilutions and a small number of replicate cultures is useful. If an accurate estimate is required, a small number of dilutions together with a large number of replicate cultures per dilution will be very helpful[53]. For the amount of dilutions and replicate cultures per dilution, Lefkovits recommended over 60 replicate cultures (optimal 120-180 cultures) per dilution, i.e., per experimental point[22]. That this would be unrealistic for many biological assays is recognized by Fazekas de St.Groth[10] and Strijbosch[54]. They stated, that it is suitable to use 20 replicate cultures per dilution, keeping the dilution in linear steps over a 10-20 fold range (Fazekas de St.Groth), or 24-72 replicate cultures per dilution (Strijbosch). The most information contained in a series of dilutions is obtained from individual dilutions with fractions of negative wells (p_0) between p_0=0.15 and p_0=0.70[10,53]. This range comprises levels of information above 90% of the maximum.

Applying this to our *in vitro* system for the detection of hematopoietic stem cells, we see that for normal bone marrow cells the highest frequencies are round 330 per 10^5 at day-7, and the lowest around 5 per 10^5 at day-28 of culture, the frequencies lying 66-fold apart. For sorted cells it can be far more (10^4-fold). In our experiments we normally use 15-20 wells per dilution. The range for the dilutions depends on the expected range of frequencies[54].

If one desires to combine data from a number of experiments, it is best to analyze all the data of all estimates as if it has been one large experiment[43]. Data from experiments which do not fit in the single-hit Poisson model have to be excluded from these calculations. However, our computer program cannot handle more than 10 dilutions in one time. Therefore, we calculate the arithmetic mean of the estimates of the individual experiments, presenting the outcome together with its standard error of the mean.

Epilogue

Recently, a paper was published that presented data on the limiting dilution analysis of long-term *in vitro* repopulating HSC[63]. This assay was based on pre-established bone marrow-derived stromal layers in 96-wells plates, on which HSC, once inoculated, could form clones that were denoted CFU-Dex. The assay supported the conclusions described in the Paragraphs 5.1 and 5.2, and in the corresponding papers, that the time of onset of clonal expansion and the life-time of a clone is a function of primitiveness of the clone-forming cell. Although the authors did excellent work in testing purified stem cell populations in their assay, several comments have to be made. These comments partially have been published[42].

We doubt the originality of the study, for it seems an almost exact copy of the study, described in this chapter. Their choice of an endpoint in their assay, CFU-Dex, is a progenitor cell that gives rise to discrete CA, which mature into colonies consisting primarily of maturing myeloid and erythroid cells. However, in addition to CA, maturing colonies without the presence of cobblestone cells are included in their criteria for a positive well. In Paragraph 5.1 we demonstrated that the latter colonies did not contain replatable CFU-C. Clones consisting of maturing (or mature) hematopoietic cells without a CA-cell present may last for weeks, without a sign of hematopoietic growth. The extinction process of a clone is delayed, resulting in a larger half-life of the clone. Including these clones for the estimation of CFU-Dex will overestimate the frequency of, mostly late appearing, hematopoietic progenitor cells. The scoring of CA is superior to scoring of hematopoietic clusters on top of the stroma, because the latter may have lost their proliferative activity days before the time of observation. The CFU-Dex frequencies are 1.8- to 4-fold higher than the CAFC frequencies after 1 week of culture

(see Table 5.7)[42]. This may be one reason for the large range of CFU-Dex frequencies per cell fraction these authors observed, which is in sharp contrast with our own observation, namely, a high reproducibility of the CAFC assay. Furthermore, in a table displaying the range of CFU-Dex frequency of several purified BMC fractions, results are shown of only one experiment, or no data were available due to the fact that not enough colonies were found to calculate the frequencies, especially of day-28 CFU-Dex.

Table 5.7 CAFC and CFU-Dex frequencies in fresh bone marrow

day	CAFC	CFU-Dex	ratio CFU-Dex/CAFC
7	214	235	1.1
14	53	117	2.2
21	12	22	1.8
28	3	7	2.3
41	0.17	0.68	4.0

Data are from several representative experiments from our own laboratory (CAFC) and from the paper of Weilbaecher *et al.* (1991) (CFU-Dex). Data contained in this table has been published previously (Ploemacher *et al.* (1992)).

Another serious drawback is the lack of statistical information in this paper. Only "Poisson statistics" is mentioned, whereas the number of dilutions, the number of wells per dilution and the method of estimation of CFU-Dex frequencies are omitted. I fear that only the zero-term of the Poisson formula has been used for a limited (only 1?) number of dilutions. This may be a second reason for the large range of CFU-Dex frequencies per cell fraction. In addition, these investigators only counted CFU-Dex frequencies on day-7, -14, -21, and -28. Doing this, one fails the accurate course of the frequency curve, which may be important, especially during day-5 to day-14.
In conclusion, we have found no evidence in the report of Weilbaecher and colleagues that suggests an advantage of the CFU-Dex assay over the CAFC assay.

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Chapter 6
Sex-mismatched bone marrow transplantation

Preface

In studies on the primitiveness of hematopoietic stem cells, transplantation experiments *in vivo* are of essential significance. Since the observation that cells from the transplanted bone marrow are responsible for the survival of lethally irradiated recipients, bone marrow transplantation (BMT) is widely used in experimental and clinical hematology. A comprehensive review of the essentials of BMT is given by Vriesendorp[45].
Clonal assessment of primitive hematopoietic stem cells in the mouse uses a transplantation model, in which a stem cell is determined with a relatively sharp definition: a cell that has the ability to form a macroscopic colony in the spleen at *n* days after transplantation into an irradiated host. The stem cells are termed day-7 CFU-S (colony forming unit in the spleen) if the macroscopic colony is visible 7 days after transplantation, or day-12 CFU-S if the colony is present after 12 days.
Recently, it appeared that these CFU-S are not the most primitive hematopoietic stem cells (HSC) (Chapter 2), and various assays have been developed to measure the more primitive pre-CFU-S activity. Two of these assays are discussed in Chapter 4. The erythroid repopulating ability (ERA) of a bone marrow graft measures the degree of reconstitution of the erythroid blood compartment 12 days after transplantation, by means of diverse erythroid parameters. Marrow repopulating ability (MRA) of a bone marrow transplant determines the number of nucleated cells, the number of CFU-C or CFU-S generated by the graft. Only MRA[CFU-C] and MRA[CFU-S] are reliable assays for pre-CFU-S function. The time span of these assays, however, is relatively short, namely 12-13 days plus the time needed to generate CFU-C or CFU-S. Also, studies on the survival of lethally irradiated mice transplanted with bone marrow cells (BMC) will suffer from measuring short-term repopulating stem cells, like CFU-S, that generate a rapid production of blood cells during the period the irradiated animal otherwise would have died from bleeding (shortage of platelets), or infection. Long-term bone marrow activity may in these cases originate from endogenous HSC that survived the irradiation. Real HSC activity, therefore, may only be measured by assessments of stable blood and bone marrow chimerism in the long term.

A large number of assays on the assessment of long-term engraftment is available (Chapter 2). Genetic disorders of erythropoiesis, congenic differences within inbred mouse strains, and transfection of hematopoietic progenitor cells with a unique marker

are examples of frequently used models for the determination of red or white blood cell chimerism. These methods, however, almost all require special techniques or breeding facilities. For the determination of long-term hematopoietic reconstitution we therefore decided to use a syngeneic sex-mismatched BMT model. With this method we are able to assess leukocyte chimerism in the same inbred mouse (CBA♂ × C57Bl♀)F_1 we have used for all our previous experiments, without having the difficulties mentioned above. The determination of partial chimerism is based on the detection of Y-chromosomes in leukocytes in blood smears and cytospin preparations of hemato-lymphopoietic organs using *in situ* hybridization with a murine Y-chromosome specific probe. The background of the Y-probe used, the technique of *in situ* hybridization and the choice of the proper donor-recipient combination in sex-mismatched BMT are the subjects for this chapter.

Parts of the data contained in this chapter will be published in:

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6.1 *In situ* hybridization with the murine Y-chromosome specific probe M34

Molecular studies of the mouse Y-chromosome have revealed DNA sequences that are specific for or enriched on the Y-chromosome[2,15,18,25,26,27,28]. Consequently, Y-chromosome specific or enriched repeated DNA sequences were used as probes[1,2,7,17,18,25,27,38,41]. Some of these probes[1,18,39] have been used in experimental hematology to discriminate between donor-derived and host-derived repopulation in sex-mismatched BMT[8,10,17,23,40,44]. Most of the hybridizations were performed on leukocyte-DNA attached to solid supports. For our experiments on the long-term repopulating ability of BMC samples *in vivo* we used *in situ* hybridization (ISH) to determine the degree of donor-type reconstitution in sex-mismatched BMT[44]. With the permission of Dr. L. Singh (Center of Cellular and Molecular Biology, Hyderabad, India) we obtained from Dr. J.W.M. Visser (ITRI-TNO, Rijswijk, The Netherlands) the mouse Y-chromosome specific repeated sequence probe M34[36,37,39]. A brief description of the origin of the probe is given.

Bkm satellite DNA, derived from the W-chromosome of the snake Banded krait (Bkm)[38] hybridizes in a quantitative male-specific manner to murine DNA[15]. The major component of this hybridization consists of repeats of the tetranucleotide GATA[38]. A male mouse genomic DNA library was screened with this Bkm satellite DNA and subse-

quently with the probe derived from this satellite DNA (consisting mostly of $(GATA)_n$), after which the lambda clone M34 was isolated. A 2.7 kb *Eco*R1 fragment showed an extensive hybridization with the murine Y-chromosome[39]. *Eco*R1 genomic blots of male and female mice of all *Mus musculus* species showed no female hybridization, but revealed a strong signal with this fragment at approximately 3 kb on male DNA. *In situ* hybridization with M34 revealed specific signals throughout the entire Y-chromosome. M34 sequences were not found in females under the hybridization and stringency condition used[39]. The 2.7 kb M34 *Eco*R1 fragment we use for our ISH, of which a nucleotide sequence will be published (Dr. L. Singh, personal communication), consists for approximately 45% of GATA repeats. These GATA repeats are only quantitatively specific for the murine Y-chromosome, which means that on autosomes and on X-chromosomes some GATA sequences are present[38] (this may be one explanation of the background signal we observe in the nuclei of cells after fluorescence *in situ* hybridization (FISH) with this Y-probe).
The 2.7 kb male specific mouse clone M34 restriction fragment 2 (p17-M34/2) is inserted in the *Eco*R1 restriction site of the plasmid pJRD 158B, also containing the gene encoding for resistance to ampicillin. *Eco*R1 digestion of the entire plasmid revealed two bands, one at 3 kb (insert) and another at 4 kb (pJRD). It appeared that in our hands the entire construct consisted of two pJRD plasmids and one 2.7 kb insert. The reason for this has remained unclear. The probe was grown in E.coli according to the standard method[31]. Plasmid DNA was isolated[31], and is kept at -70°C. Bacterial stocks containing the plasmid are stored at -70°C as well.

Labeling and detection of the Y-probe

The most commonly used labels for probe DNA are deoxynucleotide 5'-[^{32}P]-triphosphates and deoxynucleotide 5'-biotin-triphosphates. For our experiments we used nick translation for the labeling of the M34 Y-probe with biotin (vitamin H). Other labeling techniques and alternative labels are therefore beyond the scope of this paragraph.
Using nick translation[30] it is possible to label not only the insert but also the entire circular double stranded plasmid, which includes the insert that serves as the specific probe. Nick translation is an enzymatic labeling technique that utilizes the different properties of DNA polymerase I and DNase I. DNase I is an endonuclease that hydrolyzes the dsDNA of the plasmid and randomly generates nicks in the duplex, creating a complex mixture of nucleotide fragments with 5' termini. The sizes of these fragments are determined by the concentration of DNase I. DNA polymerase I uses the nicks to hydrolyze nucleotides from the 5' side of the nick and simultaneously build in new nucleotide residues at the 3' hydroxyl part of the nick, complementary to the opposite

DNA template. Thus a movement of the nick in the 5' → 3' direction takes place, replacing preexisting nucleotides with new nucleotides, while the nucleotide sequence remains unchanged (translation). For the incorporation of new nucleotide residues, nucleotides bearing a label can be used. Biotin can be covalently bound to the C5 position of the pyrimidine ring of dUTP. This biotin-16-dUTP can be used as a substrate for DNA polymerase I, although it is somewhat less effective than unlabeled dUTP[19]. The length of the spacer arm by which biotin is coupled to dUTP enhances proper exposure of biotin in the hybrid dsDNA[4]. Biotinylated probes are being used for hybridization. The hybrids of these probes have a lower stabiliy than their naive counterparts. Nick translation of a double stranded plasmid with labeled nucleotides gives rise to labeled fragments of different sizes which overlap each other partially. When these fragments are used for hybridization they tend to form hyperpolymers (networks) due to hybridization with each others overlapping complementary region. This occurs especially in the presence of dextran sulphate 500. Because not only the insert was labeled but the entire plasmid, labeled DNA fragments of the plasmid will contribute to building a tree of labeled DNA, thus amplifying the hybridization signal. Biotinylated probes have a high resolution, a good sensitivity, a long stability (> 2 years at -70°C) and allow a quick, non-isotopic detection.

The detection of hybridized biotinylated probes depends on the highly specific and very strong interaction between biotin and avidin or streptavidin. When avidin is coupled to appropriate indicator molecules, in our case the green fluorescein isothiocyanate (FITC), it can readily be detected. When necessary, amplification with a biotinylated anti-avidin monoclonal antibody can be performed, followed by a second step of avidin-FITC. After the detection steps it is recommended to keep the cells embedded in the antifading agent 1,4-diazobicyclo-(2,2,2)-octane (DABCO)[3] containing the red fluorescent propidium iodine to counterstain cellular DNA. Preparations can be maintained at -20°C at least up to 6 months.

Molecular hybridization

For the detection of the Y-chromosome in murine leukocytes we used the *in situ* hybridization technique with the M34 murine Y-chromosome specific DNA probe. Since the introduction of molecular hybridization in intact cytological preparations[5,9,14], technical knowledge and progress as well as biological and medical applications have been expanded enormously. Optimal conditions for DNA (and RNA) hybridization are largely studied on nucleic acids immobilized on solid supports (e.g., nitrocellulose (NC) paper), the so called mixed phases systems, and on hybridization in solution (single phase systems). The principles of the methodology of molecular hybridization are reviewed by

Meinkoth and Wahl[22], Coghlan *et al.*[6], Höfler[12], Matthews and Kricka[21], Höfler[13], and Maniatis[31]. Theoretical aspects concerning our protocol for fluorescence *in situ* hybridization with the M34 Y-probe are subject for this section.

Double stranded (ds) DNA - the two chains are held together by hydrogen bonds between guanine and cytosine (G-C), and adenine and thymine (A-T) - will dissociate in single stranded (ss) DNA under specific conditions, and renature when these conditions are abolished. If during the process of annealing labeled ssDNA fragments (probe) that contain sequences complementary to the target DNA are added to the native ssDNA, the probe DNA will compete for the hydrogen binding, resulting in stable combinations of native DNA and probe DNA. This hybrid DNA subsequently can be visualized. The kinetics of hybridization of DNA probes with DNA free in solution or attached to NC are alike, and it is assumed that nucleic acids within the cell behave similarly.
The rate with which ssDNA will hybridize to single-stranded probes shows first order kinetics, since the concentration of the probe is in vast excess over that of target sequences (for nick translated probes additional factors play important roles, like the formation of complex networks, and the decrease in concentration of the probe available for hybridization with target DNA). Hybridization is determined by probe strand length, base composition (percentage G-C), molecular complexity (i.e., the total number of base pairs in a non-repetitive sequence), temperature, viscosity, pH, concentration of helix destabilizing agent (e.g., formamide), and ionic strength. Within certain ranges some of these conditions have little effect. Ionic strength, mostly determined by NaCl, hardly influences hybridization as long as the NaCl concentration is kept above 0.4M NaCl, and effects of pH are small between pH 5.0 to 9.15 at the salt concentration mentioned. A high temperature causes dissociation of dsDNA, while temperatures round 37°C allows the renature of ssDNA to dsDNA. Probes with a low complexity at high concentrations will enhance the hybridization rate. Another enhancing factor is the addition of anionic dextran polymers to the hybridization mixture. Wahl *et al.*[46] measured an increment of the hybridization rate up to 100 times for hybridizations utilizing nick translated probes, when 10% dextran sulphate 500 (final concentration) was added. This effect was ascribed to an increased formation of probe networks of partially overlapping sequences of probe strands, that were generated during nick translation. In addition, the effective probe concentration is enhanced due to the volume dextran sulphate occupies.
Despite a number of mismatched bases (i.e., no G-C or A-T) a stable hybrid may form. Hybridization conditions are a compromise between associating and dissociating forces, to promote hybridization to identical probe sequences and to prevent hybridization to related but non-identical sequences. To realize a highly specific hybridization product, hybridization is performed at stringent conditions (dissociating conditions): low Na^+ concentration, the presence of formamide, and a high temperature. Monovalent kations

interact electrostatically with nucleic acids, resulting in a decreased electrostatic repulsion between the two DNA strands of the duplex when the salt concentration is increased. NaCl acts as a dsDNA stabilizing agent. Formamide decreases duplex stability. A higher level of specificity of the hybridization can be achieved by stringent washes after the hybridization period. These conditions determine the degree of mismatch that can be tolerated in a hybridization reaction. At high stringency a high percentage of homology is required.

In situ hybridization

Molecular *in situ* hybridization (ISH) is the technique with which labeled probes that contain complementary sequences, hydrogen bind to cellular DNA. It is assumed that the behavior of DNA in the cell is similar to DNA attached onto solid supports. ISH is similar to mixed phases hybridization: the cells themselves form the solid support. However, this is true to a certain extent, because intact, mostly fixed cells or tissues may contain some obstructive factors like the cytoskeleton, lipid membranes, and cross-linked proteins. Fixation of cells prior to ISH, therefore, is a compromise between keeping an intact morphology, a maximal presence of cellular target DNA (or RNA), and leaving the target DNA accessible for the diffusing probe. Especially aldehyde fixation of cells (e.g., formaldehyde), which leads to crosslinking of DNA and binding of proteins to DNA, will alter the hybridization rate and the stability of the molecular hybrid. Often pretreatment of cells and tissues is carried out with a detergent to lyse the lipid membranes (e.g., Triton X-100) or with proteinase digestion (proteinase K, pepsin) to enhance the passage of the probe and the accessibility of the target DNA. Treatment of the cells with HCl may denature proteins and partially hydrolyze the DNA. The length of the probe is another important factor in reaching the target DNA. Cellular target DNA and double stranded probe DNA have to be heated (±90°C) during 5-15 min to get denatured. As applied for ISH, lowering the salt concentration and using a larger percentage of formamide is less effective in dissociating dsDNA than it is for mixed phase systems, probably due to the biologic circumstances within the cell. BSA and herring (or salmon) sperm DNA are added to prevent nonspecific attachment of the probe to cellular components.
Together with the variables mentioned earlier, cellular conditions determine the stringency of hybridization and washing conditions necessary for the specificity of the signal. Hence, these may differ for each type of cells or tissue, although the same probe is used.

In summary, the sensitivity of ISH depends on:

- retention and accessibility of cellular target DNA
- probe construct
- hybridization conditions
- type and efficiency of probe labeling
- sensitivity of signal detection

From the above it may become clear, that every combination of probe and target DNA and the cells or tissues will have its own particular hybridization characteristics, and thus its specific hybridization conditions. The conditions described in this chapter, therefore, may be specific for the probe used, although alternative conditions for the same probe have been described.[17,37,39]

Protocol for FISH with the M34 Y-probe on blood smears

Biotinylation of the M34 Y-probe by nick translation is carried out according to the prescriptions of the nick translation kit (Bethesda Research Laboratories). One reaction vessel contains 1 μg of the plasmid. The label used is biotin-16-dUTP (Boehringer). After nick translation is stopped the reaction mixture is eluted over a Sephadex G-50 column (prepared in a 1 mL syringe containing a small wad of cotton wool) by centrifugation for 1.5 min at room temperature. This clears the biotinylated probe from free labeled and unlabeled nucleotides. The probe is then concentrated by ethanol precipitation and dissolved in 10 nM Tris-HCl/ 0.1 mM EDTA (pH 7.5). The final concentration of the labeled probe is 0.1 μg/μL. The probe is stored at -20°C, where it can be kept for months.

In situ hybridization with the M34 Y-probe is carried out on blood smears and on smears of single cell suspensions of bone marrow, spleen, and thymus in horse serum. The smears are fixed in methanol for 10 min at room temperature and stored at -20°C. FISH can be performed reliably a least up to 6 months after preparation of the smears. The protocol for FISH is adapted from Pinkel *et al.*[29], who used the protocol to hybridize metaphase and interphase nuclei with a 0.8 kb DNA probe (pY431A), specific for the human Y-chromosome. In turn, this protocol is an adaptation of the hybridization protocol by Harper *et al.*[11].

After the smears are adapted to room temperature, the cells are made permeable with 0.1M HCl/ 0.05% (v/v) Triton X-100 for 7.5 min at 37°C, and subsequently fixed in 1% (v/v) paraformaldehyde/ PBS for 15 min at room temperature. The slides are dehydrated in an ethanol series (50% → 70% → 90% → 100%) and are allowed to air dry. Then

the slides are overlaid with 5μL of hybridization mixture. This mixture consists of 2×SSC (standard sodium citrate; from a stock of 20×SSC (3.0 M NaCl/ 0.3M tri-Na-citrate.$2H_2O$; pH 7.0)), 50% (v/v) deionized formamide, 10% (w/v) dextran sulphate, 0.1% (v/v) Tween-20, 0.5 mg/mL herring sperm DNA and 2.5 ng/mL biotinylated M34 Y-probe (final concentrations; pH 7.2). The mixture is covered with a plastic cover slip. Cellular and probe DNA are denatured for 5 min at 80°C and are allowed to hybridize overnight at 37°C in a 100% humidified moisture chamber. After hybridization the slides are kept in 2×SSC at room temperature before the stringent washes. The latter consist of two washes in 2×SSC/ 50% formamide (pH 7.0-7.4) for 5 min each at 37°C. Hereafter, the slides are washed in 2×SSC for two times 5 min at 37°C and kept in 4×SSC/ 0.1% Triton at room temperature. Then the slides are incubated with avidin-FITC (5 μg/mL avidin-FITC (Vector)/ 4×SSC/ 0.1% Triton/ 3% (w/v) BSA) for 20 min at 37°C. After washing in 4×SSC/ 0.1% Triton the slides are incubated with biotinylated goat-α-avidin (2 μg/mL biotinylated goat-α-avidin (Vector)/ 4×SSC/ 0.1% Triton/ 3% BSA) for 20 min at 37°C, washed two times in 4×SSC/ 0.1% Triton, and again incubated with avidin-FITC. After two washes in 4×SSC/ 0.1% Triton the slides are dehydrated and allowed to air dry. Finally, the slides are embedded in 5 μL of an antifading medium and covered with a glass cover slip. The antifading medium consists of 18 μg/mL DABCO (Sigma)/ 90% (v/v) glycerin/ 50 ng/mL propidium iodide/ 0.02M Tris-HCl. The preparations are stored at -20°C, where they can be kept for months. All concentrations mentioned are final concentrations.

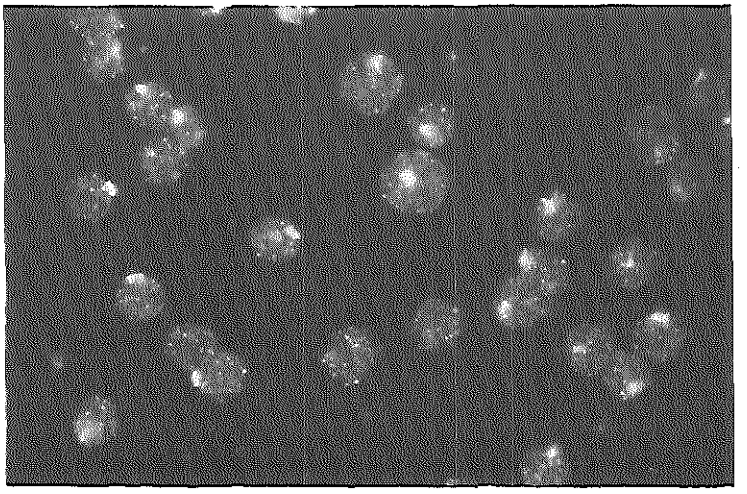

Figure 6.1. Example of blood leukocytes of a male mouse wich was transplanted with female BMC, hybridized with the M34 Y-probe, and stained with avidin-FITC (original magnification 630×).

An example of a group of periferal blood leukocytes stained with the M34 Y-probe is shown in Figure 6.1.

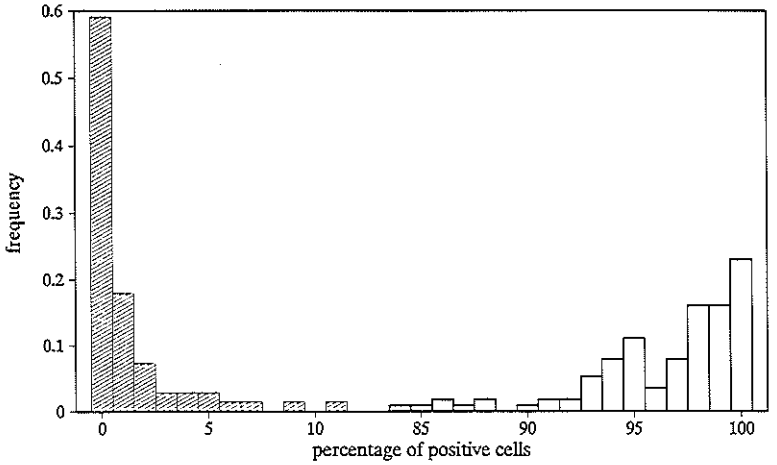

Figure 6.2. Frequency distribution of positive observations in 69 female (▦), and 117 male (□) blood smears, hybridized with the M34 Y-probe.

Y-probe hybridization in male and female blood smears

To give an impression of the sensitivity of this method to trace male cells in blood smears, Figure 6.2 shows the distribution of data on male and female smears, presenting the percentages of nucleated cells that revealed a positive signal. One hundred cells were scored in each blood smear. When a positive control preparation revealed less than 90% positivity, the entire staining series was abandoned and the staining procedure repeated on fresh slides. These results indicate that FISH using the M34 murine Y-probe is a reliable technique to discriminate male from female nucleated cells, giving quantitative repopulation data in sex-mismatched BMT models. Ninety-five percent of all observations in male blood smears was found above 88% positivity, while in female smears percentages below 6 came in the range of 95% of all observations.

6.2 Sex-mismatched bone marrow transplantation: the choice of the proper donor-recipient sex combination

The Y chromosome is not genetically inert with respect to transplantation antigens. The role of the male-specific antigen H-Y in graft rejection has extensively been studied using skin grafts (see for a review Simpson, 1982[35]). In the experiments in which

sex-mismatched BMT is the model for the measurements of LTRA of BMC samples, we had to reckon with this information to secure optimal results. Therefore, we studied which donor-recipient pair will give a true-to-nature reflection of the reconstituting capacities of transplanted HSC, without being impeded by any type of anti-H-Y reaction.

Materials and methods

Mice. Male and female (CBA♂ x C57Bl♀)F_1 mice were bred and maintained at the Laboratory Animal Center of the Erasmus University under conventional conditions, and were of SPF-5 quality. The major histocompatibility complexes for the parent strains of the used hybrid F_1 mice were H-2^q for CBA and H-2^b for C57Bl. At 10-20 weeks of age mice were used as recipients for BMT or as donors of bone marrow cells (BMC), harvested from both femora and tibiae.

Sex-mismatched bone marrow transplantation. Six samples of male or female BMC, ranging from 10^4 to 3×10^6 BMC per mouse, were infused into 30 recipients of opposite sex (5 mice per sample). Twenty-four hours before BMT the recipient mice received a sublethal dose of 8.25 Gy γ-irradiation from two opposing ^{137}Cs sources (Gammacell 40, Atomic Energy of Canada) at a dose rate of 1.06 Gy/min. Five irradiated female and 5 irradiated male mice that had not received BMC, served as controls. Blood smears were made from each individual mouse at 1, 2, 3, 4, 8, and 12 weeks after transplantation. At 12 weeks the mice were killed and cytospin preparations were made of suspensions of

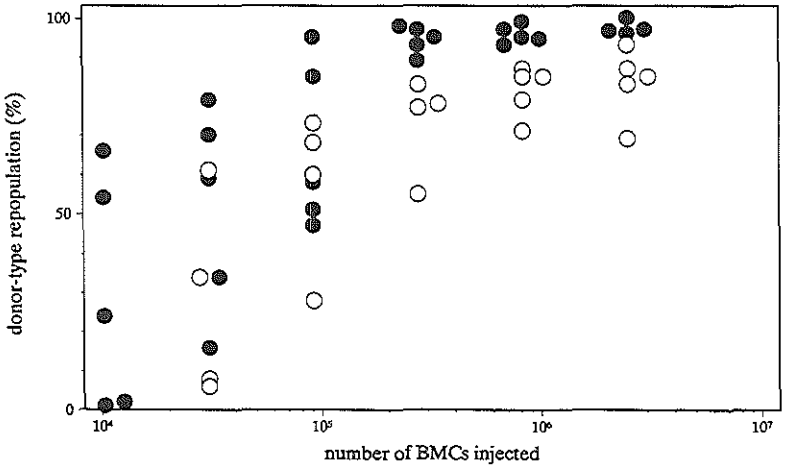

Figure 6.3. Percentages of donor-type repopulation in the blood after male-to female (○) and female-to-male (●) BMT, 12 weeks after transplantation. Data represent values of individual animals.

bone marrow, spleen, and thymus. The blood smears and cytospins were assayed for the percentage of donor-derived nucleated cells using fluorescence *in situ* hybridization with a murine Y-chromosome specific probe. Comparison of the stem cell potential to induce stable chimerism was done by using the number of BMC required for 50% donor-type repopulation. This number was calculated after logit transformation of the proportion of donor-type repopulation (logit $P = \ln(P/(1-P))$, and logarithmic transformation of the BMC input. Simple linear regression was performed on repopulation data between 20 and 80 percent, using *logit P* as dependent variable. The number of BMC required for 50% donor-type repopulation was then calculated from the equation of the linear regression, using *logit P = 0* for 50% donor-type repopulation. In addition, the 95% confidence intervals were estimated.

Results

The percentages of donor-type repopulation in the blood of female mice transplanted

Table 6.1 Number of BMC required for 50% donor-type leukocyte repopulation in the blood of female recipients transplanted with male BMC, and of male recipients transplanted with female BMC

weeks after transplantation	male-to-female*	female-to-male
1	2.41×10^6 (1.62×10^6-3.09×10^6)	6.83×10^5 (4.90×10^5-9.12×10^5)
2	6.43×10^4 (2.69×10^4-9.77×10^4)	4.97×10^4 (3.63×10^4-8.32×10^4)
3	5.48×10^4 (2.88×10^4-8.51×10^4)	5.53×10^4 (3.63×10^4-8.91×10^4)
4	1.20×10^5 (4.79×10^4-2.34×10^5)	6.43×10^4 (4.17×10^4-1.05×10^5)
8	1.65×10^5 (3.80×10^4-6.92×10^5)	3.73×10^4 (7.59×10^3-1.07×10^5)
12	9.21×10^4 (4.07×10^4-2.09×10^5)	3.76×10^4 (1.38×10^4-1.58×10^5)

* Data represent the estimation of the number of BMC required for 50% donor-type repopulation and, in parentheses, their 95% confidence intervals

with various numbers of male BMC, or vice versa, 12 weeks after transplantation, are shown in Figure 6.3. From these data the number of BMC required for 50% donor-type repopulation was determined. Table 6.1 shows that essentially similar numbers of BMC were required to achieve 50% donor-type repopulation in blood leukocytes at week 2 or later. However, more male BMC were required to obtain 50% donor-derived leukocytes in the blood of female recipients, then vice versa. In other words, male BMC gave lower percentages of donor-derived leukocytes in female recipients than did the same number of female BMC injected into male recipients. This difference in efficiency of engrafting potential of female and male BMC was confirmed when the degree of donor-type repopulation was determined in bone marrow, spleen, and thymus at 12 weeks after transplantation (Table 6.2). These differences ranged from 5 times in the blood to 10 times in the bone marrow in favor of the female-to-male combination.

Table 6.2 **Number of BMC required for 50% donor-type leukocyte repopulation in the blood, bone marrow, spleen and thymus of female recipients transplanted with male BMC, and of male recipients transplanted with female BMC, measured 12 weeks after transplantation**

organ	male-to-female *	female-to-male
blood	9.21×10^4 (4.07×10^4-2.09×10^5)	3.76×10^4 (1.38×10^4-1.58×10^5)
bone marrow	2.14×10^5 (5.01×10^4-4.17×10^5)	1.81×10^4 (3.98×10^3-5.01×10^4)
spleen	7.41×10^4 (2.00×10^4-9.12×10^4)	1.57×10^4 (1.29×10^3-8.51×10^4)
thymus	6.35×10^4 (1.12×10^4-1.32×10^5)	1.06×10^4 (3.55×10^3-3.09×10^4)

* Data represent the estimation of the number of BMC required for 50% donor-type repopulation and, in parentheses, their 95% confidence intervals

Another important feature is that for each *dilution group* we found similar mean percentages of donor-type repopulation in the blood, bone marrow, spleen, and thymus (data not shown). However, when individual mice were scored for their percentages of donor-derived repopulation, it appeared that large variations existed when blood, bone marrow, spleen and thymus were mutually compared (Figure 6.4). This indicated that the diverse differentiation lineages may not be repopulated at an equal level. More detailed study, in which the cells from all differentiation lineages are purified and checked on their origin, is necessary to verify this presumption.

In all the individual dilutions tested the differences between the male-to-female and

female-to-male transplantation groups were statistically significant ($\alpha_T < 0.05$), except when 10^5 BMC were transplanted (Student-Newman-Keuls test for multiple comparison).

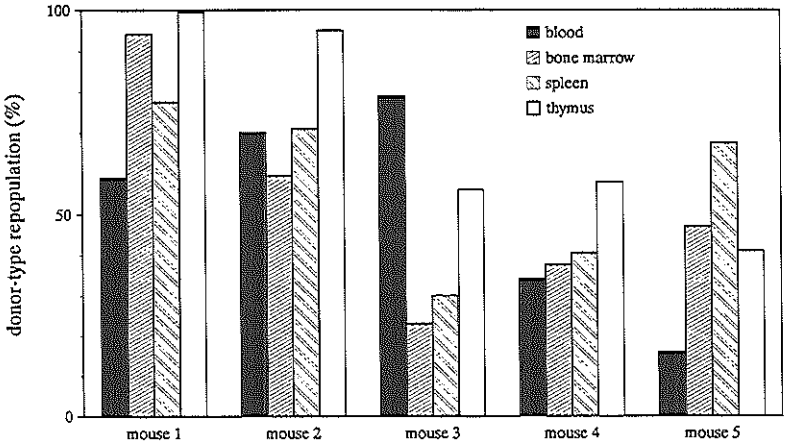

Figure 6.4. Percentages of donor-type repopulation in various hemato-lymphopoietic organs of 5 individual male mice, transplanted with 3.09×10^4 female BMC.

Discussion

Experiments on syngeneic sex-mismatched BMT have not led to unequivocal conclusions with respect to the influence of sex on transplantation results in the donor-recipient combination of choice. Some authors reported no differences between sex-matched and sex-mismatched transplantation models[24,32,33,42]. Others reported no differences between female-to-male and male-to-female BMT[16,20], while better performance of male-to-female compared with female-to-male also has been described[34]. None of these studies report the prevalence of female-to-male over male-to-female.
It was therefore surprising that we found a clear difference in donor-type repopulation based on the sex of donor-recipient pairs in favor of the female-to-male combination. A possible explanation is that the H-Y antigen of the infused male BMC may induce a host-versus-graft reaction in the 8.25 Gy irradiated female recipient, that interferes with engraftment. Immunocompetent cells surviving sublethal irradiation include NK cells, macrophages, and cytotoxic T-cells, which may be responsible for this reaction[43]. When female BMC enter a male environment, however, female T-cells will elicit a graft-versus-host reaction, which will have its positive effect on the take of the graft[45]. The net

result may be a higher degree of engraftment of female BMC in a male host compared to the male-to-female combination.
An alternative explanation for the differences between our observations and those mentioned in the literature could be a difference in antigenicity of the H-Y antigen in the various inbred mouse strains used[35], partially based on the murine histocompatibility antigen (H-2) subtype[33,35,42]. Other differences from the other reports cited are the conditioning of the recipient animals and the methods of assaying (long-term) engraftment.

Five or 6 groups of 5 irradiated mice each were given increasing doses of BMC of donor mice of the opposite sex. The cell doses were chosen around the number of BMC we thought would approximate 50% donor-type repopulation. At given time points the percentages of donor-type repopulation of each individual mouse were determined, and the number of BMC required for 50% donor-type repopulation was determined as described. With this parameter we avoid misinterpretation of data, which can be a consequence of observing marginal, physiologically insignificant donor-derived repopulation. Donor-type repopulation that has reached a plateau neither is a proper indicator for repopulating activity (see also Paragraph 2.3). Additionally, in using this method we avoid the small number of false-positive and false-negative observations that may occur around zero and one hundred percent positivity.

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Chapter 7
Loss of long-term repopulating ability in long-term bone marrow culture

Preface

A study is described on the numerical and qualitative maintenance of stem cells with long-term multilineage repopulating ability from murine bone marrow, cultured on pre-established bone marrow-derived stromal cell layers. Female bone marrow cells were cultured for a period of 1-4 weeks, and compared with uncultured cells for their ability to establish and maintain a level of 50% chimerism in the sex-mismatched bone marrow transplantation model described in Chapter 6. Chimerism was determined in nucleated cell populations using fluorescence *in situ* hybridization with the M34 murine Y-chromosome specific probe.

In recent years long-term bone marrow cultures (LTBMC) have been used in protocols for autologous bone marrow transplantation (BMT) in patients with acute myeloid leukemia (AML) and chronic myeloid leukemia (CML) as a source of normal stem cells[4,29]. This application of LTBMC arose from the observation that in LTBMC from many of these patients the growth of leukemic cells is inhibited, while normal progenitors are maintained and develop into mature cells[4,8,23].
Another recently uncovered and promising quality of LTBMC is that hematopoietic stem cells (HSC), after transfection with a foreign gene, amplify their numbers in LTBMC[1,14,20], which may lead to the application of LTBMC for gene therapy of a variety of genetic disorders using retroviruses. In both cases it is essential to know whether LTBMC allow amplification or even maintenance of the population of primitive HSC that ensures long-term hematopoietic engraftment of transplanted hosts. Weekly measurements of CFU-S produced in long-term cultures have been shown not to be proportional to the ability of the HSC in LTBMC to repopulate the erythroid blood compartment of lethally irradiated mice in the long term[17]. We therefore set out a series of experiments in which we studied the quantitative and qualitative ability of BMC cultured in LTBMC to establish and maintain stable chimerism, using a murine syngeneic sex-mismatched BMT model. In addition, the frequency of LTRA cells among cultured BMC was measured in the *in vitro* limiting dilution assay for cells with marrow repopulating and long-term repopulating cells (Chapter 5).

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Materials and methods

Experimental design. Bone marrow cells (BMC) cultured in LTBMC were compared to fresh BMC with respect to their long-term engraftment potential *in vivo* using a sex-mismatched BMT model. In addition, the frequency of marrow repopulating and long-term repopulating cells was determined using a quantitative *in vitro* assay (CAFC-assay). Female BMC were cultured for 0-4 weeks on female bone marrow-derived stromal cell layers and subsequently transplanted into sublethally irradiated male recipients, or assayed in the *in vitro* CAFC-assay. Long-term repopulating ability (LTRA) *in vivo* was determined by fluorescence *in situ* hybridization using a murine Y-probe on blood smears 1-6 months after BMT, and also on bone marrow, spleen and thymus at 6 months after BMT. LTRA *in vitro* was determined from the frequencies of clone forming cells at day-28 of the CAFC-assay.

Mice. Male and female (CBA♂ x C57Bl♀)F_1 mice were bred and maintained at the Laboratory Animal Center of the Erasmus University under conventional conditions, resulting in SPF-5 quality. The major histocompatibility complexes for the parent strains of the used hybrid F_1 mice were H-2^q for CBA and H-2^b for C57Bl. At 10-20 weeks of age the mice were used as recipients for BMT or as donors of bone marrow cells, harvested from both femora and tibiae.

Sex-mismatched bone marrow transplantation. To assay the ability of transplanted uncultured or cultured BMC to establish and maintain stable chimerism in the long term, we transplanted female BMC into sublethally irradiated (8.25 Gy γ-irradiation at a dose rate of 1.06 Gy/min) male hosts. A detailed description of this transplantation model is given in Chapter 6.

Fluorescence *in situ* hybridization. The analysis of the blood, bone marrow, spleen, and thymus with respect to the degree of donor-type repopulation in the sex-mismatched BMT model was carried out by fluorescence *in situ* hybridization (FISH) using the M-34 murine Y-chromosome specific probe. Description of this technique is given in detail in Chapter 6.

CAFC assay. The principles and methodology of the *in vitro* limiting dilution method for the quantification of murine hematopoietic stem cells are the subjects of Chapter 5. It is shown there that a relationship exists between the primitiveness of HSC and the temporal delay and the duration of clonal expansion (i.e., CA-formation) in this culture system. Since the relative frequencies of cobblestone areas at day-28 correlate with the *in vivo* marrow repopulating ability, we took the CAFC day-28 frequencies to measure LTRA *in vitro*.

Determination of LTRA in LTBMC. Fresh BMC were seeded onto pre-established stromal cell layers in tissue culture flasks, and 1-4 weeks after inoculation aliquots of entire cultures were transplanted into irradiated recipient mice of the opposite sex. One to 6 months after transplantation donor-type repopulation was determined in the blood, and at 6 months also in bone marrow, spleen, and thymus. Fresh and cultured BMC were also put into the *in vitro* limiting dilution assay, and tested on their *in vitro* repopulating ability, the day-28 CAFC content.

To establish a hematopoietically inactive stroma both femora and tibiae of 8 female mice were crunched using an aseptic mortar and pestle. BMC were harvested in LTBMC medium (10% FBS, 5% HS), while no attempts were made to prepare a single cell suspension. The BMC were divided over eight 75 cm^2 tissue culture flasks (Costar), so that each flask contained 10^8 BMC in 15 mL medium. The cultures were kept at 33°C, 10% CO_2 and 100% humidity and were weekly fed by replacing all of the culture medium. After 3 weeks the cultures consisted of a confluent, hematopoietically active stromal cell layer. At this time the cultures were given 20 Gy γ-irradiation at a dose rate of 1.06 Gy/min, which removed all hematopoietic activity. One day after irradiation the culture medium was completely changed for LTBMC medium containing 20% HS. Four days after irradiation fresh BMC were harvested from the femora and tibiae of 4 female mice, and the stromas were inoculated with a single cell suspension of 3×10^7 female BMC in 15 mL medium per flask. BMC from the same suspension were also tested for their *in vivo* LTRA in the sex-mismatched BMT model described, and in the assay for *in vitro* LTRA. The BMC were kept in culture over a period of 1-4 weeks, with a weekly change of culture medium and restitution of the non-adherent cells contained in the supernatants of the cultures.

At weekly intervals the non-adherent cells of 2 flasks were harvested and the adherent layers trypsinized. The cells in each flask were incubated with 5 mL of 0.25% (w/v) trypsin (Merck)/ PBS for 5 min at 37°C. Both the adherent and non-adherent cells were pooled and a single cell suspension was prepared in Dutton's balanced salt solution (Gibco). Cells from these suspensions were assayed for their LTRA in the *in vivo* sex-mismatched BMT assay, and in the *in vitro* limiting dilution assay.

At week 1, 2, and 3 of culture 3 groups of 5 male mice received the cellular content of either 1/20, 1/60 or 1/200 culture flask, which is equivalent to 1.5×10^6, 5×10^5, and

1.5×10^5 of female BMC initially seeded onto the stromal layers of the flask cultures respectively. At week four 1/6, 1/20, and 1/60 flask, equivalent to 5×10^6, 1.5×10^6, and 5×10^5 BMC, respectively, were transplanted into 5 male recipients each. At monthly intervals blood smears of each individual mouse were made. Six months after transplantation the mice were killed and cytospins were made from the bone marrow, spleen, and thymus of each mouse. The percentage of donor-type repopulation was determined as described.

In the *in vitro* LTRA assay uncultured and 1-4 weeks cultured female BMC were seeded in a series of 6 dilutions, 3-fold apart. For uncultured BMC the highest concentration was 27,000 BMC per well. For BMC cultured 1 to 4 weeks in LTBMC an equivalent of 30,000, 90,000, 90,000, and 600,000 originally inoculated cells per flask, per well, respectively, formed the highest concentrations. CA-frequencies were determined 5-28 days after overlay.

CFU-C assay. The supernatants of the control 25 cm^2 flasks were tested on their content of *in vitro* clonable myeloid progenitor cells, CFU-C (i.e., CFU-G, CFU-M, and CFU-GM). To do this 2×10^4 supernatant nucleated cells in 1 mL of culture medium were cultured for 7 days in duplicate 35 mm culture dishes (Costar), using a semi-solid colony assay. The culture medium consisted of α-medium, 1.2% methylcellulose (Fluka), 20% horse serum (HS), 1% BSA (fraction V; Sigma), 10% pokeweed mitogen (Gibco) mouse spleen conditioned medium as the source of hematopoietic growth factors, 80 U/mL penicillin (Gibco), 80 μg/mL streptomycin (Gibco), 8×10^{-5}M β-mercaptoethanol (Merck), 3.3×10^{-3}M L-glutamine (Merck), and 8×10^{-5}M sodium selenite (Merck). The cultures were kept at 37°C, 5% CO_2 and 100% humidity.

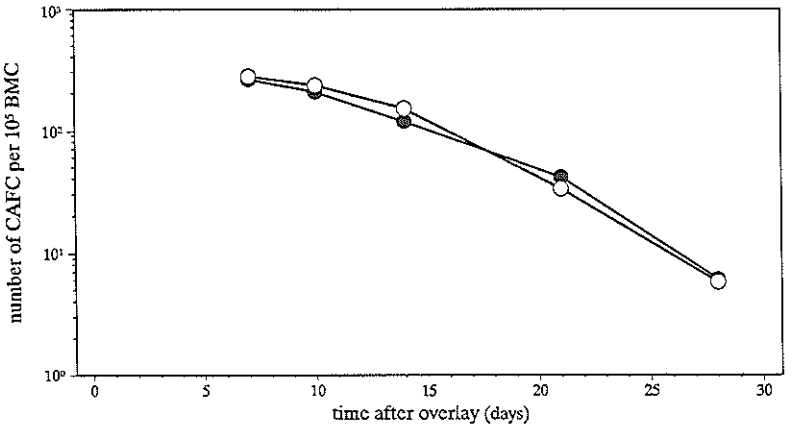

Figure 7.1. Frequencies of CAFC over a period of 28 days in fresh bone marrow (○), and BMC treated with 0.25% (w/v) of trypsin (●).

Trypsinization. To test whether trypsinization, which was required to detach the adherent HSC from the culture flasks, would influence the proliferative capacity of BMC, we incubated 5×10^7 BMC in 5 mL of 0.25% (w/v) trypsin/ PBS for 5 min at 37°C. Subsequently the trypsin activity was blocked with 5 mL ice-cold HS. The cells were centrifuged and resuspended in LTBMC medium (20% HS). The CAFC assay was performed as described. Fresh BMC that have been suspended in LTBMC medium during the trypsinization procedure of the test group served as controls. The range of cell inputs for both groups consisted of 6 dilutions of 111-27,000 BMC per well. CA-frequencies were determined at 7, 11, 14, 21, and 28 days after overlay.

Results

Trypsinization. In order to test the possible negative influence of the trypsinization protocol on the proliferative capacity of BMC, we compared fresh BMC to trypsinized BMC for CA formation *in vitro*. From Figure 7.1 it is apparent that the trypsinization procedure used to detach the adherent cells from the flasks did not lead to a loss of short-term and long-term repopulation *in vitro* as measured by the CAFC assay. Because of the good correlations of early and late appearing CA with *in vivo* CFU-S and pre-CFU-S, respectively (Paragraph 5.2), we assumed that *in vivo* LTRA cells would be equally unaffected by trypsinization.

Table 7.1 **Number of nucleated cells and CFU-C measured in the supernatants of 25 cm² flasks that were seeded with 1×10^7 fresh BMC**

time after overlay	number of cells per flask	number of CFU-GM per flask
week 1	2.10×10^6 (2.01×10^5)	8291 (2445)
week 2	5.34×10^6 (2.14×10^5)	3150 (385)
week 3	6.87×10^6 (7.68×10^5)	1065 (225)
week 4	1.41×10^6 (1.97×10^5)	2240 (337)

Data represent the arithmetic means of the values of 5 flasks (1 SEM).

Nucleated cell and CFU-GM production in LTBMC. The kinetics and extent of the weekly production of nucleated cells and CFU-C on stromal layers of the control 25 cm² flasks (Table 7.1) indicates that the quality of the cultures is comparable to that of previously published studies using LTBMC. From these data it is evident that irradiated stromal cell layers support the *in vitro* hematopoiesis properly.

Table 2 Number of fresh or cultured female BMC required for 50% donor-type leukocyte repopulation in the blood of grafted male mice

Weeks of LTBMC	months after transplantation 1	2	3	4	6
0†	2.99×10^4 (5.25×10^3-9.77×10^4)	4.61×10^4 (2.40×10^4-7.08×10^4)	4.67×10^4 (2.45×10^4-7.24×10^4)	5.38×10^4 (2.40×10^4-9.55×10^4)	4.80×10^4 (2.69×10^4-7.08×10^4)
1*	1.18×10^5 (2.40×10^4-3.39×10^5)	5.84×10^4 (1.17×10^4-1.74×10^5)	5.99×10^4 (1.23×10^4-1.74×10^5)	6.24×10^4 (1.26×10^4-1.78×10^5)	7.07×10^4 (1.66×10^4-1.86×10^5)
2	6.52×10^5 (3.31×10^5-2.57×10^6)	3.31×10^5 (1.15×10^5-6.03×10^5)	3.40×10^5 (1.48×10^5-5.89×10^5)	3.52×10^5 (1.35×10^5-6.61×10^5)	3.53×10^5 (1.48×10^5-6.31×10^5)
3	8.10×10^7 (2.09×10^6-8.91×10^8)	2.76×10^7 (ND)	1.44×10^6 (ND)	1.32×10^5 (ND)	6.72×10^5 (ND)
4	2.45×10^6 (1.26×10^6-1.05×10^7)	1.36×10^6 (2.34×10^5-7.24×10^6)	9.72×10^5 (2.75×10^5-2.29×10^6)	6.08×10^5 (9.77×10^4-2.04×10^6)	8.13×10^5 (2.04×10^5-2.04×10^6)

† Data in parentheses represent the 95% probability intervals

* Data from cultured BMC are related to the number of originally inoculated BMC on the irradiated LTBMC stromal layers.

ND 95% confidence interval could not be determined

Table 7.3 Percentages of *in vivo*[*] and *in vitro*[†] LTRA that were maintained when fresh BMC were cultured for 1-4 weeks in LTBMC

weeks of LTBMC	months after transplantation 1	2	3	4	6	CAFC day-28
0	100.00	100.00	100.00	100.00	100.00	100.00
1	25.44	78.90	77.96	86.24	67.89	25.2
2	4.59	13.92	13.75	15.29	13.62	14.3
3	0.04	0.17	3.23	4.07	7.15	8.1
4	1.22	3.39	4.80	8.84	5.91	2.7

[*] Percentages were calculated from the number of BMC, that was required for 50% donor-derived nucleated cells in the blood (see Table 7.2). The values of fresh BMC were set at 100%.

[†] Percentages were calculated from the CAFC day-28 frequencies. For fresh BMC the frequency of CAFC day-28 was 6.1 per 10^5 cells.

Maintenance of LTRA in LTBMC. BMC that were cultured on irradiated stromal layers for 1-4 weeks were compared to fresh BMC with respect to their long-term repopulating ability *in vivo*. Engraftment of these cells was expressed as the number of BMC required for 50% donor-type repopulation (Table 7.2). When the values of uncultured BMC are set at 100% (Table 7.3), it is evident that after 1 week of culturing BMC on stromal cell layers one third of the repopulating ability was lost, and that only 14% of LTRA, measured 6 months after transplantation, was left after 2 weeks of culture. Four weeks of LTBMC spared only 6% of the HSC responsible for stable chimerism over 6 months.

No loss of the *level* of chimerism was seen over the period of 6 months following infusion of cells that had been cultured, with the exception of cells that have been in culture for 3 weeks (Table 7.2). Thus, since cultured cells gave stable chimerism, as gave fresh BMC, it indicates that with decreasing LTRA numbers the quality of the remaining LTRA cells was not influenced by the culture.

Table 7.4 **Percentages of LTRA that were maintained when fresh BMC were cultured for 1-4 weeks in LTBMC, measured 6 months after BMT***

weeks of LTBMC	blood	bone marrow	spleen	thymus
0	100.00	100.00	100.00	100.00
1	67.89	86.46	157.42	42.15
2	13.62	17.49	13.62	7.80
3	7.15	14.44	12.49	7.70
4	5.91	8.18	4.39	17.13

* Percentages were calculated from the number of BMC, that was required for 50% donor-derived nucleated cells

We also measured LTRA of cultured and fresh BMC in the bone marrow, spleen, and thymus at 6 months after transplantation. The percentages of LTRA that were left after 1-4 weeks of LTBMC in diverse hemato-lymphopoietic organs are shown in Table 7.4. As for the blood, LTRA measured in these organs dramatically declined after a culture period in LTBMC. Data from bone marrow, spleen, and thymus did not differ statistically except for the values in the spleen after 1 week of LTBMC (Student-Newman-Keuls test for multiple comparison, $\alpha_T < 0.05$).

The frequencies of LTRA cells among cultured and fresh BMC were compared in the CAFC assay (Table 7.3). It shows two important features. First, as was observed for *in vivo* LTRA, the frequency of *in vitro* LTRA decreased dramatically after culturing BMC in LTBMC. Second, the *in vitro* measurements of LTRA frequencies closely resembled the values obtained by *in vivo* measurements of LTRA, which determines the ability of a

BMC sample to induce stable 50% donor-type chimerism.

Discussion

We have studied the quantitative and qualitative properties of the long-term reconstitution capacity of BMC that were cultured in LTBMC for several weeks. It appeared that only a few percent of LTRA remained after 4 weeks of LTBMC. These remaining LTRA cells, however, had an equal quality to establish stable chimerism as compared to fresh BMC.

Long-term bone marrow cultures have gained large interest for their use as a source of normal HSC in BMT. Stem cells from murine LTBMC have proven to reconstitute lethally irradiated syngeneic, semi-allogeneic, and allogeneic recipients[10,19,27]. Because of the absence of mature B- and T-lymphocytes in LTBMC[10], transplantation with cells from these cultures may be comparable to the reconstitution of irradiated hosts with T-cell depleted BMC, which diminishes the development of a Graft-versus-Host (GvH) reaction. Indeed, stem cells from LTBMC can reconstitute the lymphoid compartment of normal irradiated[22,25] or untreated SCID mice[15], without evidence for GvH disease.

It has been found that long-term cultures initiated with BMC from patients with AML, CML and ALL fail to support the growth of leukemic cells, whereas normal progenitors readily develop[4,7,8,11,12,23,26,29]. Also, the promyelocyte leukemia cell line HL60 is not supported by human LTBMC adherent layers[21]. Cells from LTBMC of leukemic bone marrow have been used successfully in autologous BMT as a source for presumably leukemia-free stem cells[3,4,5,28,29]. Some authors have reported that a prolonged culture period (> 4 weeks) is required to remove all detectable leukemic cells.

Our observations show that BMC, cultured in LTBMC, retained their quality of establishing stable engraftment of blood, bone marrow, spleen, and thymus rather than leading to transient engraftment. At the same time, however, the frequencies of LTRA cells decreases dramatically over time. This indicates the restrictions of the use of this culture system in transplantation protocols, especially when purging of leukemic cells using LTBMC would necessitate prolonged culture times.

In our culture system on pre-established stromal layers BMC do not need to form their own micro-environment. The irradiated bone marrow-derived stromal cell layers were not inferior in their support of hematopoietic growth and development, compared to unirradiated stromas, as has been reported previously[6,16,24,30,31]. Therefore, the loss of LTRA can not be attributed to the lack of a (supportive) stromal layer at the time of inoculation. In addition, the enzymatic treatment used for the harvest of the adherent layer does not alter progenitor cell growth characteristics nor frequencies of primitive and less primitive stem cells, as measured in the CAFC-assay.

Genetic disorders of the hematopoietic system may serve as clinical targets for somatic cell gene therapy. In this respect the use of LTBMC for expansion of transplantable (human) HSC *in vitro* will have therapeutic applications. Expansion of the progenitor and stem cell pool in LTBMC has been reported by several authors, using viral infection of cells either in or preceding long-term culture. Viral leukemogenesis in LTBMC leads to an increase in pool size of multilineage HSC[9,13]. Infection of murine BMC in LTBMC with a recombinant Moloney leukemia virus containing the *src* gene derived from Rous sarcoma virus resulted in a dramatic increase in the production of CFU-S and CFU-C, which had expanded self replication potential[1,20]. No evidence was found for leukemic transformation of the hematopoietic cells, but the adherent stromal cells did transform. Using human BMC that were infected with retrovirus carrying the Tk-*neo* gene, a sustained production of transfected CFU-C and BFU-E was found in both the adherent and non-adherent fractions of LTBMC up to 6 weeks of culture[18]. Using the same construct to mark murine BMC, it has been observed that pluripotent HSC self-renew in LTBMC, giving rise to daughter stem cells that could reconstitute both lymphoid and myeloid systems of multiple recipients[14].
Our present data show that the largest part of LTRA is lost when culturing fresh BMC in LTBMC. The finding by Fraser and colleagues[14], that LTRA cells self-renew in LTBMC, suggests that the initial loss of LTRA cells in culture is even more dramatic. The low percentage of the remaining LTRA that we observed after 4 weeks of culture may therefore be the result of an even lower number of surviving LTRA cells complemented by an expansion of some of these LTRA cells. However, it has not been excluded that expansion of the transfected progenitor cell pool (self-renewal) was a result of the introduction of foreign genes in hematopoietic progenitor cells[2].
Our results extend data from Harrison *et al.*[17] that indicate that the number of primitive HSC with erythroid repopulation ability decreases in LTBMC.

In Chapter 5 we describe an *in vitro* assay for the quantification of hematopoietic stem and progenitor cells. It is shown, that in this assay measurements done at day-10 of culture strongly correlates with the day-12 CFU-S content of the BMC suspension tested, and that data obtained at day-28 correlate with pre-CFU-S activity. In this chapter we show further data that suggest a close correlation between day-28 measurements *in vitro* and LTRA as determined *in vivo*. This further extend and support our contention that the CAFC assay enables *in vitro* frequency analysis of a variety of HSC subsets, including CFU-S day-12 and HSC with MRA and LTRA *in vivo*.

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Chapter 8
The role of fibronectin in the adherence of hematopoietic stem cells to their micro-environment

Preface

The extravascularly located bone marrow stroma which forms the non-hematopoietic compartment of the bone marrow and which serves as hematopoietic micro-environment, is required for specific lodgement, proliferation, and differentiation of hematopoietic stem cells *in vivo*. The importance of the stroma is demonstrated in long-term bone marrow cultures (LTBMC), in which the hematopoietic development of progenitor cells is preferentially dependent on the formation of a bone marrow-derived stromal layer. This is demonstrated by the presence of the so-called cobblestone areas (CA) in these cultures. The hematopoietic micro-environment consists of cells and extracellular matrix (ECM). Collagens, proteoglycans, and fibronectin are some of the components of the ECM. The importance of the ECM is demonstrated by a study in which inhibition of the production of ECM components by stromal cells *in vitro* abolished hematopoiesis[305]. This chapter deals with one component of the ECM: fibronectin.

Fibronectin[145,173] can be considered a prototype cell adhesion protein. Since its discovery as a fibroblast cell-surface protein that had a counterpart circulating in the plasma, a variety of functional properties of fibronectin has been revealed. Some of its functions, like the binding of heparin, fibrin, collagen, and cells, can be located on specific domains in the glycoprotein, that are resistant to proteolytic treatment. Receptors on the cell surface mediating the adherence to fibronectin or other adhesive glycoproteins belong to the receptor superfamily of the integrins. Each receptor has its own glycoprotein specificity.
Fibronectin is a substantial element of the ECM of the bone marrow and thus of the hematopoietic micro-environment. It functions as an adhesive ligand between cells and matrix components and between different ECM molecules. Since it is involved in the adhesion of cells, we studied the interaction of the diverse hematopoietic stem cell subsets to fibronectin as a part of the complex interactions between hematopoietic stem cells (HSC) and the hematopoietic stroma.
The discovery of fibronectin, its origin, structure, tissue distribution, biological activities, molecular biology, and cell attachment determinants have been extensively reviewed[113,174,206,228,234,300]. The scope of the following paragraphs is to give a characterization of fibronectin with regard to its function in the attachment of cells in general, and to its role in the attachment of hematopoietic cells to their micro-

environment in particular. In addition, a description is given of the class of cell-surface adhesion receptors that mediate the adhesion of cells to matrix components. In the last paragraph a number of experiments on the adherence of murine hematopoietic stem cell subpopulations to fibronectin is presented. This paragraph is submitted for publication as:

Van der Sluijs JP, Baert MRM and Ploemacher RE (1993). Differential adherence of murine hematopoietic stem cell subsets to fibronectin.

8.1 Fibronectin

Origin and function

Fibronectin is probably one of the most versatile proteins known, both functionally and structurally. It is synthesized by fibroblasts[235], endothelial cells[17,119,161], peritoneal macrophages[123], certain epithelial cells[45,183,219], chondrocytes[49,83], astroglial cells[274], Schwann cells[144] and hepatocytes[265]. In tissues fibronectin is an ECM molecule rather than a cell-surface protein[282], It appears as aggregates and as fibrils. It is a major component of the matrix of connective tissues, especially of loose connective tissue[257], and is widely distributed throughout the body. It is present in the basement membranes of epithelium and endothelium, in striated and smooth muscle tissue, in the sinusoidal walls of the liver as well as in platelets and plasma[158,257,304]. In the stroma of hemato-lymphatic tissues fibronectin molecules form dense networks, branching throughout the tissue, except in the germinal centers of the spleen and lymph nodes[10,254,257,288]. Morphological studies of bone marrow revealed that hematopoietic colonies are associated with fibronectin-rich areas[288], and that fibronectin is situated at sites of cellular interactions between granulocytes and fibroblastic stromal cells[254]. A similar association could be demonstrated in ultrastructural examination of LTBMC[11]. In fibroblast layers *in vitro* fibronectin forms an extensive pericellular matrix, which mediates cell-cell and cell-substratum contacts[235].

The functional properties of fibronectin are related to cell-to-cell and cell-to-substrate adhesion, spreading (morphology), migration (chemotaxis), and (embryonic) differentiation of cells. Furthermore, it plays a role in promoting phagocytosis by monocytes/macrophages[16,82,217,293], cytokine production by monocytes[57,269], the forming of ECM protein networks (together with collagen and proteoglycans), and platelet aggregation and thrombus formation. Most cells need attachment to survive and to proliferate, as shown by the fact that cells growing *in vitro* usually require an attachment factor. The serum used for cell culture provides such factors. It contains at least fibronectin and vitronectin, which each can support attachment[88,89].

The property of fibronectin to bind to cells, together with its binding capacities to a

number of other ECM proteins, like collagens[64], glycosaminoglycans and proteoglycans[200,256], and fibrin(ogen)[175,176], gives fibronectin the image of a cell attachment-promoting constituent of ECM and blood clots.

The fibronectin molecule

The fibronectin molecule is composed of two similar but not identical polypeptides, held together by two disulphide bonds near the COOH-termini, forming a dimer with a molecular weight of about 450 kD[249]. The complete amino acid sequence has been determined from cloned cDNA[139] and from the protein itself[249]. The fibronectins from various species show a high degree of sequence homology[2,5]. The general structure of

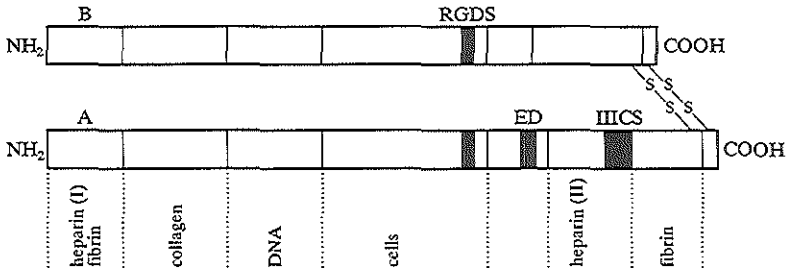

Figure 8.1. Schematic representation of the fibronectin glycoprotein. Note the various binding sites on the molecule, and the sites of alternative splicing.

fibronectin is shown in Figure 8.1. The polypeptide is composed of three different types of homologous repeating units in the amino acid sequence (homology types I, II and III)[140,141,201,251]. These appear as loop structures. Altogether, fibronectin contains nearly 2500 amino acids. The fibronectin glycoprotein contains 4-6 carbohydrate moieties, consisting of oligosaccharides linked to asparagine[188]. The carbohydrates contain terminal galactose, sialic acid, and fucose residues[35,188,301]. These carbohydrates protect the fibronectin polypeptide against proteolysis[12,303], and can affect its binding functions[303]. They do not play a role in the secretion of fibronectin[188,189].

The morphologic appearance of the fibronectin molecule is as an elongated chain when examined by standard techniques of molecular electron microscopy[61,66]. Physical measurements, however, indicate that the molecule is folded into a flexible, oblong structure[224,291].

There are structural differences between plasma fibronectin, produced by hepatocytes[265], and the so-called "cellular" fibronectin, isolated from cultured cells or from tissues[265,297]. The diverse forms of fibronectin are generated by transcription of a single gene into a common precursor mRNA which undergoes alternative splicing[13,139,184,198,242,243,265,272,278]. The regions of the protein in which this variation occurs are the ED site (the 5th type-III homologous segment, seen from the COOH-terminus), and the type-III connecting

segment (IIICS), connecting the second last and the last type-III homologies near the COOH-terminus[139,243,272] (see Figure 8.1). These differences occur only in the A chain of the fibronectin dimer. The fibronectins throughout the body, therefore, consist of a common B chain and one of the A chains that vary as a result of alternative splicing. The heterodimeric fibronectin molecules are thought to be formed from a fibronectin polypeptide pool. This assumption is consistent with the observed heterogeneity of cellular and plasma fibronectins found *in vivo*[139].
Alternative splicing of a single fibronectin encoding pre-mRNA may affect the functions of fibronectin, as we will see later. It may also protect the glycoprotein when the alternatively expressed segments contain new glycosylation sites[241,250,302]. In agreement with the small structural differences, fibronectins from different sources are remarkably similar in their functional properties.

Fibronectin can be cleaved into functional domains by controlled proteolysis. The many binding sites in the fibronectin polypeptide are located in separate domains. These include the binding site for fibrin (I), heparin (I), and Staphylococcus in one domain, and for collagen, cells, heparin (II), and fibrin (II) each in separate domains (see Figure 8.1)[85,201,233,244].

Attachment of cells to fibronectin
Binding of cells to fibronectin is mediated by a variety of mechanisms, which are discussed here in detail.
The domain of fibronectin that promotes the attachment of cells is in the middle portion of the glycoprotein. It contains the sequence L-arginyl-glycyl-L-aspartate (Arg-Gly-Asp = RGD)[¶] (see Figure 8.1), essential for cell binding. The role of this sequence was demonstrated by making progressively smaller fragments of the cell binding domain and testing these fragments together with synthetic oligopeptides reproducing the amino acid sequences of such fragments on their cell binding capacities[204,205,207,209]. When coated onto a surface, the fragments and synthetic peptides containing the RGD-sequence promote cell attachment, albeit less effective than the full fibronectin molecule[¶¶]. In solution, RGD-containing peptides inhibit the attachment of cells to a fibronectin coated surface[4,87,206,207,208,298,299]. Changes in the RGD-sequence eliminates the cell binding activity of the peptides[4,207,208]. Surprisingly, however, it has been observed that the reverse sequence, i.e., Ser-Asp-Gly-Arg (SDGR), is similar in its inhibitory activity of cell attachment to fibronectin than the forward tetrapeptide sequence[299]. The tetrapeptide

[¶] Also referred to as the tetrapeptide Arg-Gly-Asp-Ser (RGDS)

[¶¶] Most of the cell attachment experiments were performed with fibroblastic cell lines of various origin. In several studies cell types were chosen with different fibronectin-binding characteristics (e.g., baby hamster kidney fibroblasts and murine melanoma cells).

sequence in fibronectin is located in a segment of the glycoprotein that is likely to form a hydrophilic loop at the surface of the molecule, which seems to make it available to interaction with cells[41,146,207]. The RGD site has also shown to be functional in macrophage movement in fibronectin-fibrin gel matrices[149]. Moreover, macrophage phagocytosis could be inhibited by RGD peptides[294].

The RGD sequence has been found a cell recognition site of a number of other ECM and platelet adhesion proteins. These include vitronectin[88,261], collagen type I[48], fibrinogen[75,216], von Willebrand factor[216], osteospontin[187], thrombospontin[154], and possibly laminin[105]. Despite the similarity of the RGD cell attachment sequence in the various adhesive proteins, cells are able to recognize these proteins individually through their cell-surface receptors.

The RGDS containing central cell binding domain of fibronectin is recognized by the integrins $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$, and $\alpha_v\beta_5$ of various cell types (see Table 8.2).

For a limited number of cell types (such as neural crest cells and their derivatives, melanoma cells, lymphocytes, and monocytes) adhesion to and spreading on fibronectin are mediated by additional cell binding regions located in the alternatively spliced IIICS region of the protein, while other cell types will not adhere to these sites[107,108,166]. The IIICS region of human fibronectin consists of 120 amino acids and contains two spliced sites. The N-terminal CS1 region of IIICS comprises an entire spliced segment and has a length of 25 amino acids[107]. This region represents the major site of interaction of fibronectin with melanoma cells[108]. The minimal active sequence is Leu-Asp-Val (Leucine-Aspartate-Valine = LDV)[138]. This sequence is completely conserved in the IIICS regions of human, rat, bovine, and avian fibronectins[138]. The truncation of the CS1 domain to the tripeptide LDV produced a progressive loss of its high activity. This indicates that, apart from the minimal sequence, additional sequences in the regions are essential for full activity.

The other part of this cell type-specific adhesive activity located in the IIICS domain is mediated by the Arg-Glu-Asp-Val tetrapeptide sequence (Arginine-Glutamate-Aspartate-Valine = REDV), contained in the CS5 site at the carboxyl-terminus of IIICS[13,107,108,178]. This site is mutated to Arg-Gly-Asp-Val (RGDV) in rat and bovine fibronectin[243]. CS5 is only present in very low levels in the plasma fibronectin used for adherence assay[598]. The 25 amino acids residue CS1 is only 2-3 fold less active in promoting melanoma cell spreading than intact fibronectin, on a molar basis[108]. In contrast, the CS5 residue is 230-fold less effective in melanoma cell spreading than intact fibronectin. Both sites are recognized by the $\alpha_4\beta_1$ (VLA-4) integrin.

CS1 and CS5 are each found in distinct alternatively spliced regions of the IIICS, and can function separately. These sequences are unique to fibronectin. The IIICS region is only contained in the large (A-) subunit of fibronectin[198,242]. Among the different fibronectins, there are molecules containing either CS1 alone, CS5 alone, neither

peptides or both peptides.
A salient detail may be, that the pentapeptide GRGDS is able to block the spreading of murine melanoma cells on CS1 and CS5 coated substrates[¶], due to inhibition of $\alpha_4\beta_1$ interaction with the IIICS fragments[178]. Larger RGD-containing peptides, however, will not inhibit binding to and spreading on IIICS ligands, nor will CS1 peptides do on the RGD-containing central cell binding domain of fibronectin. This indicates that inhibition of adherence to fibronectin by this pentapeptide may not only act on the RGD-containing cell binding site, but also on the CS1 and CS5 mediated cell adhesion. These observations suggest that adhesion of cells to both the central cell binding domain and the IIICS fragment show similar mechanisms of interaction with $\alpha_5\beta_1$ and $\alpha_4\beta_5$, respectively, since RGD-containing oligopeptides block the function of both domains. However, in the native fibronectin molecule these cell binding sites do not compete with each other, because of their different receptor binding. In contrast with the above observations, other studies report that the IIICS receptor-ligand interactions are RGD-independent[74,156,167,285], or only slightly sensitive to RGD peptides[81]. These discrepancies may be due to the types of adherence assay and/or the concentrations of blocking peptides used. To date, it is unclear to what extent the various sites of fibronectin involved in the binding of cells interact[55].

The adhesion-promoting qualities of the mature fibronectin glycoprotein may function under complex post-transcriptional regulation. Recently, it has been reported that alternative splicing of the fibronectin pre-mRNA may be used during wound healing as a mechanism for the biosynthesis of the various forms of fibronectin that are more appropriate for the cell migration and proliferation associated with tissue repair[68]. In addition, both the RGDS-containing cell binding domain and the IIICS are required in association, each with its functional specificity, to permit effective locomotion[55]. Cell type-specific expression of alternatively spliced human fibronectin IIICS mRNA has been described[98]. The types of fibronectins located in the hemato-lymphopoietic organs, however, are unknown. Whether different types of fibronectins in the bone marrow have any function, either qualitatively or quantitatively, in the regulatory mechanisms of localization of hematopoietic progenitor cells and HSC, remains to be discovered.
Some lymphoid cell lines adhere preferentially to the central cell binding domain of fibronectin, whereas other lymphoid cells adhere mostly to the carboxyl-terminal IIICS[156]. Subpopulations of fibronectin-adherent and non-adherent cells has been found in thymocytes[32], activated T-cells[143], and pre-B lymphocytes[14].

A cell recognition sequence was found in the Hep II domain, designated H1. This site

[¶] CS1 and CS5 are coated to tissue culture plastic an CS1-IgG and CS5-IgG peptide-protein conjugates.

lies outside the regions of alternative splicing and is therefore present in all fibronectin isoforms[177]. The active site proved to be Ile-Asp-Ala(-Pro-Ser) (isoleucine-aspartic acid-alanine(-proline-serine) (= IDA(PS)). It probably resembles the LDV(PS) sequence of the active site in the CS1 segment of the IIICS. The IDA(PS) site is recognized by the $\alpha_4\beta_1$ (VLA-4) integrin.
As for LDV, IDA alone is not sufficient to support (melanoma) cell spreading, suggesting that the secondary and tertiary structures of the peptides are important for the performance of their function.
The functional relevance of H1, like for CS5, is not yet clear, since their adhesive activities are far lower that of CS1. Their function may concern 1) stabilizing of CS1-dependent adhesion (because of a close spatial relationship of CS1 with CS5 and H1), 2) transduction of different signals to the cell interior, and 3) difference in effects on migration and immobilization.

The interaction of fibronectin with cells is affected by the binding of proteoglycans[157,182,229¶] to fibronectin[118,166,200,292]. Proteoglycans contain several glycosaminoglycans (GAGs) side chains like heparin, heparan sulphate, hyaluronate, dermatan sulphate and chondroitin sulphate, by which they bind to the heparin binding sites of fibronectin. Heparin is the most highly sulphated GAG, and therefore binds more strongly to fibronectin than do other GAGs[134,232]. Despite a weaker interaction between chondroitin sulphate, hyaluronate or dermatan sulphate and fibronectin, proteoglycans containing these GAGs also interact with fibronectin[23,137,151,223]. This heparin-dependent adhesion of cells to fibronectin is mediated by two sequences in the COOH-terminal heparin-binding domain (Hep II), FN-C/H-I and FN-C/H-II[84,165,167]. These sequences bind heparin and support the attachment of some cell types in association with the $\alpha_4\beta_1$ (VLA-4) integrin. The adhesion to these sequences is probably mediated by cell surface heparin sulphate

¶ Proteoglycans are a group of complex carbohydrates which form a substantial amorphous component of the ECM, especially in connective tissues. They consist of a core protein to which one or more glycosaminoglycan (GAG) chains are covalently bound. These GAGs are made up of linear polymers of repeating disaccharide units containing a derivative of an amino sugar, either glucosamine or galactosamine. At least one of the sugars in the disaccharide has a negatively charged carboxylate or sulphate group. The major GAGs are hyaluronate, chondroitin sulphate, keratan sulphate, heparan sulphate, and heparin. The functions of proteoglycans largely depend on their GAG content. The appearance of GAGs within tissue is as components of proteoglycans. Therefore, studies on the biological role of GAGs can only be speculative in predicting their function *in situ*. To give an impression of the size of proteoglycans, a typical proteoglycan in cartilage tissue contains about 80 GAG chains, weighing each 20 kD. With an average MW of the core protein of 250 kD, the entire complex weighs about 1850 kD, and has a length of about 300 nm. Often these proteoglycans themselves are aggregated non-covalently to long filaments of amino sugars (hyaluronic acid). This binding is promoted by a small link protein. The entire complex may then have a length of several microns. These very large poly-anions bind water and cations and thereby form the ground substance of the (connective) tissue. Additionally, they have the capacity for multiple interactions with other molecules. They bind together ECM components, cells and matrix, and capture soluble molecules into the matrix and at cell surfaces.

proteoglycans. An additional site within the Hep II fragment, FN-C/H-III, binds directly to chondroitin sulphate-containing proteoglycans, and promotes the adhesion of melanoma cells in coordination with the $\alpha_4\beta_1$ (VLA-4) integrin[115].
Cell surface proteoglycans, either anchored in the cell membrane by the hydrophobic regions of the core protein[185], or attached to a proteoglycan cell-surface receptor[132,171] can augment the cell-fibronectin interaction by binding to the heparin binding sites of fibronectin[117,118,152,292], whereas soluble proteoglycans can inhibit cell attachment by interfering with this interaction[23,137,223]. Thus, cells interact with fibronectin at the RGD cell attachment site, at CS1, CS5, H1 and at the heparin binding sites. The specificity of the interaction seems to come from the RGD or CS1 site, while binding at the heparin binding sites plays an augmenting role. Both the heparin and chondroitin sulphate containing proteoglycans enhance the binding of fibronectin to collagen[120,122,232], and cause precipitation of such complexes[186].

The above described mechanisms may have contributed to the observed need for heparan sulphate for the adhesive properties of human early hematopoietic progenitor cells and the bone marrow ECM[79]. Besides, fibronectin may not be the only ECM component to which hematopoietic progenitor cells attach[44].

Participation of fibronectin in the extracellular matrix
The ECM is formed by complex interactions between a large variety of ground substance molecules, of which only a few are discussed in this chapter. The assembly of a fibronectin matrix as a component of the entire ECM is hypothesized as follows[168].
The RGD receptor initiates the deposition of fibronectin into fibrils by binding fibronectin to the cell surface. Additional steps are needed to elongate these fibrils. These include a fibronectin-fibronectin interaction, and a crosslinking of adjacent fibronectin molecules to one another by disulphide bonding. A fibronectin-fibronectin *inter*chain site has been found near the collagen binding domain[103], and the cross-linking occurs through rearrangements of *intra*molecular disulphide bonds in the NH_2-terminal domains of adjacent fibronectin molecules into *inter*molecular bonds[169].
Fibronectin binds to all types of collagen[62,63,64], a glycoprotein of great importance for the structure of tissues by its presence in all basement membranes and extracellular matrices. This binding is not inhibited by heparin and other GAGs[64,231]. Their mutual affinity is undoubtedly reflected in the well-ordered distribution of fibronectin and collagen in the ECM[21,22,273]. Additionally, fibronectin mediates effectively the attachment of cells to collagen[40,133,199]. This may reflect its function *in situ*. Furthermore, fibronectin interacts with proteoglycans, a major substance of the ECM. Proteoglycans have the ability to interact with most of the other components of the ECM. They may facilitate the assembly of macromolecular complexes of ECM proteins. Such complexing may play a role in the deposition and anchoring of fibronectin into a matrix. However,

participation of cells is also required for the matrix deposition, as is the interaction of fibronectin with cells through receptors[228].

The balance in the role of fibronectin in promoting anchorage and migration of cells appears to be delicate. A cell has to make and break its adhesive contacts constantly to be mobile. For example, fibronectin is important in embryonic cell migration[25,51,53,54,55,65]. A cell that lacks both the matrix and the receptors for matrix proteins is likely to be a circulating cell. Immature erythroid cells resident in the bone marrow possess receptors for fibronectin and attach to fibronectin-coated surfaces, whereas circulating erythrocytes do not[197]. The expression of the fibronectin receptor, and most probably other adhesion receptors, may be a determining factor in keeping the immature hematopoietic cells attached to the bone marrow stroma until they are differentiated into mature cells, to be released into the circulation. In addition, primitive HSC might temporarily loose their fibronectin receptors in favor of the repopulation of depleted bone marrow locations, e.g., after irradiation or treatment with cytostatic agents.

For *in vitro* studies, an attachment protein can be active if it is pre-adsorbed to a *substrate* (e.g., polystyrene tissue culture plastic). The non-bound protein is washed away and the substrate-adsorbed fraction is tested for its activity[174,296]. The protein mediates cell attachment to and spreading on the substratum. If present in *solution* at sufficient concentrations, the same attachment protein can theoretically saturate all cell surface receptors, resulting in insufficient numbers of free receptors to bind to substrate-adsorbed protein. This is the case for fibronectin. A similar competitive inhibition of fibronectin-mediated adhesion is found with the cell binding domain of fibronectin[298], or even with synthetic peptides like those containing the RGD(S) sequence. The competitive inhibitory activity of the fragments and synthetic peptides vary, depending on the assay system and the target cells. The amount of substrate-fibronectin has striking effects on the ability of a peptide to competitively inhibit adhesion[298].

The primary functional form of fibronectin is the insoluble protein. In this form fibronectin interacts with cells. Since the K_d of fibronectin (8×10^{-7} M $\approx$ 0.360 mg/mL) is just above the concentration of soluble fibronectin in plasma (0.3 mg/mL), there is little binding of fibronectin to its receptors in plasma[3,121,164]. However, plasma fibronectin can become incorporated into the ECM[89].

It has been reported that sialylated gangliosides interfere with the adhesion of cells to fibronectin[135]. A ganglioside-binding domain on fibronectin has been localized, distinct from the RGDS cell-binding site[268].

8.2 Integrins

The adhesion of cells to fibronectin involves multiple determinants on the glycoprotein that react with discrete cell surface receptors. These cell-surface receptors belong to the superfamily of the integrin adhesion receptors. This integrin family consists of a series of related αß heterodimers involved in a variety of cell-matrix and cell-cell adhesion functions. The name "integrin" was chosen to denote an integral membrane complex forming the physical transmembrane connection between the ECM and the cytoskeleton[264]. This transmembrane relationship plays significant roles in cellular adhesion, morphology, and migration. One or more integrin types appear on nearly all cell types, including hematopoietic cells.

The integrin molecule

All cell-surface receptors of the integrin superfamily share several structural properties[109,110] (Figure 8.2). Integrins are composed of two subunits, α (120-180 kD) and ß (90-110 kD), which are combined in a non-covalent complex. Each subunit has a short C-terminal cytoplasmic domain, a transmembrane segment, and a large N-terminal extracellular domain. The ß subunit contains areas of extensive intrachain disulphide bonding. In several cases the α subunit consists of a heavy and a light chain, linked by

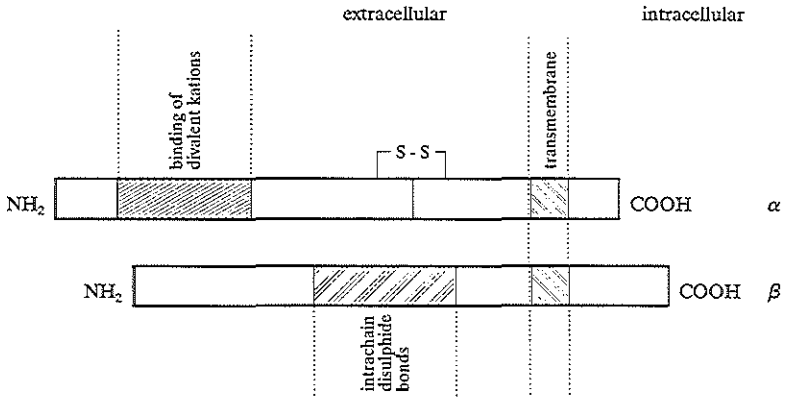

Figure 8.2. Schematic representation of an integrin molecule.

disulphide bonds. The α and ß subunits of a given integrin are not structurally related. To date, eight homologous but distinct ß subunits and fourteen different α subunits have been sequenced. The ligand-binding specificity of the αß dimer depends on the identity of the subunits present in an integrin[106,230]. Individual integrins can often bind to more than one ligand[130]. Additionally, individual ligands are recognized by more than one

integrin. However, different integrin subunits mediate different cellular responses to a common ligand[36,60,286].

From the different human α subunits the complete amino acid sequences are described, the overall similarity between these subunits being 20-30%. However, their short C-terminal cytoplasmic domains have very little mutual homology. This suggests the possibility of unique cytoskeletal interactions for each type of α subunit[92]. The individual α subunits between different species, however, are remarkably similar[20]. Between the human integrin $ß_1$, $ß_2$ and $ß_3$ subunits an amino acid sequence similarity of 44-47% exists, especially in the C-terminal half[7,69,131,153,226]. An 82-90% amino acid similarity between $ß_1$ subunits from human, chicken, frog, and mouse integrins suggests a high conservation of $ß_1$ integrin subunits across species.

Association of the α and ß subunits is required for the cell-surface expression of integrins. The synthesis of the α subunit is rate-limiting and seems to regulate the appearance of ß on the membrane[90].

Both subunits are transmembrane glycoproteins. Studies on the structural appearance of integrins revealed a model in which the N-terminal extracellular regions of α and ß subunits were present in an globular head. Each subunit has a hydrophobic transmembrane segment and a short (≤ 50 amino acids) intracellular C-terminus[34,126,180]. The cytoplasmic domains interact with cytoskeletal proteins and other cytoplasmic components[28,52,86,104,162,192,253]. An outline of the structural, functional and evolutionary relationships of the integrins has been given by Hynes (1992)[110].

Integrins are fairly resistant to proteolytic treatment of intact cells. Divalent cations are essential for receptor function in both affinity and specificity for ligands, and the association of the α and ß subunits[71,128,129]. Both α and ß subunits contribute to the ligand-binding site of the integrin that is located on the interface between the two subunits.

Integrins and the adhesion to fibronectin

The integrins have been ordered into distinct subfamilies. This distinction is based on the utilization of the ß subunits (see for a comprehensive survey: Hemler (1990)[92], and Hynes (1992)[110]). Members of the $ß_1$ subfamily each contain the $ß_1$ subunit in association with one out of nine α subunits. Three types of α subunits associate with $ß_2$, which is a leukocyte-specific ß subunit[8,9,150], and two α subunits with $ß_3$. Although another five ß subunits have been discovered, they are not important with regard to the scope of this chapter, namely, an outline of the role of fibronectin and its receptors in the attachment of hematopoietic stem cells to its micro-environment, and therefore will not be discussed here.

The β_1 integrins function as adhesion receptors for ECM and as cell-cell receptors[¶]. They contain nine different α subunits, and include the subfamily of the *very late activation* (VLA) antigens. A summary of some properties of the different VLA antigens is given in Table 8.1.

Table 8.1 Integrin subfamily of very late antigens of activation (VLA)

	subunits	ligand	distribution
VLA-1	$\alpha_1\beta_1$	col*, lam	widespread
VLA-2	$\alpha_2\beta_1$	col, lam	widespread
VLA-3	$\alpha_3\beta_1$	col, lam, fn	widespread
VLA-4	$\alpha_4\beta_1$	fn, cells	lymphocytes, monocytes, hematopoietic cells
VLA-5	$\alpha_5\beta_1$	fn	widespread
VLA-6	$\alpha_6\beta_1$	lam	widespread

* col = collagens
lam = laminin
fn = fibronectin

The name "VLA" originates from the discovery of VLA-1 and VLA-2 as antigens that appear very late (2-4 weeks) after activation of T-lymphocytes by antigens or mitogens[95,97], but is of no relevance to the other VLA proteins. In addition to the 6 VLA proteins listed in Table 8.1, three other β_1 integrins have been described. The $\alpha_7\beta_1$ integrin binds to laminin, the binding properties of $\alpha_8\beta_1$ are unknown, and the $\alpha_v\beta_1$ integrin binds to the RGD site of vitronectin and probably of fibronectin[19,280]. Nearly all hematopoietic cell types express one or more (activated) VLA molecules, with the exception of granulocytes and red blood cells[91].

For the attachment of hematopoietic cells to fibronectin our attention is drawn by VLA-3, VLA-4, and VLA-5, although also $\alpha_v\beta_1$, $\alpha_{IIb}\beta_3$, $\alpha_v\beta_3$, $\alpha_v\beta_6$, and $\alpha_4\beta_7$ are involved in the adherence of cells to fibronectin[¶¶]. Other adhesive properties of integrins, like those mediating lymphocyte homing or extravasation of circulating leukocytes, are reviewed elsewhere[15,29,30,33,56,72,110,111,190,246,258].

VLA-3 ($\alpha_3\beta_1$) appeared to be a fibronectin receptor[263,283], but it does not bind to fibronectin in the presence of VLA-5[60,126]. The cellular expression of VLA-3 is upregu-

[¶] Studies on adhesion *receptors* have been carried out mostly on fibronectin and synthetic peptides, platelets, leukocytes adhesion and in embryonic development.

[¶¶] α_v is the α-subunit of the vitronectin receptors; $\alpha_{IIb}\beta_3$ is the platelet glycoprotein GPIIb/IIIa.

lated in the presence of fibronectin[221]. VLA-3 also functions as a collagen receptor[60], and as a receptor for laminin[58,76], and is present at intercellular contact sites[125]. Publications differ as to whether or not this receptor recognizes RGD[60,112,284]. The expression of VLA-3 is independent of cell lineage, but is positively correlated with the presence of fibronectin, and probably the other ligands. Loosely adherent or circulating cells, including lymphocytes, are negative for VLA-3[222]. In human LTBMC it is expressed on fibroblasts and endothelial cells, and on CFU-F[252].

VLA-4 ($\alpha_4\beta_1$)[96] is present on about 40% of the nucleated cells of the bone marrow, both in the mouse[290] and in the human[252], and on $CD34^+$ human bone marrow cells[267]. It is highly expressed on immature blasts and erythroid progenitors[227], on T- and B- lymphocytes, and on monocytes. In addition, it is expressed on the monocytic-macrophagic cells of the adherent layer of human LTBMC[252], and on myeloid and lymphoid cells in murine LTBMC and Whitlock-Witte cultures[172]. It appears as a receptor for non-RGD cell binding sites in fibronectin[74,81,177,178,179,181,285]. The integrin VLA-4 recognizes specifically the CS1 and CS5 sites within the alternatively spliced IIICS region of fibronectin. Only cells that bear $\alpha_4\beta_1$ are capable of recognizing the IIICS, of which the minimal essential sequences are LDV (CS1) and REDV (CS5). In addition, VLA-4 binds to the H1 site of the COOH-terminal Hep II domain of fibronectin, of which the sequence IDA(PS) represents the active site[177]. VLA-4 has been described as a cell-cell adhesion receptor on lymphocytes, facilitating the attachment to the endothelium of the high endothelial venules of Peyer's patches[100,101,102]. It is a receptor for VCAM-1 (=Vascular Cell Adhesion Molecule-1), a cell surface adhesion molecule[59] found on lymphokine-activated endothelial cells and containing a LDV sequence[191]. Anti-VLA-4 antibodies block the interaction between T-helper and T-suppressor cells[80]. It is being hypothesized that VLA-4 dependent cell-cell adhesion is mediated by fibronectin, especially after the observation that T-cells synthesize fibronectin-like material[77,78].

VLA-5 ($\alpha_5\beta_1$) is the prototype fibronectin receptor[7,26,99,218]. It is the major receptor for the RGDS site of the central cell binding domain of fibronectin, although several other integrins also recognize this domain[106,230]. The murine α_5 subunit appears to be 90% homologous to its human equivalent[99], indicating cross species conservation. It is present on a subpopulation of T-lymphocytes and on monocytes[92,136,163,285], platelets[93,142,210,284], and follicular dendritic cells[238]. It is further expressed by erythroid progenitors[227,281] and by $CD34^+$ human bone marrow cells[267]. It mediates the attachment of monocytes to fibronectin[26,73,275], which can be blocked by RGD containing peptides as well as by anti-VLA-5 antibodies[26].
The VLA-5 integrin has been found to recycle in an endocytic-exocytic cycle[24]. This suggests a mechanism by which fibronectin binding cells migrate along the substrate by removing and replacing fibronectin receptors on its surface membrane.

Despite the presence of VLA-4, VLA-5 (and VLA-6) on the surface of circulating T-cells, these cells attach minimally to fibronectin or laminin[6]. Reversely, T-cells hardly express VLA-1, VLA-2 and VLA-3 on their surfaces, but still attach to collagen, albeit moderately[6]. Upon *in vitro* activation of T-cells, their ability to attach to fibronectin is increased[6,143], reflecting an increased VLA-5 expression[92]. Monocytes, however, expressing large amounts of VLA-6 and moderately VLA-4 and VLA-5, attach firmly to laminin and moderately to fibronectin, but not to collagen, despite the presence of VLA-1, VLA-2 and VLA-3[270]. Macrophages only adhere to laminin following phorbol myristate acetate (PMA) treatment[170]. Even an increased expression of a particular integrin heterodimer[260,262] does not necessarily indicate that it will be functional[58,148]. This may be due to loss of glycosylation[262,275] or phosphorylation[27,46] of the receptor, the absence of divalent cations[71,255], or the presence of gangliosides[268].

Apart from VLA-3, VLA-4, and VLA-5, another fibronectin receptor has been identified on monocytes[26]. It is involved in the enhancement of phagocytosis mediated by fibronectin. The receptor contains a β_3 subunit and its binding depends on the RGD site of fibronectin. This receptor is also found on granulocytes. Other integrins that are involved in the cellular attachment to fibronectin are IIb/IIIa ($\alpha_{IIb}\beta_3$) on platelets[202], VNR ($\alpha_v\beta_3$) on endothelium[39], and VNR_{alt} ($\alpha_v\beta_5$) on some carcinoma cells[38]. These receptors all attach to the RGD cell binding domain of fibronectin.
The expression of some integrins is upregulated in the presence of cytokines[47,90,114].

VLA-4 and VLA-5 are involved in the CD3-dependent $CD4^+$ T-cell activation through interaction with the CS1 domain and RGDS-containing cell binding domain of fibronectin, respectively[181], although in this case the VLA-5/RGDS interaction is required for the VLA-4/CS1 recognition. CS1 does not only promote T-cell adhesion, it also stimulates $CD4^+$ cell proliferation[181].
Transfection of the human erythroleukemia cell line K562, expressing VLA-5, with α_4 cDNA resulted in cells expressing both VLA-4 and VLA-5 in approximately equal levels. Their adhesion to intact plasma fibronectin, however, was almost completely inhibited by anti-VLA-5 antibodies, but not by anti-VLA-4[59]. These experiments again suggest that adherence of cells to fibronectin is dominated by $\alpha_5\beta_1$/RGDS over $\alpha_4\beta_1$/CS1, although the CS1 site can promote cell adhesion independently from the RGDS domain[179]. In this case, it has to be noted that a fibronectin dimer always contains two RGDS cell binding domains, while the CS1 site is only present in the A-chain, if not spliced out. The molar ratio of CS1 and RGDS in human plasma fibronectin, therefore, is less than 1:2. This may be one reason for the dominance of the $\alpha_5\beta_1$/RGDS interaction.

The invasion of the embryonic thymus with T-cell progenitors appears to depend on

fibronectin and laminin[237]. It can be inhibited by both RGD peptides and anti-β_1 integrin antibodies. Early thymocytes ($CD4^-/8^-$ and $CD4^+/8^+$) adhere to fibronectin[31], as do pre-B-lymphocytes during maturation in the bone marrow[14]. These capacities are lost upon release into the circulation. The same goes for maturing red blood cells, of which the capacity to adhere to fibronectin is lost together with a decrease in VLA-5 expression on the cell-surface[197].

Individual cells can vary in the expression of their integrins or modulate the binding properties of these integrins, thereby changing their adhesive properties[42,129,130]. In addition, the specificity and affinity of an integrin is not always constant. Both activation and deactivation are elegantly demonstrated in the $\alpha_{IIb}\beta_3$ integrin on resting circulating platelets[127,203], on the extravasation of circulating neutrophils and monocytes[33,190], on antigen-presentation to T-lymphocytes[56], and on the homing of T-cells[258]. Activation of integrins is triggered by activating proteins or by the ligand[50]. The activation of T-cells by antigen leads to activation of $\alpha_2\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$ without changes in surface levels[37,247,248,289]. The effect is an increased adhesion of the activated T-cells to fibronectin, collagens, and laminin. Activation is accompanied by conformational changes of the extracellular domain of the integrins, which is extended on ligand binding[70]. An integrin binds to its ligand only in the activated state. Effective activation stimuli vary depending on the integrin and on the cell type.
Also, deactivation of $\alpha_5\beta_1$ has been described[1,46]. The exact mechanisms underlying these phenomena are as yet unclear.

One of the intracellular events triggered via integrin-fibronectin binding on fibroblasts, lymphocytes, and endothelial cells is the elevation of cytoplasmic pH. This correlates with the parallel stimulation of spreading and growth of the cells on the substrate[116,239,240]. Another intracellular effect of integrin-ligand binding is tyrosine phosphorylation[245]. Furthermore, the binding of fibronectin to $\alpha_5\beta_1$ (VLA-5) on T-cells leads to the induction of the AP-1 transcription factor necessary for the transcription of interleukin-2[295].

The wide variety of integrin distribution and the versatility of its functions lead to the conclusion that integrins play many roles in many cells. They are not simply adhesive molecules on cell surfaces. When focusing only on the fibronectin-integrin interactions, lots of interactions, regulation properties and functions are possible, leading to complex mechanisms. This also includes the adherence of HSC to fibronectin.

In summary, there are seven possible sites on fibronectin to which a cell may adhere. These are listed in Table 8.2. The mutual relationships and physiological relevance of some of these sites are not clear. The most important mechanisms for cell-to-fibronectin

Tabel 8.2 Cell attachment sites on fibronectin

Cell attachment site	cellular receptor
RGDS	$\alpha_3\beta_1$(?) $\alpha_5\beta_1$ $\alpha_v\beta_3$ $\alpha_{IIb}\beta_3$ $\alpha_v\beta_5$
CS1	$\alpha_4\beta_1$
CS5	$\alpha_4\beta_1$
H1	$\alpha_4\beta_1$
FN-C/H-I	1)
FN-C/H-II	1)
FN-C/H-III	2)

1) transmembrane-bound proteoglycans
proteoglycans, attached to a proteoglycan cell-surface receptor
2) chondroitin sulfate proteoglycans

attachment are $\alpha_5\beta_1$/RGDS and $\alpha_4\beta_1$/CS1, which can function independently. However, when acting together the $\alpha_5\beta_1$/RGDS mediated adhesion will dominate. The other mechanisms may have a modulating role.

8.3 Interactions of hematopoietic cells with fibronectin

Fibronectin alone cannot support hematopoiesis[220]. It provides anchorage for stromal cells, other ECM components like proteoglycans, and hematopoietic cells both *in vivo* and *in vitro*. Apart from its key function in the structural and functional properties of the hematopoietic micro-environment, its function is very much dependent on the specific fibronectin-receptors, the integrins. Cells expressing one or more fibronectin-recognizing integrins are able to bind to the fibronectin of the hematopoietic micro-environment. Whether this binding augments specific HSC-stromal cell binding mediated by other cell adhesion molecules (CAM)[155,159] and lectin/carbohydrate interactions[266], or represents at least part of the specific interaction between HSC and stroma, has, as yet, not been clarified.

The distribution of the various fibronectin splicing variants in the bone marrow tissue may play a role in the homing and specific localization of intravenously injected HSC during bone marrow transplantation. Hematopoietic progenitor cells may utilize multiple integrin-mediated adhesion pathways to localize specialized micro-environmental niches in the marrow stroma. Of course, this can only be speculative, because little is known about the distribution of the various fibronectin splicing variants in hematopoietic

organs, although specific spatial distribution of distinct hematopoietic progenitor subsets has been reported[147,160,211].

The role and nature of the interactions of hematopoietic progenitor cells and HSC with fibronectin, a component of the hematopoietic micro-environment both *in vivo* and *in vitro*, is largely unknown. An important finding was the association of the loss of adhesion to fibronectin with the loss of cellular adhesion to hematopoietic stroma during erythroid differentiation (see below). Other studies were concentrated on the involvement of fibronectin-cell adhesion in the homing of pre-T-cells into the thymus, and their subsequent development and differentiation. Evidence is accumulating that the integrin $\alpha_4\beta_1$ (VLA-4) is involved in the adhesion of primitive progenitor cells to fibronectin.

Of all the differentiated mature blood cells only subpopulations of (activated) T-cells and B-cells adhere to fibronectin. In the case of granulocytes and monocytes, these cells only adhere to fibronectin after activation, e.g., after binding to VCAM-1 on (activated) endothelial cells. Platelets, when not activated, do not adhere to fibronectin. Mature red blood cells also do not adhere to fibronectin. In summary, most circulating blood cells do not adhere to fibronectin.

Erythroid progenitor cells have been reported to attach firmly to fibronectin-coated substrates[43,44,193,194,195,197,271,281]. This attachment could be blocked for more than 90% either by RGDS-containing peptides[197], or by monoclonal antibodies against the RGDS central cell binding domain on human plasma fibronectin[271] or against VLA-5[281]. This indicates that most of the binding of erythroid progenitors to fibronectin involves $\alpha_5\beta_1$/RGDS recognition. This assumption was extended by the observations that undifferentiated murine (MEL) and human (K562) erythroid leukemia cell lines also adhered to fibronectin using this mechanism[43,196,197,279]. Upon differentiation, however, erythroid cells gradually loose their capacity to bind to fibronectin[43,44,195,271,281]. BFU-E and CFU-E attach to fibronectin, but reticulocytes and mature red blood cells do not. Fibronectin-adhesion is lost in differentiating, morphologically identifiable erythroblasts[43,281]. Reticulocytes of erythropoietically stressed animals, however, still attach to fibronectin[194,195,197].
A recent study indicates the involvement of VLA-4 ($\alpha_4\beta_1$) in the adhesion of human erythroblastic progenitor cells to fibronectin, which could be blocked by anti-α_4 antibodies[227]. The involvement of VLA-4 in hematopoiesis was further demonstrated by the inhibition of maturation of myeloid cells contained in the so-called cobblestone areas

(CA) in murine LTBMC and of lymphopoiesis in Whitlock-Witte cultures[1] by a monoclonal antibody against VLA-4[172]. CA themselves are, however, formed, even in the presence of anti-α_4 antibodies. In contrast to the erythroid progenitors, it has been reported that myeloid progenitors (CFU-GM, CFU-G and CFU-M) do not or only weakly attach to fibronectin[43,44,271].

One study on the attachment of murine CFU-S-12 and multilineage LTRA cells to human plasma fibronectin reports that a considerable fraction of these cells attach to the isolated alternatively spliced CS1 fragment, after a two-hour incubation, and that CFU-S-12 express the α_4 subunit of the integrin VLA-4[290]. Adherence to CS1 could be blocked by antibodies directed against α_4 and against β_1. Pre-incubation of bone marrow cells with anti-β_1 inhibited the formation of day-12 spleen colonies by these cells. A striking observation was, however, that no adherence of CFU-S-12 could be demonstrated to intact fibronectin, nor to an isolated RGDS-containing cell binding domain, giving rise to some suspicion with regard to the physiological significance of the binding of HSC to CS1. In addition, CFU-S-12 did neither adhere to collagen types I, III, and IV, nor to laminin. Thirty-six percent of murine bone marrow cells stained positive with LPAM-1, a monoclonal antibody against murine α_4. When α_4-positive bone marrow cells were injected into lethally irradiated mice, CFU-S-12 -derived spleen colonies developed.

For the interaction with cultured stromal layers, human B-cell progenitors utilize VLA-4/VCAM-1[236]. Additionally, VLA-4/VCAM-1, VLA-5/fibronectin, and β_2-integrin/ICAM-1 pathways appeared to be important for the attachment of human $CD34^+$ bone marrow cells to a marrow stromal layer *in vitro*[267]. Furthermore, it appeared that $CD34^+$ cells utilize VLA-5 and, to a lesser extend, VLA-4, to adhere to fibronectin.

Murine cortical thymocytes adhere to the central RGDS-containing cell binding site of fibronectin, but not to collagens, laminin, or vitronectin[31,32]. In addition, B- and T-lymphoid progenitor cells, in different stages of (embryonic) development or differentiation, use the RGDS cell recognition site[14,237,248] or the CS1 site[14,248] for the attachment to fibronectin. The property of adherence to fibronectin is lost upon maturation, and non of the non-activated circulating T- and B-cells attach to fibronectin. However, activated T-lymphocytes adhere to fibronectin using the $\alpha_5\beta_1$/RGDS and $\alpha_4\beta_1$/CS1 recognition mechanisms, with a clear preference for $\alpha_4\beta_1$/CS1[285]. In addition, fibronectin, soluble as well as embedded in the stroma, is a stromal factor in the induction of terminal differentiation and IgG production of human bone marrow B-lymphocytes *in vitro*, toge-

[1] Whitlock-Witte culture is the B-lymphoid counterpart of the predominantly myeloid Dexter-type long-term bone marrow culture.

ther with interleukin-6. Fibronectin alone cannot exert this effect. This action is mediated by VLA-4/CS1 (Ig-secreting B-cells have the phenotype VLA-4$^+$VLA-5$^-$ [225].

In serum-free semi-solid cultures of human hematopoietic progenitor cells, fibronectin stimulated the triggering into cycle and/or proliferation of CFU-GEMM, BFU-E and CFU-E in a concentration-dependent way[287]. CFU-GM proliferation was not seen in these cultures. This erythroid growth promoting activity was blocked by RGDS containing peptides, suggesting an additional role of the RGDS central cell binding domain of fibronectin. However, it has not been ruled out that this effect is due to a fibronectin-induced release of hematopoietic growth factors by stromal cells or lymphocytes, which is suggested by the observation that fibronectin serves as a growth factor for fibroblasts[18].

8.4 Experiments on the adherence of murine hematopoietic stem cell subsets to fibronectin

Introduction

HSC require direct interaction with the components of a supportive micro-environment for optimal maintenance, proliferation and differentiation. The mechanisms underlying the homing and lodging of HSC in the bone marrow are mediated by membrane interactions of the stem cells with stromal cells and with components of the ECM. One of the ECM components which also occurs in the bone marrow, fibronectin[10,254,257,288], is involved in diverse adhesive interactions with a large variety of cell types, including hematopoietic progenitor and stem cells.
Two major sites on the fibronectin glycoprotein are involved in the adhesion of cells. First, fibronectin contains a central cell binding domain, which includes the minimal essential sequence Arg-Gly-Asp(-Ser) (RGD(S))[204,205,206,207]. This site is recognized by a number of cell-surface receptors, of which VLA-5 is the most significant[7,99,218]. VLA-5 ($\alpha_5\beta_1$) is a member of the superfamily of the integrins[92,110]. Integrins consist if two non-covalently linked subunits, α and ß. Second, the progenitor mRNA of fibronectin undergoes alternative splicing[139,198,243,265], and some of the alternatively spliced products contain the CS1 site[107,108,138,166], which is located near the COOH-terminal heparin-binding domain. This site is recognized by the integrin VLA-4 ($\alpha_4\beta_1$)[94,96,285].
Erythroid progenitor cells have been demonstrated to adhere to fibronectin-coated substrates, and this adhesion was lost upon differentiation[44,193,195,271,281]. Most of this binding involved $\alpha_5\beta_1$/RGDS recognition, indicated by the specific inibition of the interaction between these progenitor cells and fibronectin by RGDS-containing peptides[197], and by monoclonal antibodies against the RGDS-containing central cell

binding domain of fibronectin[271] or against VLA-5[281]. A recent study, however, indicated the involvement of VLA-4 ($\alpha_4\beta_1$) in the adhesion of human erythroblastic progenitor cells to the CS1 site of fibronectin[227]. The involvement of VLA-4 in hematopoiesis was further demonstrated by the attachment of day-12 CFU-S and cells with long-term repopulating ability to isolated CS1, and by the expression of the α_4 subunit on day-12 CFU-S[290]. However, day-12 CFU-S did not adhere to intact fibronectin in this study. In addition, the important pathways for the attachment of human $CD34^+$ BMC to a marrow-derived stromal layer *in vitro* appeared to be VLA-4/VCAM-1, VLA-5/fibronectin, and β_2-integrin/ICAM-1[267]. These cells seem to utilize VLA-5 and, to a lesser extend, VLA-4 to adhere to fibronectin[267]. Furthermore, anti-VLA-4 antibodies inhibit the outgrowth of myeloid cells contained in the cobblestone areas in murine LTBMC, and inhibit the lymphopoiesis in Whitlock-Witte cultures[172].

Here we have studied the property of the murine stem cell continuum, ranging from CFU-S to cells with marrow repopulating ability (MRA) and long-term repopulating ability (LTRA) to adhere to bone marrow-derived stroma, and to intact human plasma fibronectin as a component of the ECM of the bone marrow. The fibronectin-adherent and non-adherent BMC populations were tested on their content of stem cells using the CAFC-assay[214,215]. This assay measures quantitatively the primitive and less primitive HSC subsets *in vitro* by means of time-dependent cobblestone area (CA) formation. Primitive HSC adhered for a large extent to fibronectin, in contrast to the less primitive CFU-S. This adhesion could partially be blocked by RGDS-containing peptides.

Materials and methods

Mice. Male (CBA × C57Bl)F_1 mice were purchased from the Laboratory Animal Center of the Erasmus University Rotterdam. At 10-20 weeks of age the mice were killed, and bone marrow cells from both femora and tibiae were used either to prepare stromal layers, or employed in the adherence experiments.

CAFC-assay. The CAFC-assay is basically a miniaturized LTBMC under limiting-dilution conditions. It is used as a quantitative *in vitro* readout system for short-term repopulating, CFU-S-like cells, and for MRA and LTRA cells (Chapter 5). Stromal layers were prepared in flat-bottomed 96-wells culture plates (Falcon) by culturing 5×10^5 male BMC per well in LTBMC medium, without attempting to make a single-cell suspension. The medium consisted of α-medium, 10% fetal bovine serum (FBS; HyClone), 5% horse serum (HS; Gibco), 0.41 mg/mL Fe-saturated human transferrin (Hoechst-Behring), 8×10^{-5}M β-mercaptoethanol (Merck), 10^{-5}M hydrocortisone-21-hemisuccinate (Sigma), 80 U/mL penicillin (Gibco), 80 μg/mL streptomycin (Gibco),

8×10^{-5}M Na-selenite (Merck), and 1.6×10^{-3}M L-glutamine (Sigma) (final concentrations). The cultures were kept at 33°C, 10% CO_2 and 100% humidity. After 11 days the cultures were given 20 Gy γ-irradiation at a dose rate of 1.06 Gy/min, which abolished all hematopoietic activity without affecting the ability of the stromas to support hemopoiesis.

To perform a limiting dilution assay (LDA) with the BMC samples to be tested, BMC were overlaid on the irradiated stromas in a series of dilutions, 3-fold apart. Each dilution consisted of 20 wells. The cultures were fed weekly by replacing all of the LTBMC medium, now containing 20% HS instead of 10% FBS and 5% HS. Between 5 and 35 days after overlay the individual wells were scored on the presence of immature hemopoietic clones (cobblestone areas, CA). Using Poisson statistics and the Maximum Likelihood solution[67,259], the frequencies of the progenitor cells that had formed CA (cobblestone area forming cells, CAFC) were calculated. These are expressed as the number of CAFC per 10^5 BMC. We have previously demonstrated the relationship between the primitiveness of HSC and the temporal delay and the duration of clonal expansion (i.e., CA-formation) in this culture system. Excellent correlations exist in this assay between day-10 CAFC and day-12 CFU-S, and between day-28/35 CAFC and MRA/LTRA[213,214,277]. Therefore, we used the day-10 CAFC to measure the less primitive day-12 CFU-S, and the day-28/35 CAFC to assess the more primitive MRA/LTRA.

Time-dependent adherence to stroma. Pre-established stromal layers made in 96-wells plates as described above were rinsed to wash out the serum that is contained in the LTBMC-medium. Single-cell suspensions of fresh BMC in α-medium were put on the stromal layers in 6 dilutions of 20 wells per dilution, 3-fold apart. The cell inputs ranged from 27,000-111 BMC per well. The BMC were allowed to adhere to the stroma for 0.5, 1, 2, 4, and 8 hours under serum-free conditions at 33°C, 10% CO_2 and 100% humidity. After these time periods the cultures were carefully rinsed and the α-medium including the non-adherent cells was removed and replaced by LTBMC-medium (20% HS). The cultures were fed weekly. CAFC frequencies were determined at 4-28 days after overlay.

Adherence of CAFC to fibronectin. Two 25 cm^2 polyethylene tissue culture flasks (Costar) were coated with 1 mL of 250 μg/mL of purified human plasma fibronectin (Sigma) in PBS (10 μg fibronectin/cm^2) for 4 hours at 37°C. Two control flasks were coated with 1 mL of 250 μg/mL of bovine serum albumin (BSA, fraction V; Sigma). Subsequently, the flasks were washed with PBS to remove unbound fibronectin or BSA, and inoculated with 2 mL of a single-cell suspension of 1.5×10^6 BMC per mL α-medium per flask. The cells were allowed to adhere for 4 hours under serum-free conditions at 33°C, 10% CO_2 and 100% humidity. For measurements of the time-dependence of the adherence to fibronectin BMC were allowed to adhere for 1, 2, 4, and

8 hours. Thereafter, the cultures were firmly agitated and the non-adherent cells were harvested. The adherent cells were trypsinized for 5 min at 37°C using 2 mL of 0.25% (w/v) trypsin/PBS per flask. The trypsin was blocked by 2 mL of ice-cold HS per flask. The remaining adherent cells were detached using a cell scraper, and all adherent cells were harvested. The adherent cells of the 2 flasks were pooled, as were the non-adherent cells. The adherent and non-adherent cells were centrifuged, resuspended in LTBMC-medium (20% HS) and put in the CAFC-assay. The highest number of adherent BMC per well was around 10,000 BMC, for the non-adherent cells around 30,000 BMC. CAFC frequencies were determined from day-5 to day-35 of culture. The percentages of adherent CAFC were calculated from the total number of adherent plus non-adherent CAFC per flask. The latter was set at 100%. In addition, the CFU-C contents of both groups were determined using the semi-solid culture system.

Blocking of fibronectin-adherence. Two synthetic peptides containing the minimum essential sequence of the central cell binding domain of fibronectin, RGS, were used to block the adherence of CAFC to fibronectin-coated substrates. One synthetic peptide with a related but not functional sequence, GRADSP, was used as a negative control peptide. BMC were allowed to adhere to fibronectin in the presence of 10-360 μg/mL of RGDS (0.08-0.83 μmol/mL) (Sigma), 100-500 μg/mL GRGDS (0.2×10^{-3} - 1.0×10^{-3} mol/L) (Sigma), or GRADSP (1.10 μg/mL) (Telios) in α-medium (final concentrations). The adherent and non-adherent cells were assayed in the CAFC-assay as described.

CFU-C assay. Fibronectin-adherent and non-adherent cells were tested on their content of *in vitro* clonable myeloid progenitor cells, CFU-GM. To do this 2×10^4 adherent cells

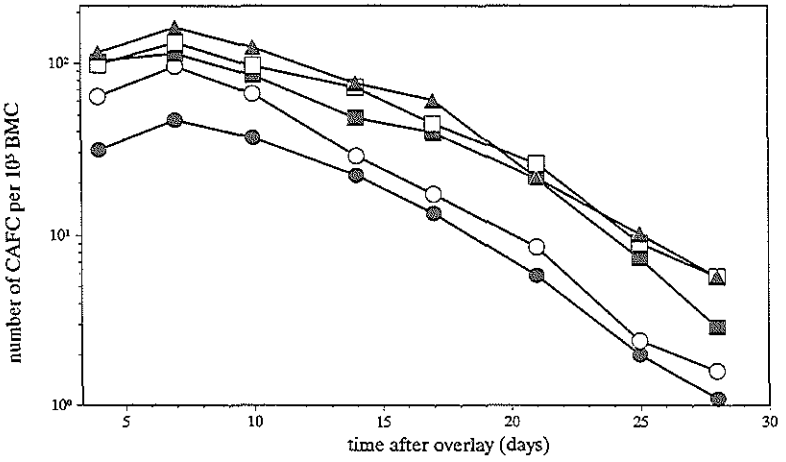

Figure 8.3. Adherence of CAFC to irradiated bone marrow stroma (means of 5 experiments). Adherence-time: 0.5 hour (●), 1 hour (○), 2 hours (■), 4 hours (□). Fresh BMC control (▲).

per mL and 5×10^4 non-adherent cells per mL were put in a semi-solid colony assay. The culture medium consisted of α-medium, 1.2% methylcellulose (Fluka), 20% HS, 1% BSA, 10% pokeweed mitogen (Gibco) mouse spleen conditioned medium as the source of hemopoietic growth factors, 80 U/mL penicillin, 80 μg/mL streptomycin, 8×10^{-5}M β-mercaptoethanol, 3.3×10^{-3}M L-glutamine, and 8×10^{-5}M sodium selenite. One mL of culture medium was put in each of two duplicate 35 mm culture dishes (Costar) per group. The cultures were kept at 37°C, 5% CO_2 and 100% humidity. Colonies consisting of more than 50 cells were counted on day 7 of culture.

Results

Time-dependent adhesion of CAFC to bone marrow stroma. To study the adherence kinetics of primitive and less primitive HSC we allowed these cells to adhere to bone marrow-derived stroma for various time periods under serum-free conditions. It appeared that of each progenitor cell type (CAFC-5 to CAFC-28) all cells attach to bone marrow stroma in 4 hours (Figure 8.3). Longer adhesion times gave results identical to 4 hours of adhesion (data not shown). Thus, primitive (CAFC-28) and less primitive (CAFC-10) stem cells show similar kinetics in their adherence to stroma.

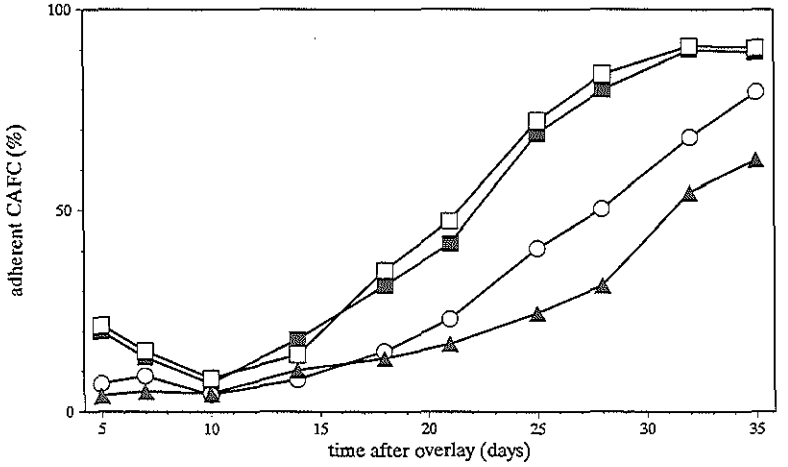

Figure 8.4. Adherence of CAFC to fibronrctin as a function of time. BMC were allowed to adhere for 1 hour (▲), 2 hours (○), 4 hours (■), and 8 hours (□).

Adherence of different stem cell subsets to fibronectin. We have studied the adherence of various HSC subtypes to fibronectin. Both adherent and non-adherent fractions of fresh BMC were tested on their frequencies of early and late appearing CA in the CAFC system, and the percentages of adherent CAFC were calculated from the

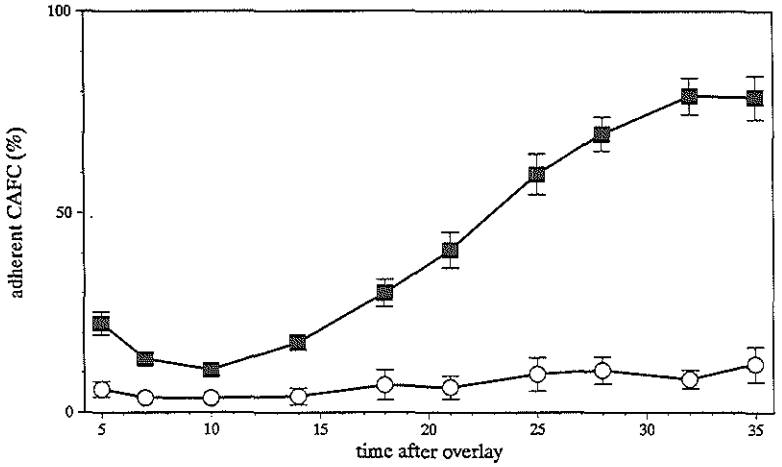

Figure 8.5. Percentages of CAFC that adhered to fibronectin (■) and BSA (○). Data represent the means of 9 (fibronectin) or 3 (BSA) experiments.

total number of adherent and non-adherent CAFC per flask. Time-dependence of fibronectin-adherence was similar to the adherence of CAFC to stroma, in that after 4 hours maximal adherence was achieved (Figure 8.4). In studies on the adherence of CAFC to fibronectin and BSA following a 4-hours adhesion time, we observed a striking difference in the fibronectin-adherent properties of the less primitive CAFC-10 and the primitive CAFC-28/35 (Figure 8.5). Of all CAFC-35, 78.9% adhered to fibronectin, whereas only 10.7% of all CAFC-10 did. In addition, CAFC that appeared earlier than day-10, and therefore are considered less primitive than the CAFC-10,

Table 8.3 **Effect of the tetrapeptide RGDS on the adherence of CAFC to fibronectin**

concentration of RGDS	CAFC-10	CAFC-28
no RGDS	10.7*	69.9
0.08 μmol/mL	10.2	60.4
0.23 μmol/mL	3.0	69.8
0.29 μmol/mL	3.7	53.9
0.83 μmol/mL	9.8	53.0

* Data represent the percentages of adherent CAFC, calculated from the total number of harvested adherent and non-adherent CAFC

showed better adherence to fibronectin than did CAFC-10. The number of CAFC that adhered to BSA compared to the total number of adherent and non-adherent CAFC per

flask was of no relevance over the entire range of early and late appearing CAFC. The percentages of adherent CFU-C per flask were 14.6 for fibronectin-adherent, and 3.6 for BSA-adherent CFU-C. The difference between these two values is statistically significant ($p < 0.05$).

Specific blocking of fibronectin-dependent CAFC adherence. To investigate whether the adhesion of CAFC to fibronectin was mediated by the central cell binding domain of the glycoprotein, we attempted to inhibit this adhesion specifically by using two synthetic peptides containing the functional sequence of this domain, RGD, in a competitive fashion. Adherence of CAFC-35 to fibronectin could be inhibited down to 41.4% of maximal adherence in the presence of 1.00 μmol/mL of the pentapeptide GRGDS (Figure 8.6), while the adherence of CAFC-10 was inhibited from 10.7% to 2.5% in the presence of the same concentration of GRGDS, a decrease in the adherence of 76.6%. The tetrapeptide RGDS inhibited the adherence of CAFC-10 to 5.5% in the presence of 0.08 to 0.83 μmol/mL RGDS (Table 8.3). A concentration of 0.83 μmol/mL RGDS inhibited the adherence of CAFC-28 to fibronectin from 69.9% without tetrapeptide to 53% in the presence of RGDS. In a concentration of 1.1 μmol/mL, the irrelevant peptide GRADSP, in which a Glycine (G) was replaced by an Alanine (A), had no effect on the fibronectin-adherence of all CAFC (Figure 8.7), indicating that the inhibitory effects of the two RGD containing peptides were not due to non-specific hinderance.

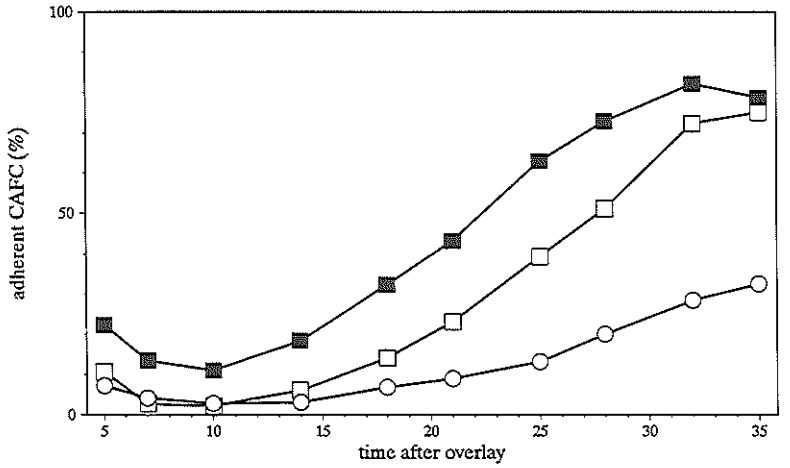

Figure 8.6. Blocking of CAFC adherence to fibronectin (■) using 0.2 μmol/L GRGDS (□), or 1.0 μmol/L GRGDS (○).

The percentages of total cell and progenitor numbers that could be harvested in all the fibronectin adherent experiments are presented in Table 8.4. It appears that of all the progenitors that were submitted to the adherence experiments, 47-73% could be harvested in the adherent and non-adherent cell fractions.

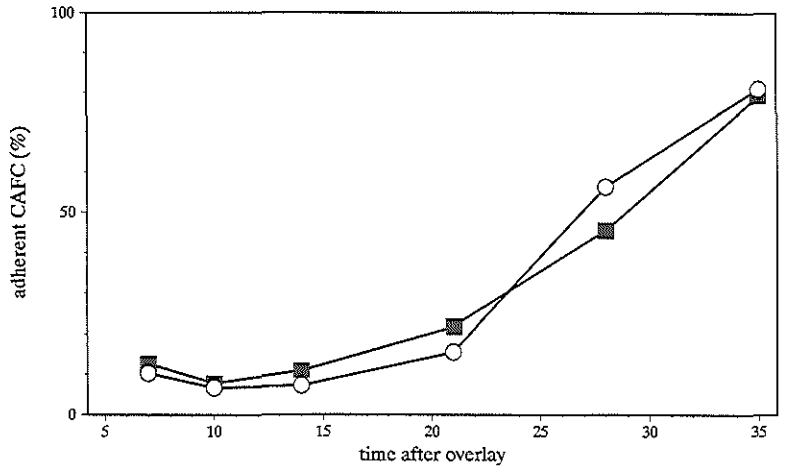

Figure 8.7. Percentages of CAFC adherent to fibronectin in the absence (■) or presence (○) of 1.1 μmol/L GRADSP.

Table 8.4 Recovery of nucleated cells and progenitors in fibronectin-adherence experiments

	cells	CFU-C	CAFC-10	CAFC-28
percentage recovery*	52.32	47.31	59.16	72.87
1 SEM	3.28	4.36	6.44	5.43
number of observations	16	14	15	15

* Data represent the fraction of adherent plus non-adherent cells and progenitors that could be harvested in all fibronectin-adherence experiments, as a percentage of their numbers inoculated

Discussion

This study describes the differential adherence of HSC subsets to bone marrow-derived stroma or to fibronectin *in vitro*. Fresh BMC were allowed to adhere to fibronectin-coated substrates, and both the adherent and the non-adherent cell fractions were tested on their content of primitive (cells with MRA/LTRA) and less primitive (CFU-S) stem cell subsets using the CAFC assay. This assay discriminates between distinct stem cell

subpopulations on the basis of time-dependent CA formation. Using the CAFC assay to quantify HSC subpopulations *in vitro*, the necessity for purifying these subsets to homogeneity, as required in adhesion studies, was thus avoided.

Our data show a striking difference between the distinct HSC subpopulations with respect to their property to adhere to the ECM glycoprotein fibronectin. A majority of primitive long-term repopulating cells, measured *in vitro* as CAFC-28/35, adhered for 80% to a fibronectin-coated substrate. In contrast, only 11% of all less primitive CFU-S-like cells, measured as CAFC-10, adhered to fibronectin. The adherence of CAFC-10 could almost entirely be blocked by the synthetic peptides RGDS and GRGDS, which both contain the minimal essential sequence of the central cell binding domain of fibronectin (RGD)[204,205,206,207], while no blocking effect was observed by the irrelevant hexapeptide GRADSP. This indicates that the subpopulation of CAFC-10 that adhered to fibronectin, adhered to the RGD-containing cell binding domain of the glycoprotein.

The adherence of CAFC-28/35 could partially be blocked by high molar concentrations of the pentapeptide GRGDS, and to a lesser extend by RGDS. This indicates that at least part of the mechanism by which primitive HSC adhered to fibronectin is by the recognition of the RGD-containing binding site of the glycoprotein. This site is recognized by several integrins, namely $\alpha_3\beta_1$, $\alpha_5\beta_1$ (VLA-5), $\alpha_v\beta_1$, $\alpha_{IIb}\beta_3$ (platelet GPIIb/IIIa), $\alpha_v\beta_3$, $\alpha_v\beta_6$, and $\alpha_4\beta_7$ [110]. Of all these integrins only $\alpha_5\beta_1$ (VLA-5) has been demonstrated to be involved in the binding of hematopoietic progenitor cells to the RGD-containing site of fibronectin[197,271,281]. However, nearly all hematopoietic cell types express one or more integrins[91]. Therefore, the role of the other RGD-binding integrins in the binding of HSC to fibronectin still has to be unraveled.

Not all the binding of CAFC-28/35 could be inhibited by GRGDS. The observation that 44% of all CAFC-28/35 that adhered to fibronectin could not be blocked even at high molar concentration of the blocking pentapeptide, suggests an additional mechanism for the adhesion of these primitive HSC to fibronectin. This is probably represented by the binding of cell-surface VLA-4 ($\alpha_4\beta_1$) to fibronectin[172,227,267,290]. VLA-4 recognizes several distinct sites on the fibronectin molecule. These sites are H1[177], and the alternatively spliced CS1[107,108,138] and CS5[108,178]. The recognition of VLA-4/CS1 is physiologically the most important, and has been described in the fibronectin-adhesion of CFU-S and LTRA cells in the mouse[290] and for erythroblastic progenitors in the human[227].

A salient detail may be, that the pentapeptide GRGDS is able to block the spreading of murine melanoma cells on CS1 and CS5 coated substrates, due to inhibition of $\alpha_4\beta_1$ interaction with the IIICS fragments[178]. Larger RGD-containing peptides, however, will not inhibit binding to and spreading on IIICS ligands, nor will CS1 peptides do on the RGD-containing central cell binding domain of fibronectin. This indicates that inhibition of adherence to fibronectin by this pentapeptide may not only act on the RGD-containing cell binding site, but also on the CS1 and CS5 mediated cell adhesion. These observations suggest that adhesion of cells to both the central cell binding domain and

the IIICS fragment show similar mechanisms of interaction with $\alpha_5\beta_1$ and $\alpha_4\beta_5$, respectively, since RGD-containing oligopeptides block the function of both domains. However, in the native fibronectin molecule these cell binding sites do not compete with each other, because of their different receptor binding. In contrast with the above observations, other studies report that the IIICS receptor-ligand interactions are RGD-independent[74,156,167,285], or only slightly sensitive to RGD peptides[81]. These discrepancies may be due to the types of adherence assay and/or the concentrations of blocking peptides used. To date, it is unclear to what extent the various sites of fibronectin involved in the binding of cells interact[55].

Altogether, the evidence suggests that the adhesion of primitive HSC to fibronectin is mediated by VLA-4/CS1 and VLA-5/RGDS interactions. How these two mechanisms relate to one another, is as yet unclear. It has been reported for the adhesion of $CD34^+$ human BMC to fibronectin that these cells utilize VLA-5 preferentially to VLA-4, but that both integrins participate in this adhesive property[267]. The preferential dominance of the VLA-5/RGDS interaction may be explained by the fact that of the fibronectin dimer only the A chain is alternatively spliced. A fibronectin molecule, therefore, always contains two RGDS cell binding sites, while the CS1 site is only contained in the A chain, if not spliced out. Thus, the molar ratio of CS1 and RGDS in human plasma fibronectin is less than 1:2.

The distribution of the various fibronectin splicing variants in the bone marrow tissue may play a role in the homing and specific localization of intravenously injected HSC during bone marrow transplantation. Cell type-specific expression of alternatively spliced human fibronectin IIICS mRNA has been described[98]. Hematopoietic progenitor cells may utilize multiple integrin-mediated adhesion pathways to localize specialized micro-environmental niches in the marrow stroma. For the moment, this can only be speculative, because little is known about the distribution of the various fibronectin splicing variants in hematopoietic organs, although specific spatial distribution of distinct hematopoietic progenitor subsets has been reported[147,160,211].

Whether the binding to fibronectin augments specific HSC-stromal cell binding mediated by other CAM[155,159] and lectin/carbohydrate interactions[266], or whether this binding represents at least part of the specific interaction between HSC and stroma, has, to date, not been clarified.

Our data are partially in agreement with an earlier study, describing the inability of day-12 CFU-S to adhere to fibronectin[290]. These authors, however, found that day-12 CFU-S did adhere to the isolated CS1 site of fibronectin. In the present study we have found that only a minority of all CFU-S adhered to fibronectin, in contrast to the majority of MRA/LTRA cells, and that the adhesion of CFU-S was due to the RGDS-containing cell binding domain on the glycoprotein.

In this study we investigated the CAFC content of both the adherent and the non-

adherent cell fractions, which gives more reliable information on the adhesive properties of all HSC. Using the CAFC assay, which makes a clear distinction between primitive and less primitive HSC, we were able to measure the adhesive properties of these stem cell subsets without the use of multiparameter sorting. Although not all progenitor cells could be harvested in the adherence experiments, the percentages of the recovery of total cell numbers, CFU-C, and early and late appearing CAFC were relatively high, indicating the reliability of the methodology and the results. It is important that studies on the adherence of cells to fibronectin are performed under serum-free conditions. Serum used for cell culture contains the attachment factors fibronectin and vitronectin, among other factors, which may inhibit the adhesion of cells to coated substrates. The concentration of soluble fibronectin in plasma is 300 μg/mL, a concentration similar to the fibronectin preparation used for coating the culture flasks.

The distinction of CFU-S and MRA/LTRA cells with respect to the property to bind to fibronectin, described in the present study, adds another argument to the discussion whether or not CFU-S and MRA/LTRA cells represent the same class of stem cells. CFU-S and MRA/LTRA cells differ with respect to affinity to WGA, retention of rhodamine 123, radiosensitivity, and a variety of chemostatic agents. Now, these cells appear also to differ with respect to their adherence to fibronectin. Our data thus support conclusions from other studies, indicating that MRA/LTRA is distinct from the majority of CFU-S[124,212,213,276].

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Chapter 9
General discussion

Studies on the heterogeneity of the hematopoietic stem cell compartment in the mouse have led to the still ongoing discussion whether the most primitive stem cells have both long-term repopulating ability (LTRA) and spleen colony forming ability[16,29,30,31,35,40]. The alternative hypothesis is that CFU-S and cells with LTRA represent two distinct entities[14,21,22]. Despite controversial opinions in the literature we obtained convincing evidence to exclude CFU-S from our definition of the most primitive stem cell, because the vast majority of LTRA can be separated from CFU-S, and vice versa[24,36]. In addition, CFU-S and more primitive stem cells differ in their sensitivity to various types of radiation or cytostatic treatment[9,17,25]. However, this does not exclude the existence of stem cells that carry both abilities. This thesis describes a number of experimental studies in the mouse on the distinct nature of CFU-S and more primitive stem cell subpopulations, namely those with marrow repopulating ability (MRA) and LTRA.

It was previously observed that sorting bone marrow cells (BMC) on the basis rhodamine 123 (Rh123) uptake revealed two subpopulations that differed in the time required for the onset of proliferation *in vivo*[21,22]. The $Rh123^{bright}$ sorted cells were enriched for CFU-S-8/12, while the $Rh123^{dull}$ cells did not form spleen colonies in the first 12 days after transplantation, but *generated* new CFU-S in the bone marrow (MRA). These observations could be mimicked *in vitro* (Chapter 4). CFU-S and MRA cells could be distinguished on the basis of the time of onset of hematopoietic growth on pre-established bone marrow-derived stromal layers, and on the duration of the production of new progenitors. $Rh123^{bright}$ sorted BMC showed a characteristic growth pattern of a rapid onset, an early peak, and a rapid decline of hematopoietic growth *in vitro*. In contrast, $Rh123^{dull}$ cells had a delayed onset and sustained hematopoietic growth of several weeks before the *in vitro* hematopoietic activity gradually ceased. In addition, the total production of CFU-C by $Rh123^{dull}$ cells in these long-term bone marrow cultures (LTBMC) surpassed that of the same number of $Rh123^{bright}$ BMC.
The different hematopoietic growth characteristics of distinct stem cell subpopulations on pre-established stromal layers formed the basis of an *in vitro* limiting dilution assay for frequency analysis of LTRA cells, MRA cells, and CFU-S (Chapter 5). This assay, which measures the time-dependent formation of cobblestone areas (CA) by cells termed cobblestone area forming cells (CAFC), quantitatively characterizes the stem cell continuum of unseparated or purified BMC samples in about 5 weeks. The property of the CAFC assay to discriminate primitive and less primitive progenitor cells by their time-dependent clone formation in a single assay offers a large variety of possibilities

for stem cell research in the mouse. Because of the specificity of the assay, characterization of HSC subpopulations can be performed *in vitro*, without the need for purification up to 100% homogeneity. Examples are given in Chapters 7 and 8. In addition, it is shown that this assay is able to predict the extent of long-term repopulation in experimental murine bone marrow transplantation (Chapter 7). Once adapted for human bone marrow, the CAFC assay will be useful in many experimental and clinical applications.

Human long-term *in vitro* hematopoiesis on pre-established marrow-derived stromal layers is used in an assay for so-called "long-term culture-initiating cells (LTC-IC)[32,34]. LTC-IC are indicated to measure a primitive human stem cell subset. In contrast to the murine CAFC assay, the assay for human LTC-IC measures the ability of human BMC to produce CFU-C, which does not allow routine frequency analysis of stem cells. The adaptation of the murine CAFC system for human BMC has, to date, not been published to be successful. The exact reason for the failure to use the CAFC assay in the human situation is not known. Human LTBMC appear to differ from mouse long-term cultures with respect to the morphology of the stroma, the morphology of CA, and the nature of the CA. CA-like structures in human cultures are not always associated with hematopoietic activity, but often appear as mature granulocytic clusters (personal observations). In addition, many hematopoietic clusters can be found on top of the stroma. Most probably, the stroma of human LTBMC does not form an optimal micro-environment for hematopoietic progenitor cells, in contrast to the murine situation. This may be due to suboptimal culture conditions, but the methods of harvesting human BMC may also play an important role. The major difference is that tiny clumps of murine BMC, which represent intact pieces of bone marrow containing little structural units including the extracellular matrix (ECM), form the basis of the murine stroma. The stromal layer develops from these clumps. This is in contrast with the initiation of human LTBMC for which single BMC suspensions are frequently used. These cells have been in contact with anticoagulantia, and have been depleted of erythrocytes using a density gradient. In the mouse single BMC suspensions give rise to scanty stromal layers that are poorly supportive[6]. This may be one reason for the failure of the development of a human CAFC system. However, primitive human hematopoietic progenitor cells can be cultured on both human and murine stromal cells, and give rise to hematopoietic growth for a number of weeks. This property of human BMC, together with an optimization of the human LTBMC with respect to BMC harvest and culture condition, will probably lead to a well functioning human CAFC system.

It will be a major step forward if the stromal layer of human and murine LTBMC can be generated from one or more stromal cell lines, that will allow proper CA formation. Recently, is has been shown that a combination of stromal cell lines derived from LTBMC forms a supportive micro-environment *in vivo*[15]. A few cell lines have been reported to support hematopoiesis *in vitro* by forming CA[12,20]. In addition, stromal cell

lines have been genetically engineered to produce hematopoietic growth factors[7,27,33,41]. Only a small number of cell lines support hematopoiesis adequately. Therefore, it is likely that hematopoiesis-supporting stromal cell lines must create proper micro-environmental conditions, like the expression of the right adhesion molecules, the synthesis of certain cytokines, and the formation of a sufficient ECM. However, a recent paper reports that physical contact between human hematopoietic stem cells and a stromal layer is not required for optimal growth of stem cells *in vitro*[37]. These findings are in contrast with an earlier study in murine LTBMC, that show that physical contact with a supportive stroma is essential for optimal hematopoietic growth *in vitro*[1]. In view of the essential requirement of a stromal layer, it will be very convenient if an artificially created stroma supports the growth of especially human HSC. This will create a constant quality of the culture method that does not depend on the quality of the bone marrow obtained from (healthy) volunteers. Some promising results have been achieved, and even human CA formation has been observed using murine bone marrow-derived stromal cell clones (Ploemacher, personal communication).

In the studies in which $Rh123^{bright}$ and $Rh123^{dull}$ sorted cells were grown on irradiated stromal layers, the enrichments of CFU-S and pre-CFU-S, respectively, can not measure up to enrichments that are achieved in later studies[24,36]. In the experiments included in this thesis we have not been able to separate MRA/LTRA completely from CFU-S activity. In recent years the purification protocols in our laboratory using counterflow elutriation and cell sorting on the basis of Rh123 uptake have been replaced by a discontinuous Ficoll-400 density gradient, followed by cell sorting on the basis of WGA binding[36]. This protocol allows an enrichment of CAFC-28/35 of 400-800 times.
In the experiments described in the present thesis we were not able to culture $Rh123^{dull}$ sorted, CFU-S -depleted, BMC in a semi-solid culture assay in the presence of a cocktail of IL-3, GM-CSF, G-CSF, and M-CSF. This is in contrast with the observations presented in other studies, in which sorted $Rh123^{dull}$ stem cell populations did form colonies *in vitro* in the presence of the cytokines mentioned, sometimes with high cloning efficiency[2,3,4,13,19,31,38,39]. It is likely that in the latter studies the sorted populations of primitive stem cells had been contaminated with less primitive CFU-S-like cells, that co-purify with LTRA cells in the sorting protocols used, which differed from the purification methods used in our laboratory. Using a combination of IL-3 and one or more early acting hematopoietically active cytokines, like IL-11, IL-12, and KL, it has been demonstrated that highly purified primitive stem cell populations can be cultured in the absence of a bone marrow-derived stromal layer (Ploemacher, personal communication)[18,23,26]. However, the presence of CFU-S-like cells has not been excluded in these highly purified stem cell populations. In fact, as indicated by the CAFC profile of WGA^{dim} sorted cells, CFU-S -like cells are always present[24,36]. Whether these are LTRA cells with spleen colony-forming ability, or plain CFU-S, remains to be clarified. This

notion is important in all studies on the characterization of HSC.

The repopulating ability of BMC samples can be determined by various methods. In this thesis two *in vivo* methods are evaluated on their proper measurement of repopulation with respect to the number of BMC that is engrafted (Chapter 4). These methods include the erythroid repopulating ability (ERA), which determines the ability of a bone marrow graft to repopulate the blood compartment in 12-13 days with erythrocytes (ERA[ery] and ERA[Ht]) or reticulocytes (ERA[reti]), and the ability of a graft to repopulate the bone marrow in 12-13 days with newly formed nucleated cells (MRA[cell]) or CFU-C (MRA[CFU-C]). It was found that ERA and MRA[cell] may dramatically underestimate the repopulation potential of a bone marrow graft. These assays are only useful in a narrow range of BMC input. ERA and MRA[cell] probably reflect the fast repopulating ability of relatively mature hematopoietic progenitor cells, that rapidly generate large numbers of end-stage cells. The blood and bone marrow compartments of these functional mature cells may be completely repopulated by the time the more primitive progenitor cells are still actively proliferating. The latter is reflected by the observation that MRA[CFU-C] can properly be measured over a wide range of normal BMC input (10^5-10^7 BMC per mouse). Therefore, it is concluded that MRA[CFU-C] is suitable for measurements of more primitive hematopoietic stem cells, while ERA and MRA[cell] measures the repopulating ability of more mature stem cells.
Similar results have been obtained in *in vitro* repopulation assays based on long-term stroma-dependent bone marrow cultures, in which the measurements of the production of CFU-C rather than the production of mature nucleated cells is directly related to the size of the BMC inoculum.
The third method to determine the repopulating qualities of a BMC sample *in vivo* is the measurement of its LTRA. LTRA measures the extent of donor-derived hematopoietic cells several months after bone marrow transplantation (BMT). In this thesis a sex-mismatched BMT model is elaborated (Chapter 6). In order to distinguish nucleated hemato-lymphopoietic cells from donor and host, cells from the blood, bone marrow, spleen, and thymus were screened on the presence or absence of a Y-chromosome, for which a fluorescence *in situ* hybridization technique with a murine Y-chromosome specific probe is used. It appeared that when female BMC are transplanted in sublethally irradiated male recipients a higher degree of donor-type repopulation is measured, compared to the male-to-female combination. This difference may be caused by an immunologic reactivity against the H-Y antigen, expressed only by male nucleated cells. Therefore, the female-to-male combination in this sex-mismatched transplantation model was the combination of choice, at least in the mouse strain we used for these experiments.

Long-term marrow cultures are being used in purging protocols prior to autologous BMT in leukemic patients. The observation that leukemic bone marrow is gradually depleted of neoplastic cells with increasing culture time in LTBMC, while normal stem cells could be maintained[5,8,28], makes LTBMC promising for clinical application. However, we obtained evidence that the maintenance of LTRA cells in this culture system decreases dramatically with culture time (Chapter 7), which was in agreements with other studies[10,11]. This may limit the clinical use of LTBMC in BMT, or may lead to the use of large grafts. It also restricts the use of LTBMC in transfection studies, in which LTRA cells are the target cell population for the insertion of foreign genetic material. The loss of stem cells in LTBMC may even be the principle mechanism behind the disappearance of leukemic cells in these cultures. Our study was performed in the sex-mismatched BMT model, in which LTRA was determined up to 6 months after transplantation. The CAFC study that was carried out simultaneously using the same BMC samples, revealed similar results in a period of 4-5 weeks, indicating the usefulness of the assay in predicting (and replacing?) BMT experiments.

Taking the advantage of the CAFC assay that allows frequency analysis of HSC subsets without the need for purification, we have set out to study the adhesion properties of CFU-S and LTRA cells to fibronectin (Chapter 8). It appeared that the majority (80%) of LTRA cells adhered to fibronectin, in contrast to CFU-S, of which only a small fraction (10%) adhered to fibronectin. The adhesion of CFU-S is probably mediated by the cell surface integrin $\alpha_5\beta_1$, while the adherence of LTRA cells may be due to interaction of fibronectin with both $\alpha_4\beta_1$ and $\alpha_5\beta_1$, present on these cells. This is indicated by the observation that maximal two-third of the CAFC-35 adherence, but more than 80% of the CAFC-10 adherence to fibronectin, could be blocked by the synthetic pentapeptide GRGDS. This pentapeptide contains the minimal essential amino acid sequence (i.e., RGD) of the classical cell binding domain of fibronectin, and is bound by cell surface $\alpha_5\beta_1$. The differences in the interactions of various HSC subpopulations with fibronectin is another piece of evidence for the distinct nature of CFU-S and LTRA cells. The possible role of the differential adherence to fibronectin in normal steady-state hematopoiesis is not known. It may reflect the existence of distinct micro-sites within the bone marrow micro-environment, possibly consisting of one or more alternatively spliced isoforms of fibronectin, which promote the adherence of primitive HSC. It also may be an indication for the existence of migratory pathways within the hematopoietic micro-environment, which may be used by stem cells for homing and lodging after BMT, for the establishment of the spatial distribution of stem cells throughout the micro-environment, and for the release of hematopoietic cells into the circulation. The different behavior of CFU-S and LTRA cells with respect to their adherence to fibronectin may also be a consequence of differences in activation state of the fibronectin-binding integrins on these cells.

HSC with long-term repopulating ability are the most primitive of all hematopoietic progenitor cells present in the bone marrow. LTRA cells should be the major target population for stem cell research with respect to characterization, bone marrow transplantation, and somatic gene therapy. Such studies require the development of *in vitro* and *in vivo* assays for frequency analysis and expression of human LTRA cells. A successful human CAFC assay will, have major experimental and clinical applications.

* * *

The elusive character of the hematopoietic stem cell has been brightly illustrated in "The Ballad of CMOMC", by Dirk W. van Bekkum. Poetry reaches to higher levels, reaches to places where even cobblestone pavements fall short, but:

The cobblestones pave the way, indeed, but not to hell
A safe way to go, we are doing well
It's breaking our bones, is that what you say?
In trust we proceed, to LTRA.

(CMOMC transcendent, revisited)

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Summary

Blood cells have a finite lifetime. To maintain the functions of the blood, its cells have to be replaced continuously. This occurs by processes of proliferation, differentiation, and maturation of very primitive hematopoietic stem cells. In the adult these processes predominantly take place in the bone marrow. Hematopoietic stem cells are organized in a hierarchic continuum of primitiveness according to their mitotic history and proliferative ability. The most primitive cells have long-term repopulating ability (LTRA), defined in the mouse as the ability to reconstitute a transplanted animal with all types of mature blood cells in the long run (≥ 6 months). These very primitive hematopoietic stem cells develop into stem cells with marrow repopulating ability (MRA), that are capable to generate new progenitors in the bone marrow. The next developmental stage is formed by the day-12 CFU-S that form macroscopic colonies in the spleen at 12 days after transplantation into a lethally irradiated mouse. The CFU-S can be largely separated from MRA/LTRA cells using a purification protocol that includes counterflow elutriation and fluorescence activated cell sorting on the basis of rhodamine 123.

Murine hematopoietic stem cells could be cultured on pre-established bone marrow-derived stromal cell layers (Chapter 4). The *in vitro* growth kinetics of MRA/LTRA cells differed from those of CFU-S. CFU-S had a rapid onset but a short-term duration of hematopoietic growth, while cells with MRA/LTRA had a delayed onset but a prolonged duration of hematopoietic growth. These characteristic *in vitro* growth patterns of MRA/LTRA cells and CFU-S on marrow-derived stromal layers formed the basis of an *in vitro* limiting dilution assay for the quantification of hematopoietic stem cells with LTRA, MRA, and spleen colony forming ability (Chapter 5). In this assay time-dependent clone formation (cobblestone areas; CA) on pre-established stromal layers is measured. The cell responsible for the formation of a CA is termed a cobblestone area forming cell (CAFC), and is further defined by the day on which the CA is present after inoculation on the stromal layer. We demonstrated that the day-10 CAFC is the *in vitro* equivalent of the day-12 CFU-S, and that MRA/LTRA cells are represented by day-28/35 CAFC. Using a single CAFC assay any given murine bone marrow cell sample can be analyzed for its content of primitive and less primitive hematopoietic stem cells in about five weeks. This assay can also be used for the characterization of stem cell subpopulations without the need for a 100% purification of these populations.

Two types of *in vivo* repopulating assays which are used for the measurement of hematopoietic stem cells in the mouse were evaluated with respect to the number of bone marrow cells that can be transplanted in a lethally irradiated mouse for proper measurements of repopulation (Chapter 4). These methods include the erythroid repopulating ability (ERA), which measures repopulation of the erythroid blood compartment with erythrocytes (ERA[ery] and ERA[Ht]) or reticulocytes (ERA[reti]) upon bone marrow transplantation (BMT), and the MRA, which measures ability to repopulate the bone marrow with nucleated cells (MRA[cell]) or with new progenitors (MRA[CFU-C]). Only the ability to reconstitute the marrow with new progenitor cells, MRA[CFU-C], appeared to be a reliable method for the measurement of the repopulating ability of the more primitive stem cells.

The *in vivo* LTRA of the most primitive murine hematopoietic stem cells was measured in a sex-mismatched BMT model (Chapter 6). The level of chimerism was determined using the detection of the Y-chromosome in blood leukocytes by fluorescence *in situ* hybridization with a murine Y-probe. In our hands, female-to-male BMT gave a higher degree of donor-type repopulation than gave male-to-female transplantation. This was probably due to an immune response against the H-Y transplantation antigen. Because of the non-restricted outgrowth of female stem cells in a male recipient mouse, female-to-male BMT in a sex-mismatched chimeric model is the combination of choice.

We further investigated the property of long-term stroma-dependent bone marrow culture to maintain the LTRA of a bone marrow cell sample that was inoculated onto the stroma (Chapter 7). It appeared that the LTRA was rapidly lost with culture time, resulting in only a 5% maintenance after 4 weeks of culture. However, the repopulating qualities of the remaining LTRA cells were not altered, as compared to the LTRA of fresh bone marrow cells. LTRA was measured in both the *in vivo* sex-mismatched BMT model and in the *in vitro* CAFC assay.

Finally, we studied the adherence of the various hematopoietic stem cell subpopulations to fibronectin, a component of the extracellular matrix of the bone marrow (Chapter 8). Both fibronectin-adherent and non-adherent bone marrow cell populations were assayed in the CAFC system. Of all day-28/35 CAFC 80% adhered to fibronectin. Two-third of this adherence could specifically be blocked by the synthetic pentapeptide GRGDS that contains the minimum essential amino acid sequence on the classical cell binding domain of fibronectin (i.e., RGD). The cellular receptor for this cell binding site is VLA-5 (integrin $\alpha_5\beta_1$). Therefore, the adherence of LTRA cells to fibronectin is probably mediated by VLA-5/RGD binding in combination with the action of another integrin, possibly VLA-4 ($\alpha_4\beta_1$), which binds to other domains on fibronectin. Only 10% of all day-10 CAFC adhered to fibronectin. This adherence could almost entirely

be blocked by GRGDS, which suggests a dominant role of the VLA-5/RGD in the adherence of CFU-S to fibronectin. The different adhesion properties of CFU-S and LTRA cells to fibronectin may function in the spatial distribution of stem cells throughout the bone marrow, in homing and lodging of stem cells after BMT, and in the release of hematopoietic cells into the circulation.

In conclusion, the experiments described in this thesis add further evidence to the notion that LTRA cells and CFU-S are distinct entities of hematopoietic stem cells. Optimal hematopoietic growth *in vitro* of the most primitive stem cells, those with *in vivo* LTRA, can only be achieved in close association with a proper *in vitro* micro-environment that is formed by bone marrow-derived stroma. The development of the CAFC assay, which uses this principle, gave the opportunity for quantitative analysis of the primitive stem cell compartment in the mouse. A future human version of this assay may have major experimental and clinical applications.

Samenvatting

Bloedcellen hebben een beperkte levensduur. Voor het behoud van de functies van het bloed moeten deze cellen dus voortdurend worden vervangen. Dit gebeurt door processen van celdeling, differentiatie en uitrijping van zeer primitieve hemopoëtische stamcellen. Deze processen vinden bij volwassenen voornamelijk in het beenmerg plaats. De hemopoëtische stamcellen zijn gerangschikt in een continuüm van primitiviteit op grond van hun mitotisch verleden en hun proliferatief vermogen. De meest onrijpe stamcellen hebben lange-termijn repopulerende capaciteit (long-term repopulating ability, LTRA). Deze wordt in de muis gedefiniëerd als de capaciteit om een bestraald proefdier na beenmergtransplantatie op lange termijn (≥ 6 maanden) opnieuw te bevolken met alle vormen van rijpe bloedcellen. Deze zeer onrijpe stamcellen ontwikkelen zich tot stamcellen met het vermogen om na transplantatie het beenmerg te bevolken met nieuwe stamcellen (marrow repopulating ability, MRA). Het hierop volgende stadium wordt gevormd door de dag-12 miltkolonie vormende cel (dag-12 CFU-S), een cel die in staat is om in twaalf dagen na transplantatie naar een letaal bestraalde muis een macroscopisch waarneembare kolonie te vormen op het oppervlak van de milt. CFU-S en cellen met MRA/LTRA kunnen van elkaar worden gescheiden op grond van de hoge, respectievelijke lage opname van het fluorochroom rhodamine 123.

Hemopoëtische stamcellen van de muis konden worden gekweekt op van het beenmerg afkomstige stromale lagen (Hoofdstuk 4). De groeipatronen *in vitro* van MRA/LTRA cellen en CFU-S zijn van elkaar verschillend. CFU-S hebben een zeer snelle hemopoëtische groei, die echter ook weer snel uitdooft. MRA/LTRA cellen komen daarentegen traag op gang, maar hebben een langdurige periode van hemopoëtische groei *in vitro*. Deze *in vitro* groeikarakteristieken van MRA/LTRA cellen en CFU-S op een van beenmerg afkomstige laag stromale cellen vormden de basis voor een *in vitro* limiting dilution assay waarin kwantitatief hemopoëtische stamcellen met LTRA, MRA en miltkolonie vormende capaciteit kunnen worden gemeten (Hoofdstuk 5). Met deze methode wordt een tijdsafhankelijke kolonievorming (cobblestone area, CA) op stromale lagen gemeten. De cel die zo'n CA vormt wordt een cobblestone area forming cell (CAFC) genoemd. Deze wordt verder aangeduid met de dag na inoculatie op het stroma waarop de CA zichtbaar is. We konden aantonen dat de dag-10 CAFC het *in vitro* equivalent is van de dag-12 CFU-S, en dat MRA/LTRA cellen kunnen worden gemeten met de dag-28/35 CAFC. Met behulp van de CAFC methode kan van elk monster van beenmergcellen in ongeveer vijf weken worden bepaald hoeveel primitieve en minder primitieve stamcellen het bevat. Tevens kan deze methode worden gebruikt voor het karakteriseren van de diverse subpopulaties van stamcellen zonder dat hiervoor een

100% zuiverheid hoeft worden bereikt.

Twee *in vivo* repopulatie methoden die worden gebruikt om het repopulerend vermogen van stamcellen te bestuderen, werden getest op het aantal beenmergcellen dat kan worden getransplanteerd in een letaal bestraalde muis om nog betrouwbaar repopulatie te kunnen meten (Hoofdstuk 4). Deze methoden zijn de erythroid repopulating ability (ERA), die de repopulatie van het erythroide compartiment van het bloed met erythrocyten (ERA[ery] en ERA[Ht]) of met reticulocyten (ERA[reti]) meet na beenmergtransplantatie, en de MRA, die het vermogen meet om het beenmerg na transplantatie te bevolken met kernhoudende cellen (MRA[cell]) of nieuwe voorlopercellen (MRA[CFU-C]). Van deze methoden bleek alleen de MRA[CFU-C] betrouwbaar te zijn voor het meten van het repopulerend vermogen van de meer primitieve hemopoëtische stamcellen.

Het lange-termijn repopulerend vermogen van de meest primitieve stamcellen werd gemeten in een man/vrouw beenmergtransplantatie model bij de muis (Hoofdstuk 6). De mate van chimerisme werd bepaald door de detectie van het Y-chromosoom in de leukocyten van het bloed, waarbij gebruik gemaakt werd van *in situ* hybridisatie met een muize Y-probe. In onze handen bleek een transplantaat van vrouwelijke beenmergcellen in een bestraalde mannelijke ontvangermuis een hogere graad van donor-type repopulatie te geven dan een man-naar-vrouw beenmergtransplantatie. De vermoedelijke oorzaak is een immuunreactie tegen het H-Y transplantatie antigeen. Vanwege de ongehinderde groei van vrouwelijke stamcellen in een mannelijke ontvangermuis, verdient de vrouw-naar-man combinatie de voorkeur in dit beenmergtransplantatie model.

We onderzochten tevens het vermogen van de lange-termijn stroma-afhankelijke beenmergkweek om de LTRA van beenmergcellen die op het stroma in kweek werden gebracht, te behouden (Hoofdstuk 7). Het bleek dat de LTRA verloren ging naarmate het beenmerg langer in kweek werd gehouden. Het resultaat was dat na vier weken kweek slechts 5% van het oorspronkelijke lange-termijn repopulerend vermogen aanwezig was. Kwalitatief deed dit restant van de LTRA niet onder voor de LTRA van normaal beenmerg. De LTRA werd zowel *in vivo* in het man/vrouw transplantatiemodel gemeten als *in vitro* met de CAFC methode.

Tot slot bestudeerden we de hechting van de verschillende typen hemopoëtische stamcellen aan fibronectine, een component van de extracellulaire matrix van het beenmerg. Zowel de aan fibronectine hechtende als de niet-hechtende beenmergcellen werden geanalyseerd met de CAFC methode. Van alle dag-28/35 CAFC hechtte 80% aan fibronectine. Tweederde van deze hechting kon specifiek worden geblokkeerd met het synthetische pentapeptide GRGDS, dat de minimaal essentiële aminozuurvolgorde op

het klassieke cel bindende domein van fibronectine (RGD) bevat. De cellulaire receptor voor de binding van cellen aan dit domein van fibronectine is VLA-5 (integrine $\alpha_5\beta_1$). Dit is waarom wij vermoeden dat de hechting van LTRA cellen aan fibronectine verloopt via VLA-5/RGD, waarschijnlijk in combinatie met een ander integrine, VLA-4 ($\alpha_4\beta_1$), dat bindt aan andere domeinen op fibronectine. Slechts 10% van alle dag-10 CAFC hechtte aan fibronectine. Deze hechting kon vrijwel volledig worden geremd door GRGDS. Dit suggereert een dominante rol van VLA-5/RGD in de hechting van CFU-S aan fibronectine. Het verschil in hechting van CFU-S en LTRA cellen aan fibronectine zou een functie kunnen hebben bij de ordening van stamcellen in het beenmerg, bij het in het beenmerg terechtkomen van getransplanteerde stamcellen en bij het vrijkomen van hemopoëtische cellen vanuit het beenmerg in de circulatie.

De experimenten die in dit proefschrift zijn beschreven geven extra aanwijzingen voor het steeds sterker wordende vermoeden dat LTRA cellen en CFU-S twee verschillende entiteiten binnen het hemopoëtische stamcel compartiment vormen. Een optimale hemopoëtische groei *in vitro* van de meest primitieve stamcel, die met *in vivo* LTRA, kan alleen worden behaald in nauwe samenhang met een geschikte *in vitro* micro-omgeving. Deze wordt gevormd door van het beenmerg afkomstig stroma. De ontwikkeling van het CAFC systeem, dat gebaseerd is op dit principe, maakt een kwantitatieve analyse van het compartiment van primitieve stamcellen bij de muis mogelijk. De toekomstige humane variant van dit assay kan leiden tot belangrijke experimentele en klinische toepassingen.

Toelichting voor niet-hematologen

De cellen in het bloed hebben een beperkte levensduur die variëert van enkele dagen (granulocyten) tot maanden (rode bloedcellen) of zelfs jaren (sommige lymfocyten). Om het bloed zijn functie te doen behouden zullen deze cellen voordurend moeten worden vervangen. Dit proces vindt bij volwassenen voornamelijk plaats in het beenmerg en wordt hemopoëse genoemd.
Een klein aantal zeer primitieve hemopoëtische stamcellen is in staat om door middel van celdeling, differentiatie (ontwikkeling in een speciale richting) en uitrijping alle in het bloed voorkomende celtypen te vormen. Om een indruk te geven: bij de mens worden dagelijks 200 miljard rode bloedcellen en twee miljard granulocyten gevormd, en dit gedurende het hele leven. De experimenten die in dit proefschrift zijn beschreven hadden tot doel een beter inzicht te verkrijgen in de eigenschappen van de meest primitieve hemopoëtische stamcellen. Een ingeteelde muizestam is hierbij gebruikt als proefdiermodel.
Omdat het aantal onrijpe stamcellen in het beenmerg erg klein is - ongeveer één tot vijf per 100.000 cellen is een primitieve hemopoëtische stamcel - en ze niet op grond van hun uiterlijk te herkennen zijn tussen de andere beenmergcellen, zijn er diverse methoden ontwikkeld om hemopoëtische stamcellen kwalitatief en getalsmatig te bestuderen. De klassieke methode is die van de vorming van miltkolonies. Wanneer een muis een dodelijke stralingsdosis krijgt toegediend en daarna wordt getransplanteerd met cellen afkomstig van gezond beenmerg, dan vormen zich in acht tot twaalf dagen zichtbare knobbels op de milt van het getransplanteerde dier[1]. Elk van deze knobbels bestaat uit een groot aantal nieuw gevormde bloedcellen, die afkomstig zijn van één stamcel. Tientallen jaren is verondersteld dat deze miltkolonie vormende cellen de meest primitieve hemopoëtische stamcellen waren. Echter, uit onderzoek van de laatste acht jaar, waarbij ons laboratorium een voortrekkersrol heeft gespeeld, is gebleken dat er diverse, meer primitieve stadia voorafgaan aan de miltkolonie vormende cel. Wanneer een dodelijk bestraalde muis wordt getransplanteerd met alleen maar miltkolonie vormende cellen, dan zijn de nakomelingen van deze stamcellen slechts gedurende enkele weken na transplantatie aantoonbaar in het bloed van het getransplanteerde dier. Wanneer echter nòg primitievere stamcellen worden getransplanteerd, dan zijn de nakomelingen van deze cellen vele maanden tot meer dan een jaar na transplantatie in het bloed aantoonbaar. Deze zeer primitieve stamcellen hebben lange-termijn repopulerend vermogen. Er zijn methoden ontwikkeld waarmee de miltkolonie vormende

[1] Zonder behandeling zal het dier in twaalf tot vijftien dagen sterven aan bloedingen en/of infecties, omdat het beenmerg niet meer in staat is nieuwe bloedcellen, waaronder bloedplaatjes en granulocyten, te produceren. Na beenmergtransplantatie zal de muis blijven leven.

cellen van de meer primitieve stamcellen kunnen worden gescheiden.

Dit proefschrift beschrijft een methode om de twee bovengenoemde typen hemopoëtische stamcellen aan te tonen buiten het lichaam, *in vitro*. Deze methode is gebaseerd op de zogenaamde lange-termijn beenmerg kweken. Beenmerg van een muis wordt uitgezet in een kweekflesje in kweekmedium. Dit beenmerg groeit uit over de hele bodem van het flesje en vormt een laag beenmergstroma[1] die bestaat uit de steuncellen van het beenmerg, waarin haarden van bloedvorming te zien zijn. Bestraling van deze beenmerg orgaancultuur heeft tot gevolg dat de hemopoëtische activiteit wordt gedood, terwijl het stroma intact blijft. Wanneer miltkolonie vormende cellen uitgezaaid worden op zo'n bestraalde stromale laag, dan geven deze een snelle hemopoëtische groei te zien die echter na enkele weken volledig is uitgeput. De meer primitieve stamcellen hebben een trage start, maar een veel langduriger periode van hemopoëtische groei *in vitro*. Deze resultaten zijn in essentie gelijk aan de observaties met experimentele beenmergtransplantaties *in vivo*. We kunnen daarom stellen dat hemopoëtische groei *in vitro*, gemeten op één week na het uitzaaien van stamcellen op een bestraalde stromale laag, wordt veroorzaakt door miltkolonie vormende cellen, terwijl de hemopoëtische activiteit die gemeten wordt op vier weken na het uitzaaien van stamcellen een gevolg is van stamcellen die primitiever zijn dan de miltkolonie vormende cellen.
Gebruikmakend van deze gegevens hebben we bovenstaand kweeksysteem 80 maal verkleind. In deze kweekmethode wordt de bloedcelvormende activiteit gemeten aan de hand van kolonies die zich *in* het stroma ontwikkelen. Deze hebben door de microscoop een andere aanblik dan kolonies die *bovenop* het stroma gelegen zijn, en die niet worden meegenomen in de beoordeling van een kweek. Een kolonie in het stroma, een zogenaamde *cobblestone area*, is het produkt van een stamcel, die wordt aangeduid als *cobblestone area forming cell* (CAFC). Deze CAFC wordt verder gespecificeerd met de dag (na het tijdstip van uitzaaien op de stromale laag) waarop een cobblestone area zichtbaar is. Zo is gebleken, dat de CAFC dag-10 het *in vitro* equivalent is van de miltkolonie vormende hemopoëtische stamcel, en dat de CAFC dag-28/35 een stamcel is met lange-termijn repopulerend vermogen. Met de CAFC-methode zijn we bij de muis in staat van elk willekeurig monster van beenmergcellen in één test te bepalen hoeveel miltkolonie vormende cellen èn hoeveel lange-termijn repopulerende stamcellen het bevat, zonder dat ingewikkelde scheidings- en zuiveringsmethoden moeten worden gebruikt. Ook heeft de CAFC methode geleid tot een enorme besparing van proefdieren, omdat experimentele beenmergtransplantaties nu *in vitro* kunnen worden gedaan.

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[1] Het beenmerg bestaat, behalve uit de hemopoëtische stamcellen en hun nog niet uitgerijpte nakomelingen, uit steuncellen die de zogenaamde micro-omgeving (stroma) vormen, waarin de stamellen kunnen delen en uitrijpen.

Beenmerg orgaankweken die zijn gebaseerd op stroma-afhankelijke hemopoëtische groei, worden in de kliniek in een experimenteel stadium gebruikt bij beenmergtransplantatie. Het is namelijk gebleken dat in veel gevallen van leukemie het leukemische beenmerg van een patiënt groeit in een lange-termijn beenmerg kweek. Na enkele weken in kweek zijn de leukemische cellen verdwenen en zijn alleen nog gezonde beenmergcellen overgebleven, waaronder ook hemopoëtische stamcellen. Het is een aantal malen beschreven, dat cellen uit zo'n kweek zijn teruggegeven aan de patiënt van wie ze oorspronkelijk waren afgenomen. De patiënt was intussen behandeld voor zijn leukemie. Deze vorm van beenmergtransplantatie is verscheidene malen succesvol gebleken.
Wij hebben onderzoek gedaan naar de mate waarin de meest primitieve hemopoëtische stamcellen zich konden handhaven in een lange-termijn beenmerg kweek. Beenmergcellen, afkomstig van vrouwtjesmuizen, werden één tot vier weken gekweekt op bestraalde stromale lagen, en vervolgens ingespoten in subletaal bestraalde mannelijke ontvangermuizen. Tot zes maanden na transplantatie zijn de percentages kernhoudende cellen in het bloed van de ontvangermuizen onderzocht op hun herkomst: afkomstig van het transplantaat of van de gastheer zelf. Dit is gedaan door in een bloeduitstrijkje te bepalen hoeveel procent van de kernhoudende cellen *niet* een Y-chromosoom bevatte, en dus afkomstig was van het (vrouwelijke) transplantaat. Het Y-chromosoom werd zichtbaar gemaakt door hybridisatie met een van tevoren gemerkt stukje DNA, dat specifiek was voor het muize Y-chromosoom (Y-probe). Slechts vijf procent van de meest primitieve hemopoëtische stamcellen met lange-termijn repopulerende activiteit bleek nog aanwezig in stroma-afhankelijke beenmerg orgaankweken. Hiermee zal ernstig rekening moeten worden gehouden wanneer lange-termijn beenmerg kweken worden toegepast als bron van primitieve hemopoëtische stamcellen bij beenmergtransplantatie.

Hemopoëtische stamcellen worden in het beenmerg ondersteund en op hun plaats gehouden door het beenmergstroma. Het stroma van beenmerg bestaat behalve uit steuncellen ook nog uit tussen de cellen gelegen structuren, die zijn opgebouwd uit combinaties van eiwitten, suikers en water. Eén van de eiwitten is fibronectine, dat als belangrijke eigenschap heeft dat het aan cellen bindt. Wij bestudeerden de mogelijke rol van fibronectine met betrekking tot de hechting van de verschillende typen hemopoëtische stamcellen aan beenmergstroma. Tachtig procent van alle lange-termijn repopulerende stamcellen bleek te hechten aan fibronectine. Daarentegen bond slechts tien procent van alle miltkolonie vormende stamcellen aan fibronectine. Het verschil in hechting van CFU-S en LTRA cellen aan fibronectine zou een functie kunnen hebben bij de ordening van stamcellen in het beenmerg, bij het in het beenmerg terechtkomen van getransplanteerde stamcellen en bij het vrijkomen van hemopoëtische cellen vanuit het beenmerg in de bloedbaan.

De experimenten die in dit proefschrift zijn beschreven geven extra aanwijzingen voor

het steeds sterker wordende vermoeden dat LTRA cellen en CFU-S twee verschillende typen hemopoëtische stamcellen vormen. Een optimale hemopoëtische groei *in vitro* van de meest primitieve stamcel, die met *in vivo* lange-termijn repopulerend vermogen, kan alleen worden behaald in nauwe samenhang met een geschikte *in vitro* micro-omgeving. Deze wordt gevormd door van het beenmerg afkomstig stroma. De ontwikkeling van het CAFC systeem, dat gebaseerd is op dit principe, maakt een kwantitatieve analyse van de primitieve stamcellen bij de muis mogelijk. De toekomstige humane variant van dit assay kan leiden tot belangrijke experimentele en klinische toepassingen.

Abbreviations

ALL	acute lymphatic leukemia
AML	acute myeloid leukemia
A	adenine
A	alanine
Ala	alanine
Arg	arginine
Asp	aspartic acid
BFU-E	burst forming unit-erythrocyte
Bkm	Banded krait
BMC	bone marrow cell
BMT	bone marrow transplantation
BSA	bovine serum albumin
BW	body weight
C	cytosine
CA	cobblestone area
CAFC	cobblestone area forming cell
CAM	cell adhesion molecule
CCE	current centrifugal elutriation
CD	cluster of differentiation / cluster of designation
CFU-C	colony forming unit in culture
CFU-Dex	colony forming unit in Dexter culture
CFU-E	colony forming unit-erythrocyte
CFU-F	colony forming unit-fibroblast
CFU-G	colony forming unit-granulocyte
CFU-GEM	colony forming unit-granulocyte/erythrocyte/monocyte
CFU-GEMM	colony forming unit-granulocyte/erythrocyte/monocyte/megakaryocyte
CFU-GM	colony forming unit-granulocyte/monocyte
CFU-M	colony forming unit-monocyte (-macrophage)
CFU-Meg	colony forming unit-megkaryocyte
CFU-Mix	colony forming unit-mixed lineages
CFU-S	colony forming unit in the spleen
CML	chronic myeloid leukemia
CMOMC	cell meeting our morphologic criteria
Con-A	concanavalin-A
CS	connecting segment
D	aspartic acid
DABCO	1,4-diazobicyclo-(2,2,2)-octane
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
cDNA	copy DNA
dsDNA	double stranded DNA
ssDNA	single stranded DNA
E	glutamic acid
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
ERA	erythroid repopulating ability
Epo	erythropoietin
ery	erythrocytes
FACS	fluorescence-activated cell sorting (-sorter)
FBS	fetal bovine serum
FISH	fluorescence in situ hybridization
FITC	fluorescein isothiocyanate
FLS	forward lightscatter
5-FU	5-fluorouracil
G	glycine
G	guanine
GAG	glycosaminoglycan
G-CSF	granulocyte colony stimulating factor
Glu	glutamic acid
Gly	glycine
GM-CSF	granulocyte/monocyte colony stimulating factor
GP	glycoprotein

GPI	glucose phosphate isomerase
GvH	graft versus host
H	histocompatibility antigen
HGF	hematopoietic growth factor
HPP-CFC	high proliferative potential colony forming cell
HS	horse serum
HSC	hematopoietic stem cell
Ht	hematocrit
I	isoleucine
ICAM	intercellular adhesion molecule
Ig	immunoglobulin
IL	interleukin
Ile	isoleucine
ISH	in situ hybridization
K_d	dissociation constant
KL	*c-kit* ligand
L	leucine
LDA	limiting dilution assay
Leu	leucine
LIF	leukemia inhibitory factor
lin^-	lineage markers negative
LP	longpass
LS	least square
LTBMC	long-term bone marrow culture
LTC-IC	long-term culture initiating cell
LTRA	long-term repopulating ability
MC	minimal chi-square
M-CSF	monocyte colony stimulating factor
MDR	multidrug resistance
MEL	murine erythroid leukemia
ML	maximum likelihood
MRA	marrow repopulating ability
MSCM	mouse spleen conditioned medium
mW	mWatt
MW	molecular weight
NC	nitrocellulose
ND	not determined
NK	natural killer
P	proline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PGK	phosphoglycerate kinase
P-gp	P-glycoprotein
PLS	perpendicular lightscatter
PMA	phorbol myristate acetate
Pro	proline
PWM	pokeweed mitogen
R	arginine
Rh123	rhodamine 123
reti	reticulocytes
RIA	radio immune assay
RNA	ribonucleic acid
mRNA	messenger RNA
S	serine
Sca	stem cell antigen
SCID	severe combined immuno-deficiency
SD	standard deviation
Ser	serine
SEM	standard error of the mean
SPF	specific pathogen free
STRA	short-term repopulating ability
T	thymine
TGF	transforming growth factor
TNF	tumor necrosis factor
VCAM	vasular cell adhesion molecule
VNR	vitronectin receptor
dUTP	deoxyuridine triphosphate
VLA	very late antigen of activation
WGA	wheat germ agglutinin

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Curriculum vitae

1 juli 1963	: Geboren te Nijkerk
juni 1982	: Gymnasium ß, Corderius College, Lambert Heynricsstraat 23, Amersfoort.
september 1982	: Aanvang studie Geneeskunde, Erasmus Universiteit Rotterdam
januari 1985 - mei 1985	: Student-assistent Afdeling Anatomie, Faculteit der Geneeskunde, Rotterdam
oktober 1985 - december 1986	: Student-assistent Instituut Celbiologie 1, Faculteit der Geneeskunde, Rotterdam
juli 1986 - december 1986	: Student-assistent Inst. Celbiologie 2, Faculteit der Geneeskunde, Rotterdam
mei 1987	: Doctoraal Geneeskunde, Erasmus Universiteit Rotterdam
mei 1987 - oktober 1987	: Keuze-onderzoek Instituut Celbiologie 2, Faculteit der Geneeskunde, Rotterdam, o.l.v. Dr. J.P. de Jong
november 1987 - december 1992	: Assistent in Opleiding, Instituut Celbiologie 2 / Instituut Hematologie, Faculteit der Geneeskunde, Rotterdam, o.l.v. Dr. R.E. Ploemacher
1 maart 1993 -	: Dienstplichtig militair, onderzoeksfunctie Laboratorium voor Experimentele Chirurgie, Faculteit der Geneeskunde, Rotterdam, o.l.v. Dr. C. Ince

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