PREVENTION OF BLOOD TRANSFUSIONS IN ONCOLOGIC SURGERY

VOORKOMEN VAN BLOEDTRANSFUSIES
IN DE ONCOLOGISCHE CHIRURGIE

PROEFSCHRIFT

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PREFACE

The three traditional modalities in cancer treatment are: surgery, radiation therapy and chemotherapy. Surgery has the longest history and still is the most successful treatment modality. Approximately two thirds of all cured cancer patients are cured by surgical resection alone. Yet, even with the best available therapy, 50 percent of all newly diagnosed cancer patients will die of metastatic cancer. Colorectal cancer for example is one of the leading causes of cancer related deaths in the Western World. It is treated primarily by surgical excision. However, recurrences both distant or local are frequent, and can not be prevented or treated with chemotherapy or radiotherapy.

Therefore, attempts have been made to develop immune-therapies for the treatment of colorectal cancer. The hope behind these studies was that a general increase in the host immune reactivity might lead to a concomitant increase in the immune response against a growing tumor. In patients with a Dukes' Stage B or C carcinoma, incurability is likely to be due to residual cancer existing in an occult and probably microscopic stage. Since there is considerable experimental evidence that immune-therapy has its greatest effects in situations with a small tumor burden, studies have been conducted to boost the post-surgical immune-response.

Hoover and colleagues(1) conducted a randomized trial in patients with Dukes' Stage B2 and C colon cancer. Patients were immunized with 10^7 irradiated autologous tumor cells at weekly intervals for 3 weeks with 10^7 BCG organisms given with the first two injections. The vaccinated group had significantly fewer recurrences and significantly fewer deaths than the control group. About two-thirds of the vaccinated patients expressed impressive delayed-type hypersensitivity responses upon skin testing with their own tumor. Moertel et al(2) recently published their results of adjuvant treatment with 5FU and Levamisole in Dukes' stage B and C patients. The overall death rate was reduced in Dukes' C patients by 33 percent compared both to untreated patients and patients treated with levamisole alone. Rosenberg has reported some cases of partial and complete remissions in metastasized colorectal cancer patients after LAK-cell treatment(3): Thus, there is considerable evidence that colorectal cancers are sensitive to both specific and non-specific immune-therapy.

If boosting the immune response prolongs survival, the other side of the coin may be that immune-suppression shortens survival. It has already been known for a long time that anesthesia and surgery suppress immune reactivity(4). In addition to this, experiences from transplantation medicine have shown that blood transfusions are also able to modulate the immune response. Retrospective studies have provided evidence that the immune-suppressive effect of blood transfusion is clinically significant for colorectal cancer patients. A recent meta-analysis, including 14 studies, showed that the overall 5-year survival is 31 percent in transfused and 52 percent in non-transfused patients(5). We started a randomized trial in 1986 to study this so-called "blood transfusion effect" prospectively: patients were randomized between predeposited autologous- and allogeneic (homologous) blood transfusions. Furthermore, we evaluated whether perioperative treatment with Recombinant Human Erythropoietin could diminish the need for allogeneic blood transfusions in patients with gastro-intestinal cancer.

The central theme of the studies presented in this thesis is: prevention of blood transfusions in oncologic surgery and the consequences of such a strategy. The two investigated methods (both in men and rats) are predeposited autologous blood donation and treatment with Recombinant Human Erythropoietin.

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PART I: INTRODUCTION

CHAPTER 1

RISKS ASSOCIATED WITH BLOOD TRANSFUSIONS, WITH EMPHASIS ON ITS IMMUNE-SUPPRESSIVE PROPERTIES

Blood transfusion therapy became a safe procedure after 1901 when Landsteiner discovered the existence of the ABO blood groups. At that time transfusions were given immediately from the blood donor into the vascular system of the recipient. When Lewisham introduced the "citrate method" for collecting blood without clotting in 1915, it became possible to store and transport blood. During the first world war especially, the advantages of transfusions in shock therapy became clear and resulted in the creation of blood transfusion services.

Increasing knowledge of blood groups, blood chemistry, and technical advancement has led to ever increasing numbers of blood transfusions being administered in the practice of modern medicine. It has resulted in the success of many surgical procedures and improved patient care. However in recent years, coincident with greater clinical use of blood transfusion, awareness of the possibility of associated morbidity has increased.

The incidence of transfusion reactions is largely unknown. They are probably very much underestimated, and depend on the type of transfusions given. Walker(1) has estimated that 20 percent of all transfusions result in some type of adverse effect. Fatal outcome is estimated to be 1:100,000 transfusions, if death due to viral infections is excluded(2). The presently known risks of using allogeneic blood are given in Table 1, and some are discussed in the following paragraphs.

Immunologic reactions after blood transfusions

The plasma membrane surface of leukocytes, platelets and red cells is covered with specific antigens, which can provoke the formation of antibodies when introduced into the circulation of recipients who lack them. The most well known antigens are the ABO-, Lewis-, Rhesus- and HLA-antigens.

Allo-antibodies against red cells can lead to hemolytic transfusion reactions. These

reactions may be acute (occurring within 24 hours after transfusion) or delayed (between 4 to 10 days). If blood is carefully cross-matched and screened for antibodies prior to transfusion, reactions from red cell incompatibility will be rare. A delayed hemolytic reaction is often caused by antibodies which were not demonstrable at the moment of transfusion but were reactivated by the transfusion.

Production of antibodies against white blood cells is the most common immunologic complication of blood transfusion. Approximately 10 percent of transfusion recipients develop anti-HLA-A,B,C antibodies(3), which may cause febrile non-hemolytic reactions in the transfused patient. Plasma proteins and allergens may also cause an antibody response: Antibodies directed against IgA, may for example, cause an anaphylactic reaction in IgA deficient persons.

Transmission of infection by blood transfusion

The most common disease transmitted by blood transfusion is hepatitis Non-A Non-B (NANB). A recent prospective Dutch study in patients undergoing open heart surgery, revealed that 2.3 percent of the patients were infected after surgery with hepatitis NANB(4). The incidence of post-transfusion hepatitis NANB in recipients receiving blood products is 2 to 4 percent in the Netherlands, 14 percent to 18 percent in Southern Europe and 9 to 11 percent in the USA(5). The likelihood of developing chronic hepatitis for these patients is probably 50 percent, of which 20 percent of cases will result in liver cirrhosis. Rubin and Tolkoff-Rubin(6) calculated that 0.5 percent to 1 percent of all transfused patients can be expected to develop liver cirrhosis in the USA: a total of 15,000 to 30,000 patients each year! Recently, tests have been developed to identify donors with hepatitis NANB(7). The specificity of the tests seems to be high, yet the predictive value of these test is only 16.2 percent(8). These tests are now introduced to screen all blood donors.

The introduction of HIV antibody screening has resulted in a very low incidence of AIDS due to blood transfusions in the western world. The risk of infecting a patient is primarily caused by the time gap between the onset of infection and the appearance of antibodies against HIV in the blood, the so called "window period"(9). Since the incidence of positive blood donors in the Netherlands is only 0.003 percent (1988)(10), the chance of infecting a recipient with HIV blood during the window period is 1 in 1 million(11).

Table 1. Risks of blood transfusion therapy

Immunologic Reactions

- . Acute hemolytic reactions
- . Delayed hemolytic reactions
- . Leucocyte and platelet allo-immunisation
- . Anaphylactic hypotensive reaction (AB against IgA)
- . Allergic reactions to plasma components
- . Graft versus host reaction
- . Febrile non-hemolytic reaction

Transmission of Infections

. Viral Infections

- . Hepatitis B
- . Hepatitis Non-A Non-B (Hepatitis C)
- . HIV I & II
- . HTLV I(12)
- . Cytomegalo virus
- . Epstein Barr virus
- . Parvo virus

. Bacterial Infections(13)

. Parasitic Infections

- . Malaria
- . Toxoplasmosis
- . Chagas' disease
- . Trypanosomiasis

Overloading of the Circulatory System

Iron Overloading

Suppression of Erythropoiesis

Immune-suppression

Immune-suppression

In contrast to the above mentioned hazards of blood transfusion therapy, blood transfusions are also known to have a beneficial effect on transplant outcome by their immune-suppressive action. The current clinical question is whether blood transfusions also influence the immune response to tumor cells or infectious agents. The clinical and experimental evidence of this so-called "blood transfusion effect" will be discussed, and the underlying immunologic mechanisms will be discussed.

TRANSFUSION INDUCED IMMUNE SUPPRESSION IN TRANSPLANTATION PATIENTS

The first successful renal transplant was performed in 1954. The advances in histocompatibility testing and immune-suppressive drug therapy made renal transplantation a clinical reality in the 1960's. Because of the awareness that blood transfusions might share antigens with organ donors, transfusion of blood was avoided if possible, because immunisation to those antigens might lead to accelerated graft rejection. In 1972 and 1973, Opelz et al(14) showed in a large, retrospective study that, in contrast to their expectation, recipients who had received blood transfusions before the engraftment, showed better graft survival. This beneficial effect of blood transfusions has been confirmed by many experimental and clinical studies, finally leading to deliberate preoperative treatment with both donor-specific transfusions (DST) as well as random-donor transfusions (third party) in patients waiting for a graft.

TRANSFUSION INDUCED IMMUNE-SUPPRESSION AND INFECTION

Both animal and human studies have indicated that host defence against bacterial infection is impaired after transfusion of blood. Tartter et al(15) studied prospectively the relationship between peri-operative blood transfusion and post-operative infectious complications in 343 patients undergoing surgery for colorectal cancer. The incidence of infectious complications was 4 percent in non-transfused patients compared with a 25 percent incidence in patients who did receive blood transfusions ($p=0.001$). Patients who developed infection received a mean of 2.31

transfusions compared with 0.74 in patients not developing post-operative infections ($p=0.0001$). The association of transfusion with infection was highly significant (Stepwise logistic regression, $p<0.0001$) after controlling for age, sex, blood loss, procedure, tumor differentiation, stage, admission hematocrit, duration of surgery and tumor size. Similar results were seen in patients undergoing surgical resection for Crohn's disease(16), the infection rate being 26 percent in patients who had received two or more units of blood, compared with 8 percent in patients who had received zero to one transfusion. Retrospective studies in burns patients(17), patients with colon injury(18) and abdominal trauma patients(19) all showed that patients who had received transfusions in the peri-operative period had a higher infection rate than those who had not received any blood products.

It may be argued that in these retrospective studies the more seriously ill patients required more transfusions, and that there is no causal relation between transfusions and a high incidence of infections. However, animal studies indicate that there is a causal relation. Waymack and others showed in an infectious burn(20) and in a peritonitis model(21) that both the mean survival time and the survival rate were significantly lower in allogeneic transfused rats compared to syngeneic transfused rats.

TRANSFUSION INDUCED IMMUNE-SUPPRESSION AND CANCER

The effect of peri-operative blood transfusions on the prognosis of cancer patients

Gannt(22) speculated in a letter in 1981, on a possible relationship between blood transfusions and prognosis of surgically treated cancer patients. The proposed analogy of blood transfusion leading to prolonged graft survival in organ transplant patients and a decreased survival in cancer patients undergoing surgery is not a sound one, since the relationship between immune-suppression and cancer is still unclear, as will be discussed later in this chapter. Nevertheless, many retrospective studies concerning patients with colorectal, lung, breast, renal, and other cancers have been published. Studies concerning colorectal cancer patients are summarized in Table 2.

Table 2. Studies on colorectal cancer outcome and transfusion.

Author	Patient N	BT (%)	DFSR/SR	5-Yr Survival(%) no BT	BT	S
Burrows(23)	122	60	DFSR	86	52	S
Bentzen(24)	468	66	SR	60	47	ns
Blair(25)	98	67	SR	44	65	ns
Blumberg(26)	197	65	DFSR	92	48	S
			SR	97	65	S
Corman(27)	281	84	SR	79	58	S
Creasy(28)	68	49	DFSR	53	28	S
Foster(29)	146	45	SR	68	51	S
Francis(30)	87	61	DFSR[a]	74	64	ns
Frankish(31)	174	59	DFSR[b]	74	70	ns
Lawick(32)	164	71	SR &	80	68	S
Nathason(33)	366	54	SR[a]	57	43	ns
Parrott(34)	517	72	DFSR	63	40	S
Ross(35)	159	60	SR	47	34	ns
Ota(36)	207	78	SR	77	72	ns
Voogt(37)	113	76	SR	74	48	S
Weiden(38)	171	60	DFSR	74	82	ns

BT Blood transfusions; DFSR Disease free survival rate; SR Survival rate
S Significant ($p<0.05$); [a] Overall rates; [b] Rates at 3 1/2 years

The disease free survival and 5-year survival of 164 patients from our hospital were studied retrospectively by van Lawick et al(32). The five year survival was 68% in the 117 patients who had received peri-operative blood transfusions, compared to 80% survival in the non-transfusion group. Multivariate analysis, adjusting for 11 relevant parameters showed that only tumor stage and the administration of blood transfusions were significantly associated with a decrease in survival. Blumberg and Heal(39) recently published a meta-analysis of 14 retrospective studies in colorectal cancer patients. Taken together the 5-year recurrence rates was 31 percent in the non-transfused and 52 percent in the transfused patient group. Transfusion was found to be an independent, significant factor in 5-year survival in 4 out of 9 studies. Taken together 41 percent of the transfused and 32 percent of the non-transfused patients died of cancer. The pooled data suggested a marked advantage for non-transfused patients. Using the log rank and Cox proportional hazard regression analysis, some studies showed that peri-operative blood transfusion has

an independent influence on prognosis, which is a strong suggestion that this relationship may be causal. Studies on other cancers have also been published. The evidence for a transfusion effect is poor in breast cancer(38),(40),(41), but stronger in renal(42),(43) and lung cancer(44),(45).

The effect of blood transfusions and tumor growth in experimental studies

Experimental models to study the blood transfusion effect have the advantage that the tumor load, the transfusion regimen and the surgical trauma can be controlled. Nevertheless, results in animal studies are conflicting.

Jeekel et al(46) (1982) studied the effect of blood transfusion both on the growth of both subcutaneous (sc) tumors, and the number of established pulmonary metastases after intravenous (iv) injection of tumor cells, in the WAG rat. Transfusion with 1 mL BN blood 7 to 14 days prior to tumor inoculation significantly inhibited the growth of sc adenocarcinomas but had no effect on sc basal cell carcinomas. The opposite was observed when the tumors were injected iv: blood transfusions inhibited the number of lung metastases in basal cell carcinomas and had no effect on adenocarcinomas.

Marquet et al(47) studied the effect of a single blood transfusion on the formation and outgrowth of experimental lung metastases in two different tumor models in rats. The formation of experimental lung metastases with a non-immunogenic tumor (LS 175) in a BN rat was not influenced by preceding blood transfusions, whereas inhibition was found with a highly immunogenic tumor (BC1618) in WAG rats. A transfusion given one week after tumor inoculation accelerated tumor growth in the BN rat but it neither stimulated nor depressed tumor growth in the WAG rats.

Clarke and Tarin(48) studied the effect of transfusions on growth and metastatic behaviour of two different tumors in mice. The growth rates of the primary tumors were not altered by transfusing the animals with allogeneic blood 14 days before tumor inoculation. However, the spontaneously metastasising ability of the tumors was augmented and accelerated for both tumor types, the effect being dependent on the strain of the blood donor.

Zeller et al(49) studied 5 different mouse models and were unable to find differences in the take rate, induction time, incidence, and growth rate of the tumors.

Shirwadkar and colleagues(50) showed in their tumor model that the number of inoculated tumor cells dictated whether tumor growth was stimulated (2.5×10^5 tumor cells) or inhibited (3.5×10^5 tumor cells) by allogeneic transfusions.

These seemingly conflicting experimental studies suggest that the effect of blood transfusions varies with the animal species, the animal strain of both the donor and the recipient of blood, the method of tumor administration, the immune-genicity of the tumor, the number of tumor cells given, and the type of blood product used. In fact, these studies bear a striking resemblance to the retrospective patient studies, in which "the blood transfusion effect" also depends on the type of tumor studied and the type and number of transfusions given.

The ability of blood transfusions to prolong graft survival is easily and reproducible demonstrated in numerous animal models, whereas the effect of blood transfusions on tumor growth is highly variable. This may reflect a fundamental difference in the antigenicity of transplanted and neoplastic cells. Factors that clearly influence the immune system and alter the outcome of tissue allograft may have little or no influence on the outcome of cancer. The specific and natural immune mechanisms against tumor cells will be discussed and will be related to possible mechanisms of transfusion induced immune-suppression affecting tumor growth, in the following paragraphs.

Specific immune response and control of tumor growth

The immune system is capable of recognizing and eliminating substances that are "non-self" and in general will not react to "self" antigens. Malignant transformation may be accompanied by phenotypic changes in the cells involved, such as loss of normal cell surface antigenic components and gain of neo-antigens. These new antigens can be specific for the tumor cells, which is for example often seen in experimentally virally- , chemically- or physically induced tumors (Tumor Specific Antigens). However, non-induced but spontaneously arising tumor cells seldom express antigens that are not present on any normal cell(51). Although the expression of these so-called Tumor Associated Antigens (TAA) by malignant cells may be much higher than expression of the same antigen by normal cells. They are usually weakly antigenic and do not provoke a strong immune response.

T-lymphocytes only recognize foreign antigens with their receptor in the context of the Major Histocompatibility Complex (MHC). T cell activation includes the generation of helper (Th)-, suppressor (Ts)- as well as cytotoxic T Lymphocytes (CTL). Lymphokines

produced by Th cells, such as Interleukin-2 (IL2) and Interferon (IFN) are important factors in the recruitment and activation of other cells, such as macrophages, Natural Killer cells and CTL's. Classic CTL's have been successfully cultured from many resected human cancers(52)(53), including cancers of the colon, breast, kidney, and melanoma. Some of these in IL2 cultured Tumor Infiltrating Lymphocytes (TIL-cells) have an unique specificity for the tumor from which they were derived alone. This may indicate that T-cells play a role in limiting tumor growth.

Yet, the tumor incidence data of immune-suppressed patients indicate that the role of T-cells is limited. The incidence of common tumors is not higher in transplant patients treated with immune-suppressive drugs. However, the incidence of tumors of the lymphoid system, carcinomas of the cervix uteri, the skin and the lip is much higher than in the normal population (54)(55). These tumors are probably virally induced and therefore may express viral antigens on their cell surface. This would mean that only immunogenic tumors develop, which normally would be recognized as non-self and attacked by the immune system.

Natural immunity and control of tumor growth

Natural immunity is effected by cells capable of lysing target cells spontaneously, that is, without prior sensitization. This function can be carried out by polymorphonuclear and mononuclear phagocytes as well as Natural Killer (NK) cells.

Macrophages have a remarkable ability to distinguish tumor cells from normal cells. Lymphokines produced by T-cells may activate the macrophages and render them directly cytotoxic to tumor cells(56). On the other hand, they can produce factors themselves that either inhibit tumor growth [e.g. Tumor Necrosis Factor (TNF)], or that stimulate the growth of tumor cells(57). In addition, macrophages play a key role in regulating the immune response by producing a variety of different lymphokines.

NK-cells represent a subset of lymphocytes distinguishable from T- and B- lymphocytes by their morphology and functional capacity to kill tumor or virus-infected cells spontaneously. They can lyse these targets without previous sensitization and without MHC restriction.

NK-cells isolated from normal healthy persons lyse only a limited range of highly

sensitive target cells in vitro. Stimulation with either Interferon (IFN) or Interleukin-2 (IL2) activates NK-cells to lyse a broad range of target cells both in vitro and in vivo. Nowadays, NK-cells are thought to be a first line of defense against the metastatic spread of blood born tumor cells. Evidence for this emerges from several studies:

- Suppression of NK-cell activity in mice or rats with cyclophosphamide(58), 17B-estradiol, or anti-asialo GM1(59), results in an increased numbers of pulmonary metastases.
- Augmentation of NK-cell activity with the interferon inducer poly I:C increases resistance to metastases(60), and adoptive transfer of cloned NK-cells protects against the formation of metastases(61).
- Recently a specific monoclonal antibody against rat NK-cells has been developed(62). Selective depletion of NK-cells with this antibody results in decreased survival after intravenous injection of mammary adenocarcinoma cells, but it does not influence the growth of tumor cells injected subcutaneously(63).
- NK-cell activity correlates with survival time or time to develop metastases in patients with solid cancer(64),(65).

Mechanisms of immune-suppression by blood transfusions

Despite much research the mechanisms behind the blood transfusion effect have not been completely elucidated. In transplant patients two mechanisms seem to play a role: induction of specific unresponsiveness by sharing of antigens between transfused blood and transplanted tissue, and non-specific inhibition of the immune system. In different animal models several specific mechanisms have been demonstrated to prolong graft survival, such as clonal inactivation(66), anti-idiotypic network generation (67),(68) and generation of suppressor cells(69).

It is not very likely that these specific mechanisms can cause a blood transfusion effect in post-operative patients and cancer patients. If there is any effect it should come from non-specific immune-suppression. Some authors have prospectively studied the effect of 1 to 3 transfusions on the immune system of man prospectively. They showed that after blood transfusions:

- Atypical lymphocytes appeared in the circulation, originating from both the recipient and the donor(70). These cells were probably activated (increased ^{3}H-thymidine uptake) by the antigenic challenge(71).
- The mitogenic response to PHA (T-cell function) was markedly decreased, but remained normal after autologous blood transfusion(72).
- The mitogenic response to ConA decreased after 1 week, followed by an increase in week 3(73).
- The number of CD4 cells increased when blood donor and recipient were mismatched for class-II antigens, but decreased in patients who were matched for 1 or more class-II antigens. In both situations class-II expression on lymphocytes decreased and the number of CD8 cells increased(74).
- NK-cell activity decreases(75).

Animal studies showed, further, that blood transfusions can induce:

- An impairment of macrophage bactericidal function, phagocytosis and killing of bacteria(76), and macrophage migration(77).
- An increased production of prostaglandin E, thromboxane and prostacyclin, of which prostaglandin E is known to be especially immune-suppressive(78).
- A decreased IL2 production by the splenic cells(79).

Conclusion and hypothesis

Blood transfusions can depress both the specific and the non-specific immune response. In cancer patients who will often receive blood transfusions during surgery, immune-suppression may influence tumor outcome in several ways:

1) During surgery tumor cells may enter the circulation(80). A depression of the NK-cell activity or a decreased lymphokine production by T-cells may result in a less effective clearance of these tumor cells.
2) After surgery tumor load is small, which appears to be an ideal situation for the immune system to attack the remaining tumor cells. Animal studies have indicated that immune-therapy has its greatest effect in animals with the smallest tumor burden(81).

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CHAPTER 2

ERYTHROPOIESIS DURING ACUTE ANEMIA

Introduction

Erythropoietin, a glycoprotein hormone produced in the kidney, is the primary regulator of red blood cell formation in humans and mammals. Low concentrations of the hormone are constantly present in order to maintain a relatively constant circulating red cell mass; under physiological conditions the rate of production of new erythrocytes equals the rate of destruction. Under hypoxic conditions such as anoxia, high altitude, hemorrhage or hemolysis an increased production of erythropoietin will take place and hematocrit will rise.

Anemia can be due to loss of red cells by hemorrhage or hemolysis, or from a decreased red cell production by the marrow. Excessive destruction or loss of red cells will be followed by increased levels of erythropoietin and an increased erythropoiesis. A decreased marrow production of erythrocytes can be accompanied by either an increased or inappropriately low erythropoietin level. Increased levels are found in patients with aplastic anemia and vitamin B12, folate or iron deficiencies, the anemia is due to a primary injury or deficiency of the marrow. In anemic patients with low erythropoietin levels in relation to the degree of their anemia, a reduced supply of erythropoietin may be partially responsible for the anemia.

Erythropoiesis in acute anemia after acute blood loss, such as gastrointestinal blood loss, trauma or operative blood loss will be discussed in detail in this paper.

Erythropoietin production in acute anemia

Since the work of Jacobson et al(1), evidence has accumulated that the kidney is the major source of erythropoietin production; however the liver also accounts for a small part of its production(2). Recent studies(3) using *in situ* hybridization techniques showed that the producing cells in the kidney are peritubularly located and

clustered in the outer cortex and medulla, the most vascularized areas of the kidney. There are no preformed stores of erythropoietin(4) and an increased demand for the hormone is satisfied by *de novo* synthesis; regulation of the production of erythropoietin occurs largely through control of accumulation of mRNA-erythropoietin(5).

Shuster(6) showed that mRNA-erythropoietin could be detected within 1 hour after initiation of hypoxia and continuously accumulated until the hypoxic stimulus was discontinued. It was found that mRNA-erythropoietin was produced faster and 12 times higher in mice suffering from severe anemia (hematocrit of 20%) compared to mice with a moderate anemia (hematocrit of 30%)(7); additional cells seemed to be recruited rather than an increase in production per cell. With an increasing severity of anemia, total renal mRNA-erythropoietin levels and serum erythropoietin concentrations showed an increase that correlated with the number of renal erythropoietin producing cells.

Erythropoietin levels of normal individuals and of patients with simple anemia (such as iron deficiency) measured by either bioassays or radioimmuno assays are related to the hematocrit(8). Normal erythropoietin levels are between 10-25 mU/ml, but at a hematocrit of 30% erythropoietin levels are elevated and range from 30 to 300 mU/ml. This broad range probably reflects the influence of respiratory and cardiovascular compensating mechanisms on hypoxia. Because patients with acute anemia are mostly treated with blood transfusions, little is known about the rate of erythropoietin production and erythropoietin levels following acute blood loss in men. However there are data available about erythropoietin production after acute hypobaric hypoxia and after autologous blood donation.

Eckhard et al(9) studied erythropoietin levels in volunteers exposed to simulated altitudes of 3,000 and 4,000 meters for 5.5 hours. Erythropoietin levels were significantly elevated after 2 and 1.5 hours, respectively, and were significantly different between the two altitudes; erythropoietin production rate increased exponentially when alveolar pO_2 decreased. The erythropoietin levels rose continuously during the period of investigation, after 5.5 hours of hypoxia the maximum erythropoietin level reached was 30 U/l. Presumably erythropoietin levels would have risen still further after prolonged hypoxia; mountaineers staying on 3,500 meters for 1 day can attain erythropoietin levels of 60 U/l(10).

In autologous blood donation programs, blood donations are permissible only when hematocrit is above 35%. Kruskall et al(11) studied the effectiveness of autologous blood donation programmes and showed that 25% of all scheduled donations were cancelled due to deferrals, the most important of these being anemia. Kickler and Spivak(12) demonstrated that serum immune-reactive erythropoietin levels increased with successive phlebotomies although not out of the normal range. In a separate study Spivak and Hogans(13) showed that there was an inverse linear correlation between erythropoietin and hemoglobin when the hemoglobin was below 11 g/l. It was concluded that within the hematocrit range permissible for autologous blood donation, the degree of anemia experienced is insufficient to initiate an adequate rapid increase in erythropoietin production.

These results suggest that following acute blood loss, the capacity to deliver oxygen, as represented by hematocrit, is the major regulator of erythropoietin production. Because of the need of *de novo* synthesis it takes some time before substantial amounts of erythropoietin are produced. erythropoietin formation increases poorly with moderate anemia or slightly reduced alveolar pO_2 levels in hypobaric hypoxia, but is strikingly enhanced when potentially harmful oxygen tensions are reached. In moderate hypoxia compensating mechanisms are probably capable of intercepting part of the hypoxia by cardiovascular and respiratory adjustments. In the early 1970s Messmer and colleagues(14) showed in studies on hemodilution that a maximum oxygen delivery to the tissues was established when the animals were hemodiluted to a hematocrit of 30%. The viscosity is then lowered, the flow through the venule is enhanced and venous return and cardiac output are increased.

Erythropoiesis during acute anemia

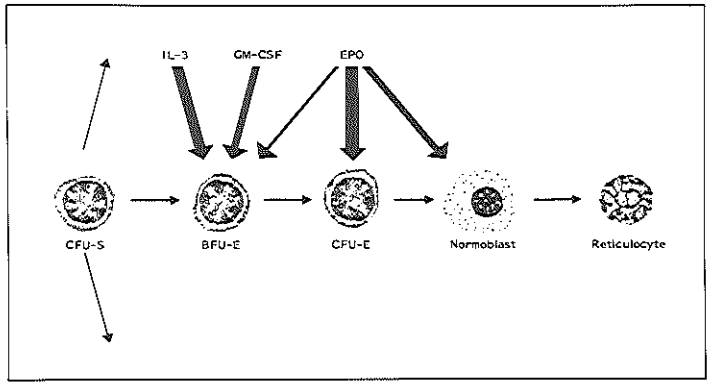
The earliest erythroid progenitors cells arising from the pluripotental stem cell (CFU_s), are the Burst Forming Unit-erythroid cells (BFU_e), which are only distinguishable *in vitro* through their growth in culture. They are succeeded by the Colony Forming Unit-erythroid cell (CFU_e) and the first morphologically distinct erythroid precursor is the pro-erythroblast (see fig 1).

Early erythropoiesis is regulated by the interaction of BFU_e with auxiliary bone marrow cells, particulary fibroblasts endothelial cells, macrophages and T-

lymphocytes(15). These cells are capable of producing interleukin-3 (IL-3) and granulocyte-macrophage colony stimulating factor (GM-CSF); hemopoietic growth factors which both stimulate BFU_e formation *in vitro* from the pluripotent precursor (Figure 1)(16). The CFU_e has an absolute requirement for erythropoietin in order to form colonies in culture and does not appear to be dependent on other growth factors(17). Proerythroblasts mature under the influence of erythropoietin in a 48 to 72 hour period(18). At the end of this time the nucleus is extruded and the erythroid cell remains as a reticulocyte within the marrow for another 2 days.

The normal total marrow transit of erythroid cells is 7 days. At high erythropoietin concentrations the reticulocyte pool will be shifted to the peripheral blood within 1 day and newly formed red cell will appear in the circulation within 5 days(19). If the bone marrow is capable of normal response, the rate of erythropoiesis after severe anemia will increase 6- to 9-fold(20). This response, however, is dependent not only on sufficient erythropoietin levels but also on the local production of growth factors and a sufficient supply of nutrients necessary for red cell generation.

Figure 1. Schematic representation of erythropoiesis in the bone marrow. Early progenitor cells, burst forming unit erythroid (BFU_e) are dependent on growth factors, such as interleukin 3 (IL-3) and granular-macrophage colony stimulating factor (GM-CSF), produced in the accessory bone marrow cells. The primary target cell for erythropoietin is the colony forming unit-erythroid (CFU_e) but at high erythropoietin levels late BFU_e are also responsive to erythropoietin.



The role of bone marrow interactions

In vitro many mediators have been described with stimulating or inhibiting effects on bone marrow cells; these include prostaglandins, interleukin-1 (IL-1), IL-2, IL-3, IL-6, interferon alpha, interferon gamma, GM-CSF and tumor necrosis factor alpha. T-cells, especially T-helper cells, are an important source of the hemopoietic growth factors IL-3 and GM-CSF. Some T-lymphocytes, especially those having the OKT 8 or T gamma phenotype, seem able to suppress erythropoiesis by releasing inhibiting lymphokines, such as interferon(21). It thus appears that red blood cell production disorders can arise from either deficient or excessive T-lymphocyte activity.

There is some clinical evidence that T-lymphocytes and other immune lymphocytes are directly or indirectly responsible for some red blood cell production disorders, e.g. aplastic anemia(22). Some investigators claim that bone marrow interactions play a role in the anemia of rheumatoid arthritic patients(23). These patients have elevated erythropoietin levels(24) but their bone marrow seems to be non-responsive. Some authors have reported that peripheral T cells from anemic rheumatoid arthritis patients inhibited CFU_e growth in normal subjects(25), which was not the case when the T cells of normal subjects were used.

There are indications that erythropoiesis is hampered post-operatively(26). Many studies demonstrated that surgery is immmunosuppressive; cellular immunity especially is depressed after surgical procedures(27). It can be speculated that this results in a diminished production of hemopoietic factors or in increased production of suppressive factors in the bone marrow. Eschbach and Adamson(28) reported two patients who under prolonged therapy with recombinant Human erythropoietin (r-HuEPO) treatment became unresponsive to treatment for a period. One patient developed a pericarditis, while receiving r-HuEPO 196 U/Kg, reticulocytosis decreased as did the hematocrit. The response to the continued r-HuEPO did not return for 6 weeks, when the inflammatory process had resolved. One other patient underwent a surgical hip replacement and despite continued r-HuEPO administration, post-operatively hematocrit fell from 40 to 18 percent.

Nutrients

The most important nutrients for red cell formation are proteins and iron. In 1940 Whipple and Robscheit-Robbins(29) were among the first to demonstrate clearly the importance of protein for optimal erythropoiesis. In protein-deficient animals,

anemia seems to be caused primarily by impaired erythrocyte production. A study of Anagnostou et al(30) indicated a diminished erythropoietin response to hypoxia after even a single day of protein deprivation.

In 1950 Finch et al(31) published a study stressing the importance of sufficient iron stores. During a period of seven weeks, 500 ml blood per week was taken from volunteers. The decrease in hematocrit in the first week averaged 4.5%, thereafter, the drop in the hematocrit was related to the ability of the individual to mobilize iron. Hillman and Henderson(32) examined erythroid marrow production in normal men with varying degrees of anemia. With daily phlebotomy a hemotocrit of 32-37% was established (moderate anemia). When a maximum constant marrow production plateau had been attained for at least two weeks each subject was subsequently phlebotomized to a hematocrit of 25 to 30% which was maintained for periods of 3 to 5 weeks (severe anemia). In moderate anemia marrow production rapidly increased over a 10 days period to reach a production plateau of 1.8 to 3.5 times normal. Production did not increase further when this level of anemia was maintained for 2 to 4 weeks. During severe anemia marrow production response was correlated with serum iron levels. At serum iron values below 70 ug/100 ml production was only about 2.5 to 3.5 times normal; between 70 and 150 ug/100ml marrow production was increased 4 to 5 times.

Treatment of post-operative acute anemia

Blood loss should be prevented by good operation techniques, erythropoiesis should be supported by a sufficient supply of nutrients and blood transfusions should be given only if strictly necessary and preferably be of autologous origin(33). In the future R-HuEPO may play a role in stimulation of post-operative erythropoiesis.

Support of erythropoiesis

For adequate erythropoiesis over a short time period considerable amounts of iron and protein are required. Post-operatively, protein intake will often be restricted and iron stores can be limited due to the underlying disease. Iron stores should therefore be investigated by monitoring serum iron, iron binding capacity and ferritin levels. Oral iron administration in post-operative patients is not always possible and seems to be less adequate in supporting blood production than intravenous iron. Hilman and

Henderson(32) calculated that during severe anemia 60 to 80 mg iron was delivered to marrow precursors for red cell production when the iron was administered orally. However, in patients treated with iron dextran injections, 80 to 160 mg of iron was used for red cells production as reflected by a subsequent increase in the rate of erythropoiesis.

Dudrick et al(34) published the clinical data from 6 patients recovering from severe acute blood loss, who had refused the administration of blood or blood products. All patients were treated with intravenous hyperalimentation (for protein supply) supplemented with iron dextran, the maximal total dose of iron dextran administered intravenously was 140 ml, infused over 5 days. In a mean period of 23 days Hb concentration increased from a mean value of 50 g/l to 106 g/l.

Administration of blood transfusions?

As allogeneic blood transfusions are associated with considerable risk such as immunisation, viral infections and immune-suppression, they should be given only when the patient's circulating hemoglobin level is low enough to compromise oxygen delivery to tissues. At rest the maximum flux of red cells occurs at a hematocrit of 30% (hemoglobin 100 g/l)(14), above this value the rising viscosity and reduced perfusion compensates for the greater oxygen carrying capacity. The hemoglobin above 100 g/l is in reserve for exercise when the rheological and oxygen delivery characteristics are considerably different. Studies on pre-operative and intra-operative hemodilution have clearly demonstrated that a hematocrit level as low as 27-30% is acceptable during and after surgical procedures, stroke volume and cardiac output increase, while heart rate remains constant if normovolaemia is preserved and the hematocrit level does not fall below 25%(35). Czer and Shoemaker(36) showed that a hematocrit value of 30% is acceptable for critically ill patients in the post-operative period. Indeed there are no indications that wound healing is improved or infections prevented when a post-operative patient has a normal hemoglobin level.

Therefore, blood transfusions should not be given to patients with a hematocrit above 30%, unless one can assume that patients are deficient in compensating facilities, or that hypoxia is likely to occur. When hematocrit values fall below 30% patient characteristics, like sex(37) and general fitness should be considered before any decision on the need for transfusions is taken. It should be noted that transfusing patients will often suppress erythropoiesis.

Allogeneic blood transfusions can be prevented by using autologous techniques. In autotransfusion, blood lost during surgery is collected and transfused to the patient; directly as whole blood or after processing the red cells. The application of hemodilution during surgery can diminish actual hemoglobin loss, especially when blood is taken before surgery, replaced by a plasma substitution and transfused after the operation. In pre-operative autologous blood donation (PABD), patients undergoing elective surgery donate, in the period before surgery, the number of blood units that they are expected to need during the operation. An extra advantage of this procedure is that erythropoiesis is already stimulated in the operative period.

Treatment with recombinant Human erythropoietin (r-HuEPO)

Winearls et al(38), and Eschbach et al(39) were the first to successfully treat the anemia of patients with end-stage renal failure with r-HuEPO. As a result of this success other types of chronic anemia are now treated experimentally with r-HuEPO. Preliminary, but favourable results have been published concerning r-HuEPO treatment in patients with cancer(40), Gaucher's disease(41) and Rheumatoid arthritis(42).

Peri-operative treatment with r-HuEPO may overcome the physiologic time gap between onset of the anemia and red cell production. Studies with baboons(43) whose hematocrit is reduced to 15% have shown that the administration of rHuEPO (1000 U/Kg) both pre-operatively and post-operatively produces a faster recovery of the hematocrit than if no treatment was given. In addition, those baboons that were treated post-operatively with r-HuEPO achieved baseline hematocrit values significantly faster (12.6 versus 28.2 days). These results are very promising because they indicate not only that the physiologic time gap can be overcome, but also that post-operative stimulation can take place. In the near future data are expected from human trials. During 1989 two clinical studies were published(44),(45) that indicated that more units of blood could be obtained if patients were treated with r-HuEPO.

Discussion and conclusions

Acute anemia resulting from acute bleeding, operative blood loss or acute hemolysis

results in hypoxia; which in turns stimulates kidney cells to synthesise erythropoietin. Within 2 hours of the onset of hypoxia newly synthesised erythropoietin can be located in the circulation. Erythropoietin levels rise exponentially with an increase in severity of anemia. Erythropoietin has been shown to stimulate the late red precursor cells in the bone marrow, with red cell production increasing 6- to 9-fold and the marrow transit time shortening to 5 days. The early progenitor cells are directly under influence of locally derived hemopoietic factors produced by accessory bone marrow cells. T-cells are capable of producing both stimulating factors (IL-3) as suppressive factors (interferon-gamma); the effect of these cells on erythropoiesis after acute blood loss is unknown.

Since allogeneic blood transfusions are associated with considerable risk such as immunisation, infection and immune-suppresion, they should be given only when oxygen delivery to the tissues will be affected. Moreover the capacity of hypoxia-compensating mechanisms, such as cardiac and lung function and any underlying complaints and degree of invalidism must always be taken into account. Erythropoiesis must always be supported by a sufficient protein intake and iron administration.

The weight of evidence suggest that in the future there will be a role for r-HuEPO treatment, especially peri-operatively. The physiologic time gap before new red cells appear in the circulation might possibly be overcome by administering r-HuEPO 5 days pre-operatively. In addition, post-operative administration of r-HuEpo can possibly maintain adequate erythropoietin levels. Randomized studies are running in an attempt to support this new treatment modality.

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CHAPTER 3

PURPOSE AND DESIGN OF PATIENT STUDIES

Introduction

It was discussed in chapter 1 that blood transfusions may suppress the response against tumor cells by their immunosuppressive action. Blood transfusions given during cancer surgery may influence either the fate of blood born metastases during operation, or the outgrowth of small numbers of already settled tumor cells; eventually resulting in a decreased survival.

Both retrospective and animal studies provided suggestive evidence for a tumor promoting effect of blood transfusions. Yet, definite proof for such a transfusion effect can only come from randomized prospective clinical studies. There are three possibilities for such a trial, that is to say: randomization between allogeneic blood and 1) Leukocyte-free transfusions, 2) Autologous blood transfusions, 3) Treatment with Recombinant Human Erythropoietin (r-HuEpo).

Leukocyte free transfusions

The immunological mechanisms behind the so called "blood transfusion effect" remain unclear. It has been argued that HLA antigens present on the transfused leukocytes are responsible for the induced immunosuppression as seen in transplant patients. Persijn et al(1) showed in a prospective study that survival of the graft did not improve if patients were transfused with leukocyte free blood. To extend these results from transplant patients to cancer patients does not go without saying because:

1) Non-specific immunosuppression by blood transfusions may play a more important role in cancer patients compared to transplant patients. The role of the leukocytes and the HLA-antigens does not need to be important in this process.

2) Animal studies done by Singh et al(2) showed that both allogeneic red cells and white cells transfusions did promote tumor growth, whereas syngeneic blood transfusions did not promote tumor growth.

3) In some retrospective studies(3) the role of plasma in inducing the blood transfusion effect was pointed out. March and colleagues(4) recently published a study concerning 131 colon cancer patients. The relative risk of recurrence for patients who received any whole blood or plasma was 2.39 ($p=0.021$) compared to patients who received no transfusion, while this risk for patients who received red cells was only 0.95 (ns).

Autologous blood transfusions

There are more arguments to do a prospective trial with randomization between autologous- and allogeneic blood transfusions. Autologous blood can be obtained either by blood donation prior to surgery (predeposited autologous blood), or by suctioning the blood lost during surgery. The latter procedure is, however, contra-indicated in cancer patients because of the hazard of re-infusing spilled tumor cells.

R-HuEpo treatment

Clinical studies with r-HuEpo showed that the anemia of patients with renal failure can be treated successfully by r-HuEpo. Recently, it has been shown in several studies that r-HuEpo therapy increases the number of collected autologous blood units. R-HuEpo therapy might play a role in the future to decrease the post-surgical need for blood transfusions.

Clinical studies presented in this thesis

The possibility of autologous blood transfusions and r-HuEpo therapy to prevent the need for allogeneic blood transfusions in cancer patients was studied. In addition we looked in this patient population and in healthy controls upon the effect of cancer, blood donation, blood transfusion and surgery on some immune parameters.

The autologous blood transfusion trial

This trial was designed in 1986 to study the effect of blood transfusion on the immune

response and tumor recurrence in colon cancer patients. It was a multi-centre prospective randomized trial in which >10 centres in the Netherlands participated. The study started in september 1986 and by now 5 years later patient accrual has been completed. Patients were randomized for autologous (predeposited) or allogeneic blood transfusions (autologous-, allogeneic patients).

Aim of the study

- To ascertain the effect of peri-operative blood transfusions on recurrent disease and survival in colorectal cancer, by comparing the disease free- and 5-year survival of autologous randomized patients with allogeneic randomized patients.
- To determine the feasibility of an autologous blood donation program in cancer patients.
- To evaluate the effect of blood donation, operation and blood transfusion on immunological parameters.

Inclusion criteria

- A histological proven colorectal carcinoma or a radiological lesion suspected for malignancy.

Exclusion criteria

- Previous malignancy except basal cell carcinoma of the skin or in situ carcinoma of the cervix uteri.
- Criteria for Autologous Blood donation, according to the rules of the American Association of Blood Banks(5).
  - Severe cardiovascular or severe respiratory disease.
  - History of convulsions after infancy.
  - Hemoglobin preoperatively <7.5 mMol/L (123 g/L) or Hct < 0.35.
- History of colitis ulcerosa or polyposis coli.
- Fixed rectal carcinoma requiring preoperative irradiation.
- Emergency operation with the exception of the construction of a proximal stoma in obstructive disease.
- Blood transfusions during preoperative work up.
- Karnofsky index >70.
- Patients with a presumably bad compliance or difficulties to ensure adequate treatment and follow-up.

Design of the study

Patients were randomized for either allogeneic- or autologous blood and were treated according to the following rules:

- Autologous patients donated 2 units of blood and started with iron supplementation before the first donation (i.e. ferrofumaraat 200 mg three times daily). The first donation was planned 14 to 12 days before the operation, and the second donation 3-4 days later. Every donation of whole blood (450 ml ± 50) was processed as packed red cells and fresh frozen plasma.
- Per- and post-operative blood loss was measured accurately. Two blood transfusions (autologous or allogeneic) would be given if blood loss exceeded 500 ml, or if the hemoglobin level was below 6.5 mMol/L. Additional allogeneic blood transfusions would be given if the hemoglobin level fell below 6.5 mMol/L.

Follow-up

Follow-up was performed according to a fixed schedule after curative resections. Evidence of recurrent disease was accepted only if one of the following criteria were present:

- Histological or cytological evidence.
- Lung metastases on chest X-ray.
- Liver metastases on ultrasound or CT-scan of the liver.
- Bone metastases on X-ray.

The r-HuEpo trial

This trial was written in 1990 and patient accrual took from 1 May 1990 to 1 May 1991. It was an open randomized study in the Dijkzigt hospital in 30 patients with proven malignant disease who needed surgical treatment.

Aim of the study

- To assess the safety of r-HuEpo therapy.
- To determine the effectiveness of r-HuEpo on erythropoiesis if administered in the perioperative period.
- To determine whether r-HuEpo can diminish or prevent post-operative anemia thus reducing the need for post-operative blood transfusions.

Inclusion criteria

- Patients must have proven malignant disease which needs extensive surgical treatment with expected blood loss >500 ml.
- Age > 18, both males and females. Females must be post-menopausal or must be practising birth control.
- Laboratory values within the following parameters:
  - Hb > 7 mMol/L (115 g/L)
  - Leukocytes > 500 cells/mm^3
  - Platelets > 75.000 cells/mm^3
  - Creatinine < 2.0 mg/ml
  - Serum Calcium < 3 mMol/L
  - Direct Coombs test: negative

Exclusion criteria

- History of any primary hematologic disease
- Cerebral metastases
- Uncontrolled hypertension
- History of seizures
- Evidence of folate-, Vit B12-, or Iron deficiency
- Acute illness within 7 days of study entry
- Corticosteroid or other immunosuppressive treatment
- Administration of an experimental drug within 30 days of study entry.
- Bone marrow depression by chemotherapy

Design of the study

Fifteen patients were randomized to receive r-HuEpo and 15 patients for no treatment with r-HuEpo. They were treated according to the following rules.

- After randomization patients received either no treatment or r-HuEpo 200 U/kg intravenously (iv) once a day for 9 days (day -5 through day +3).
- Blood loss due to operation was measured carefully and allogeneic blood transfusions were only given if blood loss exceeded 1000 ml or hemoglobin (Hb) levels dropped below 6.0 mMol/L after a 24 hours post-operative recovery period.
- The hematologic status was measured frequently until day +10.

Medication

Recombinant-Human Erythropoietin (EPREX 4000 U/ml, CILAG, Brussels, Belgium) was formulated as a sterile, buffered solution containing 2.5 mg/ml human serum albumin. 200 U r-HuEpo iv was administered daily starting 5 days before surgery (day -5) and continued until 3 days after surgery (day +3). On the day of surgery (day 0) drug was given after surgery. If possible, patients received 200 mg iron from the day of study entry until at least day + 10.

Design of the immunologic studies

Different groups of cancer patients and healthy blood donors were tested, to study the effect of cancer, blood donation, blood transfusion and operation on the proportion and numbers of lymphoid cells and their functional capacity. The groups tested are:

1) Colorectal cancer patients (n=36). Patients of the Dijkzigt hospital who were entered into the autologous blood transfusion trial, were monitored before donation, before operation, three to four days after operation and 10- 12 days post-operatively.
2) Cancer patients participating in the Epo trial (n=17). Patients were tested at study entry.
3) Metastasized cancer patients (n=33). Patients participating in a phase II trial, towards the effect of psychotherapy on tumor prognosis (Prof M de Vries, Helen Dowling Institute, Rotterdam) were tested before study entry. All these patients had measurable metastatic disease of different types of cancer.
4) Healthy blood donors (n=33). The effect of blood donation on immune parameters was tested in healthy blood donors, from the Red Cross Blood Bank (Rotterdam). Donors older than 40 years were asked to participate in this study. In the first group of 18 donors (group 4A) blood was sampled before, directly after, and 1 and 4 days after donation. The second group (group 4B), consisting of 15 donors, were tested, before donation, and 4 and 11 days after donation.

Tests performed

We studied the mitogenic responses to PHA and Con-A; the NK-cell activity; and did Flow cytometric analysis. Not all tests were done in each patient group, as is indicated in Table 1.

Table 1. Immunological studies performed in the different patient groups.

Test	Patient group[1] 1	2	3	4A	4B
Flow cytometry	X[2]	X		X	
NK-cell activity	X		X	X	X
PHA-,Con-A test	X		X	X	

[1] Group 1: Colorectal cancer patients; Group 2: Patients from r-HuEpo trial; Group 3: Metastasized cancer patients; Group 4: Healthy blood donors (A:tested in '86 B: tested in '90)

[2] Part of the patients were tested

Materials & methods

Depending on the tests being done between 5 - 40 ml heparinized blood was drawn. In order to prevent possible time fluctuations blood was always drawn between 8:00 and 10:00 AM.

Flow cytometry analysis

Cells were analyzed with a fluorescence activated cell scanner (FACSCAN, Becton and Dickinson, Mountain View, Cal., USA). SimulSET™ software was used for lymphocyte gating and determination of the different proportions of lymphocyte subsets.

One hundred uL of heparinized blood was mixed and incubated during 30 minutes at room temperature with 20 uL of the reagents described in Table 2 in separate tubes. Erythrocytes were lysed during 10 minutes with FACS® lysing solution, the remaining cells were washed three times in HBSS/PBS and analysed. The first sample was used to identify the proportion of lymphocytes, monocytes and granulocytes. This was done by a combination of antigen expression (CD45 and CD14) and light scatter characteristics. The lymphocytes were gated out, and this gate was used in all subsequent samples of that patient. The antibodies used in the other samples are given in Table 2 and identified proportions of B-, T-, and NK cells.

Absolute numbers of these subsets were calculated by multiplying the proportion of positive cells with the leukocyte count (Coulter counter) and the proportion of lymphocytes as assessed by SimulSET™.

Table 2. Description of monoclonal antibodies used in flow cytometry.

Sample	Antigen	Antibody
1	CD14	anti-leu M3-PE
2	KLH	Ig-G1-FITC
2	KLH	Ig-G2-PE
3	CD 3	anti-leu4-FITC
3	CD 19	anti-leu12-PE
4	CD 4	anti-leu3a-PE
4	CD 8	anti-leu2a-FITC
5	CD 16	anti-leu11-PE
5	CD 8	anti-leu2a-FITC
6	CD 16	anti-leu11-PE
6	CD 57	anti-leu7-FITC
7	CD 3	anti-leu4-PE
7	CD 57	anti-leu7-FITC

Lymphocyte cell preparation

Peripheral blood mononuclear cells were isolated using standard Ficoll Isopaque gradient, washed three times and resuspended with Hepes buffered RPMI 1640 (Gibco) containing 10% fetal calf serum (FCS).

Natural Killer-Cell Activity Assay

Natural killer cytotoxicity was assessed in a 4-hr assay using as target chromium 51-labelled (^{51}Cr) cells from the K562 myeloid tumor cell line(6). The assay was carried out as follows: 1 x 10^4 ^{51}Cr-labelled K562 cells were mixed in triplicate with 6.25, 12.5, 25, and 50 x10^4 peripheral blood mononuclear cells in round bottom microtiter plates (Nunc, Denmark). Cells were incubated for 4 hours at 37° C in a humidified 5% CO_2 incubator. To harvest cells, plates were centrifuged for one minute at 150 g and the supernatants were removed using the method described by Hirschberg(7) (Skatron supernatant collection system). The release of ^{51}Cr (experimental release: ER) was determined by counting radioactivity (counts per

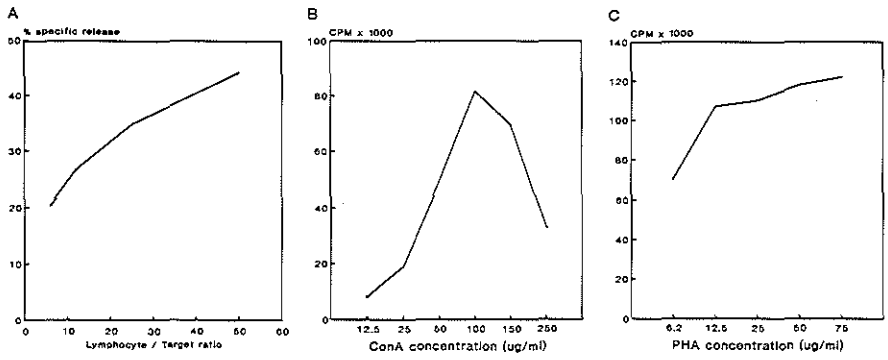
minute: cpm) in a gamma counter (LKB Wallace Ultragamma II).

Spontaneous release of ^{51}Cr (SR) from the target cells was determined in the precense of medium alone, maximal release (MR) was determined in the precence of 10% cetavlon. The percentage of lysis was calculated according to the following formula:

[cpm(mean ER)-cpm(mean SR)]/[cpm(mean MR)-cpm(mean SR)]x100%.

In the next chapters NK-cell activity will be given from the 50:1 lymphocyte target ratio because the highest values were reached with this concentration (Figure 1A).

Figure 1. The mean NK-cell activity for the four studied lymphocyte target ratio's (A) and the mean mitogenic responses to the different concentrations of ConA (B) and PHA (C).



PHA and CON A stimulation assay

Lymphocyte concentration was adjusted to 7.5 x 10^6 cells per ml in RPMI-1640 medium (Gibco) containing 10% FCS and 10^5M Beta-Mercapto ethanol. Triplicate cultures of 20 uL lymphocyte suspension and 20 uL of medium (control), different concentrations of phytohemagglutination (PHA): 6.25, 12.5, 25, 50, 75 ug/ml and

concanavalin (Con-A): 12.5, 25, 50, 100, 150, 250 ug/ml were incubated with 150 ml medium for 3 days at 37°C in a 5 % CO_2 humidified incubator. Six hours prior to termination, each culture was labelled with 0.8 uCi of methyl-3-H-thymidine (^{3}H-Tdr, specific activity 2Ci/mmol;Amersham, UK). Harvesting was performed using an automatic system (microtiter-automash, Dynatech, Holland) collecting cells on fiber-glass filters. After drying, the filters were placed in scintillation vials and scintillation fluid was added. The uptake of ^{3}H-Tdr was determined with a liquid scintillation counter (B, Searl isocap II). Activity was expressed by subtracting cpm of non-stimulated test from cpm in stimulated cells.

Because highest counts were found after stimulation with 150 ug/mL ConA and 75 ug/mL PHA (Figure 1B & 1C), the values of ConA and PHA stimulation will be given in the next chapters from these concentrations.

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PART II: IMMUNOLOGY

CHAPTER 4

THE IMMUNE STATUS OF CANCER PATIENTS

Summary

The immune status of patients with local gastro-intestinal cancer, patients with advanced cancer, and normal healthy elderly blood donors was compared. Responses to PHA and ConA were lower in both groups of cancer patients compared to the group of blood donors. The mitogenic responses to ConA were 34 x 10^3 cpm (counts per minute) in colorectal cancer patients and 28 x 10^3 cpm in patients with distant metastases and 78 x 10^3 cpm in blood donors. The NK-cell activity was only depressed in patients with metastatic disease. The mean specific release was 41 % in colorectal cancer patients, 46 % in blood donors and 22 % in patients with advanced disease.

Lymphocyte subsets were only analyzed in patients with local gastro-intestinal cancer and in healthy blood donors. The mean proportion of lymphocytes was 19.3 in the cancer patients and 32.6 in healthy blood donors ($p=0.0001$). The mean absolute number of T cells was 1517 x 10^6/L in blood donors and 957 x 10^6/L in cancer patients ($p= 0.015$).

So, there was a clear immune-suppression seen in gastro-intestinal cancer patients at the moment before surgery. This may render them more suspectible for post-operative infections, but may in addition affect survival. We were, however, not able to show with our data that the T-cell or NK-cell function had a prognostic meaning in cancer patients.

Introduction

Theoretically, immune reactions may be important in preventing the initial appearance of tumors (the idea of immuno-surveillance) or may limit tumor growth. Immune-suppression at the moment of surgery may lead to an increased metastatic potential of tumor cells or an increased outgrowth of small number of tumor cells already settled. The tumor-load after surgery is low, a situation in which immune cells probably have a better change to work effectively(1). NK-cells are thought to play a role in the prevention of blood-born metastases(2). Their activity is regulated by lymphokines, such as Interleukin-2, that are produced by T-cells.

In this chapter the immunologic status of cancer patients with local disease, will be compared with the immune status of metastasised patients and healthy older blood donors. In addition attempts will be made to correlate the immune status with survival.

Patients and tests

Patient groups

Four different groups were analyzed: 1) Colorectal cancer patients participating in the Autologous Blood Transfusion Trial (n=36). Patients were randomized for autologous- or allogeneic blood transfusions (autologous- or allogeneic patients). 2) Patients with various gastro-intestinal cancers participating in the r-HuEpo trial including; 5 esophagus, 5 cardia, 1 colon and 6 pancreatic cancer patients (n=17). 3) Patients with disseminated malignant disease who participated in the psychotherapeutic intervention trial (n=33). 4) Blood donors (2 groups) aged over 40 years (n=33).

Patients from group 1 and 2 all were operated with a curative intention. They were evaluated before operation and there were no indications for distant metastases. In some analysis, these two groups will be taken together as patients with gastro-intestinal local cancer.

Tests performed

Blood was taken in all patients between 8:00 and 10:00 AM to eliminate diurnal variability. All patients except allogeneic patients were bled at the polyclinics or at

the blood bank. Allogeneic patients were tested directly after admission to the hospital. NK-cell activity, mitogenic responses to PHA and ConA and enumeration of lymphocyte subsets were studied. Not all tests were done in all patient groups as can be seen in Table 1.

Staging and survival in colorectal cancer patients

In the colorectal cancer patients tumor staging was based on the Dukes' classification modified by Turnbull. Invasive cancer only partially involving the bowel wall, without any metastases was defined as Dukes' A. Cancers that penetrated the full thickness of the bowel wall, but still without signs of metastatic disease were classified ad Dukes' B. Finally, those cancers with nodal involvement but without evidence of distant metastases were defined as Dukes' C.

There was a standard 3 monthly follow-up in which CEA was assessed every 3 months, ultrasound of the abdomen was performed every 6 months and a colonoscopy was done yearly. The follow-up period ranged from 9 to 46 months, with an average of 23 months.

As will be shown in the next chapter, NK-cell activity decreases after blood donation, and probably during admission in the hospital. In order to compare the NK-cell activity of colorectal cancer patients with the 2 other groups, only predonation values of autologous patients were used.

Immune functions were related to survival and tumor recurrence; in autologous patients this was done with predonation values and in allogeneic patients with presurgical values.

Table 1. Different test performed in the different patients groups.

	Patient group[1]				
Test	1	2	3	4A	4B
Flow cytometry	X[2]	X		X	
NK-cell activity	X		X	X	X
PHA-,Con-A test	X		X	X	

[1] Group 1: Colorectal cancer patients; Group 2: Patients from r-HuEpo trial; Group 3: Metastasised cancer patients; Group 4: Healthy blood donors (A: tested in 1986; B: tested in 1990)

[2] Part of the patients were tested (n=13)

Survival in metastasised cancer patients

Survival data of all but 1 patient from the psycho-intervention trial were known. Seven patients were still alive on the first of January 1991; survival ranging from 16 to 39 months (mean survival 24.6 months). The other 25 patients died within this period; the average survival was 12.5 months.

Statistical analysis

For each assay, means and standard error of the mean (SEM) were determined. Differences between the groups were tested with a one-way variance analysis: the Student Newman Keuls test (SNK). Both the overall differences between the three groups and differences between the separate groups were determined and will be given in the tables ($p<0.05$). The Student' T-test (T-test) was used when two patient groups were compared. Each variable was studied for significant differences between sexes and for correlation with age. Because donors were not age- and sex- matched multivariate regression analysis, with immunologic parameters as the dependent factor and tumor stage, sex and age as independent variables, were carried out.

Results

The immune status of patients

The immune status of cancer patients was studied as defined by function assays or lymphocyte cell-surface markers. The data of patients with local cancer and from patients with advanced disease were compared with values of healthy blood donors. Table 2 displays the means and SEM for the function assays; Table 3 shows the proportion of the various leukocyte- and lymphocyte subdivisions; and Table 4 shows the significant differences of both the proportion and the enumeration of the lymphocyte subsets for the 2 groups of patients with local disease taken together in comparison with blood donors.

Function assays

No significant correlation was found between age and the three lymphocyte function assays. Significant differences between men and women were only found for the NK-cell activity in colorectal cancer patients. The mean specific release being 43.9 % in men and 26.4 % in women (T-test: $P=0.025$).

The mitogenic responses to PHA and ConA were depressed to the same extend in colorectal cancer patients and metastasised patients. The NK-cell activity was only depressed in patients with metastatic disease (Table 2). Multiple regression analysis showed that all these differences were still significant after adjustment for differences in age and sex between the three groups.

Leukocyte- and lymphocyte subset proportions (Table 3 & 4)

The proportion of leukocytes and lymphocytes did not vary with age and sex. The lymphocyte, monocyte and granulocyte percentages were significantly different between blood donors and patients participating in the r-HuEpo and blood transfusion trial (Table 3). Data are summarized for the two groups of cancer patients taken together in Table 4. The proportion of lymphocytes and monocytes were significantly higher in healthy controls, while the proportion of granulocytes was higher in cancer patients.

The proportions of the various lymphocyte subsets as defined by their markers (Chapter 3, Table 2) were not significantly different. The CD4/CD8 ration was 1.70 in donors and 1.61 in cancer patients (not significant).

Table 2. Mitogen responses and NK-cell activity (Mean ± SEM) in blood donors and cancer patients.

Patient group	age	male/ female	ConA[1]	PHA[1]	NK activity[2]
1 Colorectal	65.9 ± 2.2	21/15	33.9 ± 4.5[3]	64.7 ± 6.1[3]	41.4 ± 3.6[3]
3 Metastasised	51.2 ± 2.0	10/23	28.2 ± 4.4	51.2 ± 8.1	21.7 ± 2.6
4 Blood donors	50.3 ± 1.3	20/13	78.5 ± 7.4[4]	122.8 ± 9.5[4]	45.6 ± 4.3
SNK test all groups:			<0.0001	<0.0001	<0.0001
1 vs 3			ns	ns	s
1 vs 4			s	s	ns
3 vs 4			s	s	s

[1] Counts per minute (x 10^3) of ^{3}H-Thymidine.
[2] Percentage specific release, effector:target ratio 50:1.
[3] Only data of autologous randomized patients measured before blood donation.
[4] Only 16 donors were tested (mean age 50.5 years)

Table 3. Comparison of leukocyte and lymphocyte subsets of cancer patients and blood donors.

	Different patient groups						
	Colon(1)	Epo(2)	Donors(3)	S	1/2[1]	1/3	2/3
Patient (n)	13	17	15				
Age (mean)	59.5	62.7	49.5	0.025	n	n	s
Age (range)	31-82	44-74	43-60				
Sex (M/F)	7/6	14/3	10/5				
Leukocyte[2]	6.46	8.18	6.33	0.054			
Lympho[2]	1.50	1.20	2.03	0.03	n	s	s
Granulo[2]	4.60	6.63	3.87	0.001	s	n	s
Lympho %	24.1	15.7	32.6	0.0001	s	s	s
Mono %	5.6	4.4	7.2	0.001	n	s	s
Granulo. %	70.2	79.9	60.3	0.0001	s	s	s
B cells %	12.8	8.9	11.3	0.31			
T cells %	69.3	74.1	74.6	0.17			
CD4 %	41.6	41.8	45.5	0.24			
CD8 %	26.2	30.6	29.4	0.34			
CD4/CD8	1.69	1.54	1.70	0.61			
NK cells %	16.9	16.1	12.9	0.31			
Leu 11^+7^-%	8.8	8.9	7.5	0.65			
Leu 11^+7^+%	8.0	6.9	5.3	0.15			

[1] Significance between the three separate groups.
[2] Numbers x 10^9/L

Leukocyte- and lymphocyte subset enumerations (Table 3 & 4)

Both the absolute number of lymphocytes and granulocytes were significantly different between the three groups (Table 3). Besides the granulocyte count, no differences were seen between the two patient groups with local gastro-intestinal cancer. When these groups were taken together, the lymphocyte count was 1.33 x 10^9/L in cancer patients and 2.04 x 10^9/L in donors ($p=0.01$, Table 4). Of the numbers of circulating B-,T- and NK-cells, only T cells were significantly depressed in cancer patients with an equal and significant reduction of both CD4 positive and CD8 positive cells.

Table 4. Significant differences between blood donors and gastro-intestinal cancer patients with regard to leukocyte and lymphocyte subsets proportions and numbers.

	Cancer pts	Blood donors	T-test	MR[1]
Number pts	30	15		
Age	61.2	49.5		
Male/Female	24/9	10/5		
Lymphocyte %	19.3 ± 1.44	32.6 ± 2.26	0.0001	0.0002
Lymphocyte numbers[2]	1333 ± 91.0	2035 ± 226.2	0.010	0.018
Monocyte %	4.9 ± 0.38	7.2 ± 0.47	0.001	0.025
Granulocyte %	75.7 ± 16.1	60.3 ± 22.1	0.0001	0.0001
Granulocyte numbers	5754 ± 443.6	3869 ± 427.0	0.004	ns
T cells numbers	957 ± 65.9	1517 ± 174.8	0.015	0.0023
CD4 cells numbers	552 ± 40.1	945 ± 125.2	0.008	0.013
CD8 cells numbers	391 ± 39.1	582 ± 67.0	0.022	0.0096

[1] Multiple regression analysis. The p-value indicates the effect of having cancer or not after adjustment for age and sex.
[2] Cells $x10^6$/L.

Immunologic status and prognosis

1) Metastasised patients: Seven patients out of 32 were still alive on the first of January 1991, their survival ranging from 16 to 39 months after being tested. No correlation existed between the mitogenic responses to ConA and PHA or NK-cell activity measured at study entry and the survival of patients in months. In Table 5 the mean baseline values of the assessed parameters are given for patients who were still alive and for patients who died. No significant differences were noticed, also not if the mean values for patients who had died within 9 months were compared with the mean values of patients who died after 9 months (Table 5).

2) Colorectal cancer patients: The mean mitogenic responses and NK-cell activity measured before operation did not differ between the different Dukes' stages (Table 6). In a follow-up period ranging from 9 to 48 months, 8 out of 36 patients showed recurrent disease (mean disease free period: 11.3 months) of whom 5 died (mean survival period: 30.0 months). The immune status of the patients who died or of those who developed metastases was not significantly different from the patients who remained disease free (mean follow-up 22.8 months) (Table 6).

Table 5. Mitogenic response to PHA and ConA and NK-cell activity in metastasised cancer patients.

Patients	N	ConA	PHA	NK-cell act.
Alive	7	23.2 ± 6.7	52.0 ± 21.9	30.7 ± 6.4
Died	25	29.2 ± 5.2	51.0 ± 8.9	19.4 ± 2.8
died within 9 months	13	32.2 ± 9.1	42.1 ± 9.8	21.4 ± 4.9
died after 9 months	12	28.7 ± 5.8	63.7 ± 14.8	17.7 ± 2.8

Table 6. Function tests and prognosis in different colorectal cancer patient groups.

Test	alive		metastasised		Dukes' classification		
	yes (31)	no (5)	no (28)	yes (8)	A (9)	B (15)	C (10)
ConA	39.5 ± 5.4	26.0 ± 6.7	40.5 ± 2.8	27.6 ± 6.8	28.2 ± 8.8	37.8 ± 7.7	40.6 ± 9.4
PHA	71.3 ± 6.9	56.2 ± 8.9	75.3 ± 7.1	46.3 ± 8.5[1]	65.3 ± 12.7	77.9 ± 10.8	62.6 ± 9.6
NK	36.0 ± 3.4	50.5 ± 14.6	36.2 ± 3.5	45.2 ± 10.2	37.0 ± 6.5	35.4 ± 6.1	38.3 ± 5.0

[1] P=0.056 (T-test)

Discussion

We showed in this study (Table 7) that in patients with local disease, T-cell numbers and responses to PHA and ConA were depressed, whereas in patients with metastasised disease the NK-cell activity had dropped in addition. Numerous other investigators(3)(4) showed in cancer patients a diminished response to mitogens or antigens. Dilman et al(5) showed in 72 patients with advanced cancer depression of responses to PHA- and ConA, depression of the percentage of lymphocytes and the number of T-cells. They found in addition a decreased CD4/CD8 ratio compared to controls. Braun and his group(6) showed similar results in a group of metastasised patients, while patients who were curatively resected showed no signs of immune-suppression. Also abnormalities in NK-cell level or function have been reported in cancer patients; NK-cell activity was found to be depressed in patients with advanced disease, while normal values were seen in

patients with local disease(7) or minimal disease(8). Balch et al found in colorectal cancer patients normal NK-cell activity, while the proportion of NK-cells, as defined by anti-leu-7 mAb, was significantly reduced (9.7 % in comparison with 16 % in controls)(9). Guanti and colleagues showed that patients, who had been curatively operated for cancer in the past, had normal NK-cell activity but significantly lower levels of NK-cells (measured with the mAb 8.28).

A depression of the proportion and the number of NK-cells as defined by the anti-Leu-11 mAb was not seen in our patient groups: 16.9 % of the PMNC in colorectal cancer patients were stained by the mAb, compared to 16.1 % in patients in the r-HuEpo trial, and 12.9 % in donors. These divergent results with regard to levels of NK-cells may depend on several factors, such as cell purification technique (we used full blood), counting technique (Microscopic counting/ Facs analysis) monoclonals used (anti-leu-7, anti-leu-11 or MAb 8.28) and different patient- and control groups studied.

A depression of the immune system in cancer patients may be a factor that contributes to the development of cancer, but may also result from cancer. We were not able to show a relation between the immune status of the patients and survival neither in metastasised patients nor in colorectal cancer patients. Of the 36 colorectal patients studied, 8 patients had recurrences in the follow-up period of whom 5 died. The preoperative values of these patients were not significantly different from patients without recurrent disease (Table 6). The mean follow up was however short; ranging from 9 to 46 months and the total number of patients studied was small.

Table 7. Summary of the results.

	Patients with local cancer	Patients with metastases
NK-cell activity	=	↓
T-cell responses	↓	↓
NK-cell numbers	=	not done
T-cell numbers	↓	not done
CD4/CD8 ratio	=	not done

There was no relation between NK-cell activity and the advancement of the disease according to the Dukes' stage. This has also been found in other studies(10),(11), and may reflect that depression of NK-cell activity is not related to tumor load. Tartter et al(12) were able to show in colorectal cancer patients that the pre-operative NK-cell activity is together with tumor stage, an independent prognostic factor. In his study the 18 patients with recurrences had significantly lower pre-operative NK cytotoxicity than the 84 patients who remained disease-free. Multivariate analysis indicated that it was the sole pre-operative variable besides stage that was significantly related to recurrence and its prognostic significance was independent of stage, age, sex and tumor size.

Our data confirms that patients with local disease and metastasised disease are immune-suppressed. The patients with gastro-intestinal cancer were operated with lower numbers of circulating T-cells and a depressed T-cell response. This may increase the risk for postoperative infections, but it also may affect the fate of blood-borne metastasis. The effective clearance of tumor cells from the circulation is thought to be under control of NK-cells(13). Although, both the numbers of NK-cells and the *in-vitro* NK-cell function were not different from healthy blood donors, a decreased lymphokine production by T-cells may affect the activity of NK-cells *in-vivo*.

We can not address the question whether the observed immune-depression results from cancer, and/or attributes to cancer development or tumor growth. The study of Tarrter showing a relationship of NK-cell activity and survival has been confirmed by some other studies. Pross and Baines(14) showed in patients with solid cancer and Schantz et al(15) in patients with head and neck tumors that patients with low NK-cell activity at diagnosis were at increased risk for developing metastasises. Low NK-cell activity was an independent prognostic factor in these patients.

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CHAPTER 5

EFFECT OF BLOOD DONATION, TRANSFUSION AND SURGERY ON THE IMMUNE SYSTEM

Summary

Disturbances of the immune response during blood donation, blood transfusion and surgery may promote tumor cell metastases. NK-cell activity, T-cell responses to PHA and ConA, and lymphocyte subsets were studied in colorectal cancer patients in the peri-operative period. These patients participated in the autologous blood transfusion trial and were randomized for either predeposited autologous blood transfusions or for homologous (allogeneic) transfusions. The effect of blood donation was studied also in voluntary blood donors.

The NK-cell activity in blood donors was only 70 % of the baseline value, five days after donation of 1 unit blood. While the NK-cell activity was normal again twelve days after donation, it was still depressed in colorectal cancer patients who had donated 2 units of blood. Surgery itself had no effect on the NK-cell activity, but the administration of homologous blood transfusions resulted in a depressed NK-cell activity, if compared to non-transfused or autologously transfused patients, in the post-operative period.

The overall effect of blood donation and blood transfusion on the NK-cell activity was that autologous randomized patients were operated with a lower NK-cell activity compared to homologous randomized patients, but that post-operative values were equal. If the NK-cell indeed plays a role in the prevention of blood borne metastases, such a transient depression may affect the survival of the patients that were randomized for autologous blood transfusions.

Blood donation did not affect the T-cell response, but surgery did. The T-cell response was depressed in both the homologous and the autologous randomized patients after surgery. No additional effect of blood transfusions could be demonstrated.

Introduction

Disturbances of the host defense system during surgical procedures may enable bacterial invasion and tumor cell metastases just at the moment when risks from invading pathogens and tumor cells are maximal. The effect of surgery on the immune response was noted as early as 1904(1). Surgery includes anesthesia, trauma, blood loss and blood transfusions, and all of these factors in itself have shown to modulate the immune system(11-16)(19). We are looking upon the effect of blood transfusions upon the survival of colorectal cancer patients in a prospective randomized study; patients are randomized for autologous- or homologous blood transfusions. This randomization enabled us to study the effect of blood transfusion separately from the effect of surgery, by comparing the immune response of autologous and homologous transfused patients.

Rat studies done at our laboratory indicated that tumor growth may not only be stimulated after homologous (allogeneic) transfusions(2), but also after blood loss(3). Therefore, we studied in addition the effect of blood donation in the autologous randomized patients and in healthy blood donors.

Patients and methods

Effect of blood donation

The effect of blood donation on immune parameters was studied in 33 voluntary blood donors who were all older than 40 years, and in 16 colorectal cancer patients participating in the "autologous blood transfusion trial".

The blood donor group consisted of 21 men and 12 women all older than 40 years. They were recruited at random from voluntary blood donors visiting the Red Cross Blood Bank in Rotterdam. The first group of 18 donors was tested before; immediately after blood donation (day 1); on day 2; and on day 5. The results indicated that the next group of 15 donors could better be tested on day 5 and on day 12. On day 12 the data could be compared with those of the colorectal cancer patients.

Patients scheduled for elective colorectal cancer surgery fulfilling the criteria from the Association of American Blood Banks for autologous blood donation were randomized for either homologous or autologous blood transfusion therapy. The

patients randomized for autologous blood transfusion (autologous patients) donated two units of blood, about 14 and 11 days before scheduled surgery. Trial patients from our own center were asked to participate, in addition, in this study: 40 ml blood was sampled 2 or 3 days before the first donation (± 500 ml) at the outpatients' clinic and about 9 days after the second blood donation, being the first or second day of admission to the hospital.

The first group of healthy donors, and the autologous randomized cancer patients were tested for mitogenic responses to PHA and ConA, and NK-cell activity. In the second group of donors mitogenic responses were not longer studied, we analyzed the lymphocyte subsets in this donor group and in part of the colorectal cancer patients. The methods of the tests performed are described in chapter 3.

Effect of surgery and transfusion

The effect of operation and autologous- or homologous- blood transfusions was studied in colorectal cancer patients. Patients were tested 1 or 2 days prior to surgery (day -1), 3 to 5 days after surgery (day 4) and 9 to 12 days after surgery (day 10).

To study the effect of blood transfusion, three groups of patients were distinguished: 1) Autologous randomized patients who had received two units of autologous blood (2 auto). 2) Homologous randomized patients who had received no blood (0 homo). 3) Homologous randomized patients who had received 2 homologous blood transfusions (2 homo) (Table 1).

Table 1. Number of autologous and homologous randomized patients receiving blood transfusions (BT).

Randomization	0 Transfusions	2 Transfusions	> 2 Transfusions
Autologous	2	11*	3
Homologous	10*	12*	6

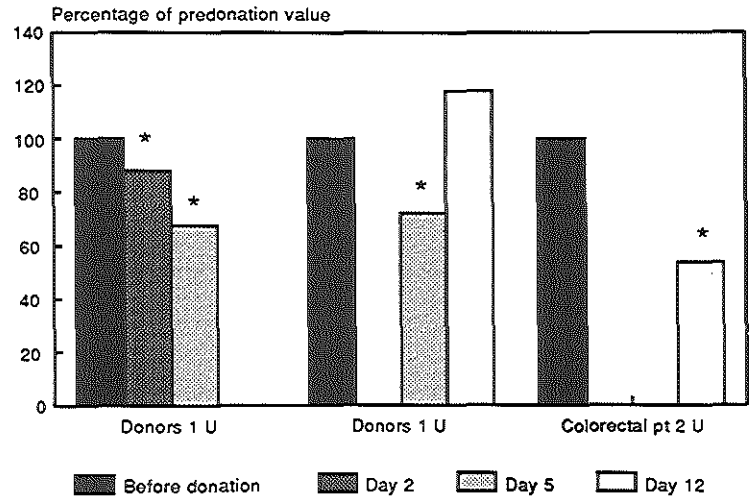
* These three groups will be used in the studies in which the effect of blood transfusions is studied separately (Figure 3C,4C,5C).

Statistic methods

Data are given in the tables and figures as mean ± Standard Error of the Mean (SEM). In order to compare the NK-cell activity in the three different studied groups, the percentagewise decrease in NK-cell activity per patient was calculated (NK-cell activity day N / baseline value x 100 %). The effect of surgery and blood transfusion within each studied group will be presented as the mean decrease/increase compared to pre-surgical values.

Because of the non-normal distribution of the data, differences between groups were studied for significance with the Mann-Whitney test (MW), while decreases and increases were studied with the paired Wilcoxon test (Wx). Decreases or increases in the three differently transfused group were also compared with each other with the MW test. All analyses were performed on the computer with the help of SPSS package.

Figure 1. Effect of blood donation on NK-cell activity in healthy blood donors, donating 1 unit of blood and in colorectal cancer patients donating 2 units of blood (*: $P<0.05$ Wx).



Results

The effect of blood donation on the lymphocyte function

Donation of 1 unit of blood by voluntary elderly blood donors or donation of 2 units by colorectal cancer patients did not result in significant decreases in the mitogenic responses to PHA or ConA during the first week after blood donation (Table 2).

However, the NK-cell activity was depressed after blood donation. It was 104 % of the baseline value directly after donation, 88% on day 2 (Wx: p=0.02), and 68 % on day 5 in the first group of tested blood donors (Wx: 0.0012)(Figure 1). A similar depression of the NK-cell activity was noticed on day 5 in the second group of donors (Wx: p=0.017), while NK-cell activity was back to normal values on day 12.

Colorectal cancer patients were tested 12 days after their first donation, which is 9 days after their second donation. The NK-cell activity was 54 % of the predonation value at that moment (Wx p=0.02). Figure 3A shows the absolute values of both homologous and autologous randomized patients. It shows that: 1) the NK-cell activity just prior to surgery is significantly lower in autologous patients compared to homologous donors (MW p=0.05), and 2) the NK-cell activity measured before surgery in homologous patients is lower compared to predonation values of autologous randomized patients (MW p=0.056).

Table 2. Effect of blood donation on mitogenic response to PHA and Con A (counts $x10^3$ /minute).

Group	timing of test	PHA mean ± SEM	range	Con A mean ± SE	range
Donors	before donation	122.8 ± 9.5	19-190	79.6 ± 7.1	49-167
	after donation	127.7 ± 8.1	49-195	76.4 ± 8.4	27-158
	day 2	126.4 ± 9.3	42-186	87.1 ± 6.8	46-179
	day 5	116.8 ± 7.3	73-177	82.4 ± 6.9	34-177
Colorectal pts	before donation	70.1 ± 11.9	25-130	25.6 ± 5.5	5-60
	day 12	49.0 ± 13.1	9-112	17.8 ± 4.8	4-43

Figure 2. Effect of blood donation on lymphocyte subpopulations (numbers). A: In normal blood donors donating 1 unit of blood; B: In colorectal cancer patients donating 2 units of blood.

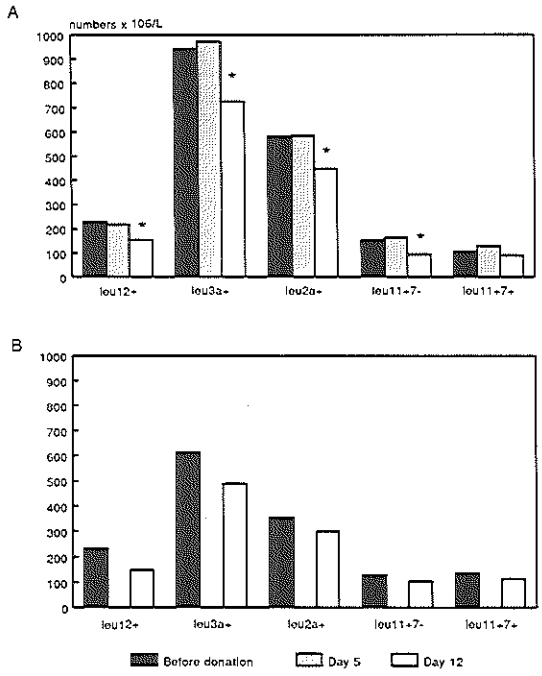
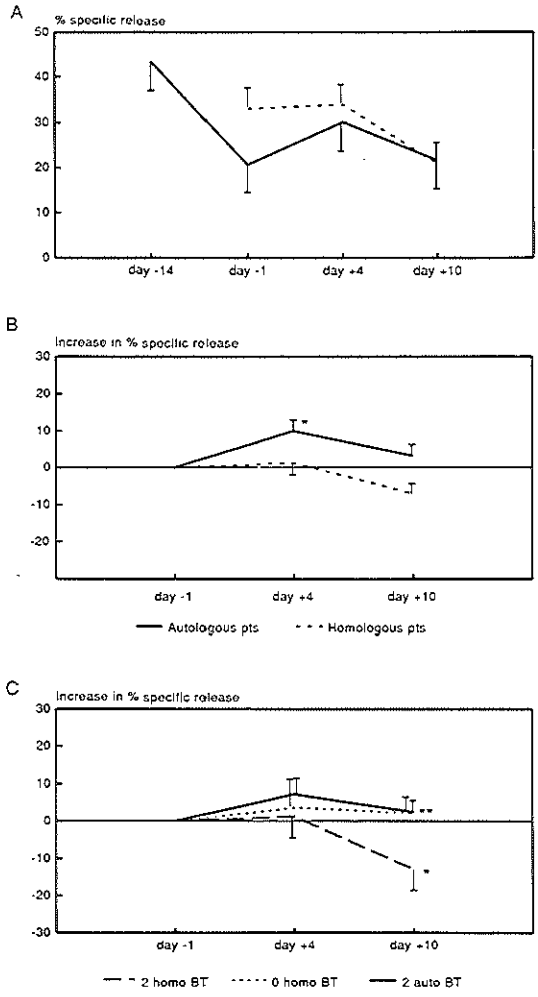


Table 3. Effect of blood donation on lymphocyte proportions.

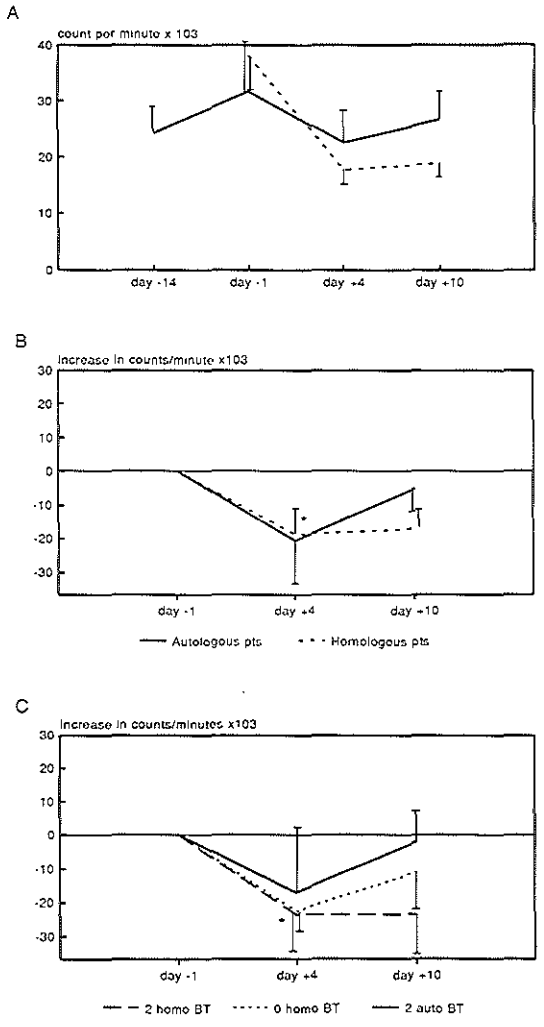
		Lympho %	% B-cells (Leu-12$^+$)	% T-cells (Leu-4$^+$)	% NK-cells (Leu-11$^+$)
Donors	before donation	32.6	11.3	74.6	12.9
	day 5	33.3	10.6	75.1	13.5
	day 12	25.4	10.2	76.1	12.5
Colorectal pts	before donation	23.2	15.8	65.2	18.7
	day 12	19.8	12.2	69.5	17.8

Figure 3. NK-cell activity (% specific release of ^{51}Cr) before and after blood donation and surgery in autologous and homologous randomized patients. A: Mean absolute values +/- SEM. B: In- and decreases from pre-surgical values for the two patient groups (mean +/- SEM). C: In- and decreases from pre-surgical values for patients who received 2 autologous (2 auto); 2 homologous (2 homo); or homologous patients who did not receive homologous transfusions (0 homo)(mean +/- SEM).



*:significantly different from baseline with the Wilcoxon test
**: significant different between groups with the Mann Whitney test

Figure 4. Mitogenic response to ConA before and after blood donation and surgery in the autologous and homologous randomized patients. A: Mean absolute values +/- SEM. B: In- and decreases from pre-surgical values for the two patient groups (mean +/- SEM). C: In- and decreases from pre-surgical values for patients who received 2 autologous (2 auto); 2 homologous (2 homo); or homologous patients who did not receive homologous transfusions (0 homo)(mean +/- SEM).



*:significantly different from baseline with the Wilcoxon test
**: significant different between groups with the Mann Whitney test

The effect of blood donation on lymphocyte subsets

In the 15 blood donors analyzed, the leukocyte count remained stable after donation. The mean lymphocyte percentage dropped from 32.6 % before donation and 33.3 % on day 5 to 25.4 % on day 12 (Wx p=0.02) (Table 3). As a consequence the absolute number of circulating lymphocytes was depressed at day 12 (Wx p=0.01). The proportions of B-, T- and NK-cells subsets did not change, nor did the CD4/CD8 ratio (Table 3); the absolute numbers however were all significantly lower than predonation values (Figure 2A).

Only 6 colorectal cancer patients were analyzed both before and after donation. The same trends were seen as described above but differences did not reach significance (Table 3, Figure 2B). The lymphocyte percentage dropped from 23 to 20 %, while absolute numbers dropped from 1.49 to 1.17 x 10^9/L.

Correlation between NK-cell activity and proportion of NK-cells before and after blood donation

There was a very significant correlation between the NK-cell activity and the proportion of NK-cells before blood donation. The correlation was 0.61 (p=0.016) with the proportion of Leu-11^+7^- cells, and 0.71 for Leu-11^+7^+ cells (p=0.0027). Taken together the correlation was 0.69 (p=0.0042). There was no correlation between the proportion of Leu-11^-7^+ cells and NK-cell activity; these cells appeared to be positive for CD3 markers, so probably were T-cells.

While NK-cell activity dropped significantly after donation the proportion of NK-cells did not change. There was no longer a significant correlation between them. The correlation between the total population of NK-cells (Leu-11^+7^- + Leu-11^+7^+) and NK-cell activity was 0.44 on day 5 (p=0.10), and 0.35 (p=0.22) on day 12.

The effect of operation and blood transfusion on mitogenic responses

By coincidence more homologous patients than autologous patients were included in this study (Table 1). Blood loss was similar for the homologous- and autologous-transfused patients (1150 and 1020 ml respectively), indicating that these groups were analogous to each other with regard to surgical stress. Patients who did not receive any blood transfusions lost significantly less blood during surgery (380 ml).

The absolute T-cell responses were not significantly different between the homologous and autologous randomized patients in the peri-surgical period (fig 4A & 5A). Taking both groups together, the mean decrease in ConA response, when

compared to pre-surgical values, was 19.260 CPM on day 4 (Wilcoxon: p=0.03) and 12.230 on day 10 (Wilcoxon: p=0.056). The response after stimulation with PHA was reduced with a mean of 23.330 CPM (Wilcoxon p=0.04) at day 4 and differences were still significant at day 10.

Studying the two randomized groups separately revealed that the experienced decreases were only significantly depressed compared to baseline values in the homologous randomized patients (Figure 4B & 5B). In addition to this, the response to PHA and ConA was significantly lower compared to baseline values only in patients who had received 2 homologous transfusions during surgery. However, when the mean decreases were compared between the three differently transfused groups, no significant differences were seen (Figure 4C & 5C).

The effect of operation and blood transfusion on the NK-Cell activity

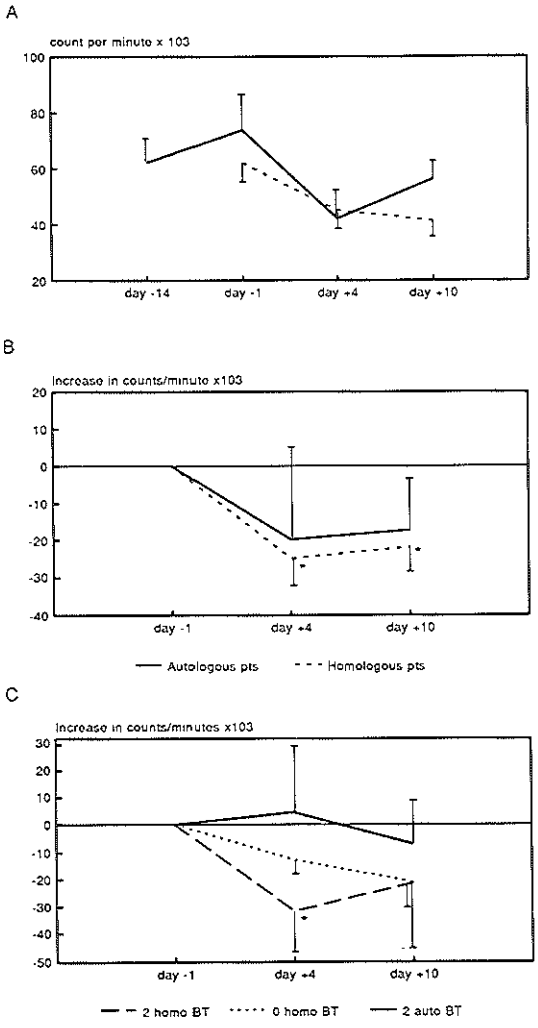
Autologous patients tended to have lower pre-surgical values compared to homologous patients (p=0.056). However, no significant differences were seen between the two randomized groups in the post-operative period (Figure 3A). This may be due to the fact that the NK-cell activity of the autologous randomized patients increased significantly after surgery. The mean specific release being 10 % higher on day 4 compared to before surgery (Wilcoxon: p=0.04) (Figure 3B).

The mean decreases for the 3 distinguished transfusion groups are given in Figure 3C. The NK-cell activity was only depressed on day 10 compared to the pre-surgical value in patients who had received 2 homologous transfusions (Wx p=0.036), while it remained stable in autologous transfused patients and in non-transfused patients. Comparison of the mean decreases between the 3 groups revealed that this depression was significantly different from the increase as seen in autologous transfused patients (MW p=0.032) and in non-transfused patients (p=0.045).

Discussion

Surgical resection is the most commonly employed therapeutic modality in patients with solid neoplasms. There have been numerous reports indicating that surgery may be immune-suppressive(11-14). We studied the effect of blood donation, surgery and blood transfusion on in vitro immunological parameters in a group of colorectal cancer patients, who were randomized for predeposited autologous- or homologous

Figure 5. Mitogenic response to PHA before and after blood donation and surgery in the autologous and homologous randomized patients. A: Mean absolute values +/- SEM. B: In- and decreases from pre-surgical values for the two patient groups (mean +/- SEM). C: In- and decreases from pre-surgical values for patients who received 2 autologous (2 auto); 2 homologous (2 homo); or homologous patients who did not receive homologous transfusions (0 homo)(mean +/- SEM).



*:significantly different from baseline with the Wilcoxon test
**: significant different between groups with the Mann Whitney test

blood transfusions. This randomization enabled us to study the effect of transfusion therapy separately from the effect of surgery. The effect of blood donation was also studied in voluntary blood donors. The results are summarized in Table 4 and discussed below.

Blood donation resulted in a temporary decrease of NK-cell activity, both in blood donors donating 1 unit and in colorectal cancer patients donating 2 units of blood. In the latter group two phenomena may play a role: 1) effect of blood donation. 2) effect of admission to the hospital. We conclude this because homologous randomized patients, who were only tested just before operation, had lower NK-cell activity before surgery than autologous patients who were tested before blood donation, but significance was not reached ($p=0.056$). This decreased NK-cell activity may be caused by stress; stress regardless of its nature, stimulates secretion of adrenocortical- and many other hormones, a number of these may mediate immune-modulation(4).

Table 4. Summary of essential results.

	NK-cell Activity	T-cell response	Lymphocyte subsets	
			proportion	counts
Blood donation	↓	=	=	↓
Surgery	=	↓↓	Not Done	
Blood transfusion	↓	=↓	Not Done	

To our knowledge Lasek et al(5) published the only study that looked upon the effect of multiple donations on NK-cell activity. While we studied the effect of donation prospectively, they compared the NK-cell activity of voluntary donors who had donated blood in the past, with the NK-cell activity of new blood donors. They showed that NK-cell activity was significantly decreased in voluntary blood donors, the effect being dependent on the total volume of blood donated. We have no definite explanation for the significant decreases in NK-cell activity seen in the 3 groups studied, but several mechanisms may play a role:

1. Hemorrhage is a potent stimulus for enhanced secretion of corticosteroids(6). This or alterations of serum levels of catecholamine, endorphin or interleukins after stress may influence NK-cell activity (4).

2. Besides their cytotoxic activity NK-cells are known to be engaged in processes regulating erythropoiesis(7). Retention from the peripheral blood in hemopoietic tissues during enhanced hematopoiesis may result in depression of their activity in peripheral blood.

3. It may be assumed that following blood donation there is a loss of a certain number of mature cells having NK-cell activity. Subsequent release into the blood of less differentiated cells could result in a decrease in NK-cell activity.

Our data refutes the latter two explanations: while NK-cell activity was depressed on day-5, both the proportion- and the absolute number of NK-cells remained stable. There was also no evidence for a shift towards more immature NK-cells, the proportion of Leu-11$^+$ cells, cells known to represent the mature NK-cell population(8)(9), had not changed. The function per NK-cell was depressed, the correlation between the percentage of NK-cells and NK-cell disappeared completely.

Donation of 1 unit blood resulted in a drop in the proportion of lymphocytes 12 days afterwards; consequently all absolute numbers of all lymphocyte-subsets were significantly decreased at that moment. We can not explain this, others(10) studied the effects of repeated lymphocytopheresis; in which procedure much more lymphocytes were removed (41×10^9) in a 12 day period. No significant decreases in lymphocyte counts mitogenic responses to PHA, ConA, and Pokeweed Mitogen were noticed in this study.

The responses to PHA and ConA were significantly depressed after surgery. This is in agreement with numerous other studies (11)(12)(13)(14). Some investigators looked in addition to the effect of blood loss and blood transfusions. Jubert et al(12) found the degree of immune-suppression to be associated with the amount of blood loss. Roth et al(13) showed that cancer patients who were transfused during surgery had a significantly lower Con-A response, compared to those who were not transfused.

We were able to study the effect of blood transfusion separately from surgery and blood loss, because our patients were randomized between homologous and autologous blood transfusions. Three different transfusion groups were distinguished: patients who received 2 autologous (2 auto), patients who had received 2 homologous (2 homo) and patients who had not received any blood transfusions (0 homo).

T-cell responses only were depressed significantly compared to pre-operative values in patients who received 2 homologous transfusions. However, the decreases as seen in each group of differently transfused patients were not significantly different from each other (fig 4C & 5C).

Other studies showed that only a single transfusion can give a depression of responses to ConA(15) and PHA(16). Therefore, we speculate that both surgery and homologous blood transfusions depress the T-cell responses but that the effect of surgery is too strong to find a clear additional effect of blood transfusions.

Surgery had no effect on the NK-cell activity. Autologous randomized patients even had significantly enhanced NK-cell activity on day 4. This increase does not have to be related with surgery but may reflect the recovery of the drop in NK-cell activity after blood donation. Griffith(17) studied patients with benign and malignant gastro-intestinal disease. e showed enhanced NK-cell cytotoxicity after surgery, whereas, Tonnesen et al(18) found no changes in NK-cell cytotoxicity during premedication, anaesthesia or surgery in patients undergoing minor gynaecological surgery.

Comparison of the three differently transfused groups revealed that the NK-cell activity 10 days after surgery was clearly affected in the homologous transfused group, while the NK-cell activity of non-transfused patients and autologous transfused patients increased slightly. Drops in NK-cell activity after blood transfusions (without surgery) have also been reported by others(19).

Clinical importance of the findings

A depression of the T-cell response after surgery may represent a decreased specific T-cell response against bacteria and tumor cells, and/or a diminished lymphokine production, resulting in less stimulated macrophages and NK-cells. This depression occurs just at a moment when risks from invading pathogens and tumor cells are maximal. Tartter et al(20) showed that there is a highly significant association between transfusions and infectious complications in colorectal cancer patients. There

is also some evidence from retrospective studies that the percentage of colorectal cancer patients with a 5-year survival is smaller in transfused patients than in non-transfused patients. Blumberg and Heal(21) recently published a meta-analysis of 14 retrospective studies in colorectal cancer patients: the 5-years survival was 52 % in the non-transfused- and 31 % in the transfused patients.

The role of T-cells in tumor growth in spontaneously arisen tumors is still under doubt, because these tumor cells seldom express antigens that are not expressed on any normal cell(22). Nevertheless specific autologous directed CTLs have been cultured from for example colon cancers(23).
NK-cells appear to play a role in the first line of defence against blood borne metastases(24). Tumor cells are released into the circulation during surgery(25) and especially during manipulation(26) of the primary tumor. A transient depression of the NK-cell activity in colorectal cancer patients may affect the effective clearance of these circulating tumor cells. Recent studies in rats with selective depletion of NK-cells showed for example that the outgrowth of iv injected tumor cells was enhanced in these rats, while outgrowth of subcutaneous tumor cells was normal(27).

The NK-cell activity was unknown in the colorectal cancer patients who had donated blood at the moment of surgery. It was depressed 2 days before surgery and was similar to the NK-cell activity of homologous patients on day 4. Comparison of the 5-year survival of autologous and homologous randomized patients will tell us in the future, whether the risks of blood donations outweighs the benefits of autologous blood transfusions.
Since NK-cells also play a role in the first line defence against viral infections(28), depression of the NK-cell activity during 1 week in donors may theoretically lead to an enhanced vulnerability for viral infections. To our knowledge this has never been reported.

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CHAPTER 6

THE INFLUENCE OF BLOOD LOSS ON TUMOR GROWTH; EFFECT AND MECHANISM IN AN EXPERIMENTAL MODEL

Summary

Retrospective studies have provided indirect evidence that allogeneic blood transfusion may adversely influence the prognosis of cancer patients. This effect may be prevented by using autologous blood transfusions. However, this involves preoperative donation of blood, the consequences of which are still unknown. The aim of the present study was to investigate the possible effects of blood loss on tumor growth and on NK-cell activity. An artificial lung metastasis model was used in the BN rat from which 20 percent of the blood volume was taken at different time intervals. The results showed that blood loss, one day prior to tumor challenge, had a profound stimulating effect on tumor growth. After blood loss, the number of lung metastases was doubled as compared to controls. This tumor-promoting effect could be prevented by an immediate plasma transfusion, but not by evoking a normal hemoglobin level after blood loss by pretreatment with recombinant Human Erythropoietin (r-HuEpo). The NK-cell activity of spleen cells was significantly depressed 24 hours after blood loss. At a 200:1 lymphocyte-to-target ratio, the NK-cell activity dropped from 41.2 percent in controls to 27.0 percent in experimental animals. Since NK-cells are assumed to play a role in the clearance of tumor cells from the circulation, the enhanced tumor growth observed after blood loss might be caused by this depression.

Introduction

Blood transfusions have numerous consequences not envisaged in the past. Besides transmission of infections and sensitization to foreign antigens(1), it has become clear in the last two decades that blood transfusions also can suppress the immune system. In transplant patients this leads to an improved graft survival(2). However, transfusion induced immunosuppression may also have negative effects, such as increased susceptibility to postoperative infections(3) or acceleration of tumor growth. From clinical data, involving over 25 retrospective studies, it has become evident that the prognosis of transfused cancer patients may be poorer compared to patients who do not receive peri-operative transfusions(4). Animal studies, carried out in our laboratory(5)(6), provided further evidence for a deleterious effect of blood transfusions.

In order to study this possible adverse effect of blood transfusions prospectively, we started a randomized clinical multicentre study in 1986, with patients suffering from colorectal cancer. The patients were randomized between predeposited autologous and allogeneic blood transfusions. Because the immunologic consequences of blood donation are largely unknown, we first studied the effect of blood donation in voluntary blood donors and in a small number of autologous trial patients. We found that, after blood donation, the NK-cell function was depressed in both groups of donors (chapter 5). Because NK-cells are thought to play a role in preventing blood born metastases(7), such a decreased NK-cell activity at the time of surgery might influence the clearance of tumor cells released from the tumor during surgery.

In the present study we therefore investigated the consequences of blood donation on tumor growth and on NK-cell activity, using the same rat model as employed earlier for studying the tumor-promoting effect of blood transfusions (5)(6).

Materials and methods

Animals

Male rats of the inbred BN strain were used. The animals were 16-20 weeks old and were bred under specific pathogen-free conditions.

Tumor

Tumor LS 175 is a spontaneous, non-immunogenic sarcoma in BN rats. The tumor is maintained as a stationary culture in RPMI medium, supplemented with 10 percent fetal calf serum (FCS). To obtain cells, free floating LS175 clumps were harvested from tissue culture flasks and, after washing, resuspended in Hank's balanced salt solution (HBSS). Single cells were prepared by rinsing the suspension through a pipette. Viability was assessed by trypan blue exclusion and usually was between 90 and 95 percent.

Lung colonies assay

Under ether anesthesia 1 x 10^6 tumor cells, suspended in a volume of 1 ml, were injected intravenously. The number of colonies developing in the lungs was counted after 28 days. The rats were sacrificed and the lungs excised, rinsed in tap water and subsequently fixed in Bouin's solution. Tumor nodules on the lung surface, visible to the naked eye, were counted. The number of experimental metastases varied from test to test, if in a certain experiment many metastases were present, only the left lungs were counted. If more than 60 colonies were present on one lung, the number was scored as 60.

Blood loss

The blood volume of the rat is about 8 percent of its body weight(8). Under ether anesthesia 20 percent of the calculated blood volume was taken via incision of the tail. This resulted in a drop in the mean arterial tension from 100 to 60 mmHg. The blood pressure was again 75 after 10 minutes and 100 after 30 minutes. Control rats had only ether narcosis.

Recombinant human Erythropoietine (r-HuEpo) treatment

Rats were treated with 40 U r-HuEpo sc (Cilag, Brussels). We will show in chapter 7 that treatment during 5 days with 40 U r-HuEpo sc, followed by 20 percent blood loss, results in hemoglobin levels not significantly different from pretreatment values.

Experimental design

The effect of blood loss on tumor growth was studied by bleeding the rats one day before (day -1), or on day 3 or 7 after the injection of 1 x 10^6 tumor cells (day 0). In the next experiments the effect of blood withdrawal, 24 hours before tumor

inoculation was assessed in detail. Firstly, it was studied whether the tumor-promoting effect of blood taking could be abolished by: 1) an immediate transfusion of fresh syngeneic heparinized plasma, and 2) by treating rats in advance with r-HuEpo, in such a way that the hemoglobin level after blood withdrawal would remain normal. Control experiments were done to exclude an effect of heparin and r-HuEpo. Rats were not bled but received 40 U of heparin (corresponding with the number of units given with the plasma transfusion), 24 hours prior to tumor cell injection; or they were treated with r-HuEpo from three days before to seven days after injection.

NK-cell assay

The NK-cell activity of spleen cells was assessed in control rats and in rats who were bled 24 hours earlier. The NK-cell assay has been described in detail elsewhere(9)(10). Briefly: target cells (YAC-cells) were labelled with ^{51}Cr. Spleen lymphocytes were isolated and incubated in different concentrations with a standard number of YAC-cells. After a 4 hour incubation, supernatants were collected and measured for radioactivity. The specific release of ^{51}Cr was calculated per lymphocyte-to-target ratio.

Statistics

The number of lung colonies were not normally distributed. Therefore, data are given as median number and range. The Mann-Whitney test was used for comparison of two groups and the Kruskall-Wallis test for comparison of more groups. The number of colonies varied from test to test, and should therefore not be compared mutually. Differences in the NK-cell activity were also analyzed with the Mann Whitney test.

Results

Effect of blood loss and blood transfusions on tumor growth

In the first experiment rats were either bled one day prior, or on day three or seven after the inoculation of tumor cells. The only group showing a significantly higher number of metastases compared to controls was the group bled on day -1 (median: 9.5 lung colonies versus 23, respectively). In all following experiments blood therefore was taken on day -1.

Effect of blood loss, plasma transfusion and r-HuEpo treatment on tumor growth

To investigate whether shock might be the reason for the tumor-promoting effect of blood withdrawal, rats were transfused immediately after blood donation with syngeneic plasma. It was found that plasma transfusions could completely annihilate the tumor-promoting effect of blood loss; the median number of lung colonies was 46 in animals which were bled only, and only 11 in the group which, in addition, had received plasma (Table 1).

Table 1. The effect of blood loss, blood loss combined with r-HuEpo therapy, and blood loss combined with a plasma transfusion, on tumor growth.

Exp	Treatment	N	Number of lung colonies Median	Range	Sign*
I	Controls	12	13	0-60	1/4**
II	Bled	11	46	0-60	2/4
III	Bled & r-HuEpo	11	57	2-60	3/4, 3/1
IV	Bled & plasma	10	11	1-60	4/2, 4/3

* Groups are significantly different according to the Krusskal-Wallis test ($p < 0.01$).
** Significant different (MW test)

Table 2. The effect of r-HuEpo and heparin on tumor growth.

Exp	Treatment	N	Number of lung colonies Median	Range	Sign
I	Controls	11	12	3-23	ns
	r-HuEpo treatment (40 U, day -3 - +7)	12	19	1-60	
II	Controls	12	28	5-60	ns
	Heparin (40 U, day -1)	10	36	12-60	

Rats were also pretreated with r-HuEpo during five days in order to investigate whether the decrease in hematocrit after blood loss might be a crucial factor. Pretreatment with r-HuEpo resulted in a normal hematocrit despite blood loss; however, the number of lung colonies remained high (Table 1). In addition, control experiments showed that heparin, given 24 hours before tumor inoculation, or r-HuEpo treatment alone had no effect on tumor growth (Table 2).

Effect of blood loss on NK-cell activity

The NK-cell activity of the spleen lymphocytes from rats bled 24 hours earlier was significantly depressed at all lymphocyte-target cell ratios studied (Table 3). At the 200:1 ratios the activity was only 50 percent from that of controls.

Table 3. NK-cell activity of spleen cells at different lymphocyte-target ratios.

	Lymphocyte-target ratio			
	1:25	1:50	1:100	1:200
Control group	8.8 %	25.3 %	39.4 %	41.2 %
Bled group	0.2 %	9.3 %	21.6 %	27.0 %
Significance	0.016	0.009	0.016	0.047

Discussion

The major finding of the current study is that blood taken 24 hours before i.v. injection of tumor cells resulted in an increased number of lung colonies. The same rat tumor model has been used earlier by Singh et al(11). They showed that, after withdrawal of 30 percent of the blood volume at day -7, no effect was seen on tumor take, while bleeding the rats on day 7 resulted in a profound stimulating effect on tumor growth. We repeated the latter experiment but observed no significant effect. However, the animals in our experiments were bled less vigorously and were given more tumor cells; this might explain the difference in results(12).

Other studies on the effect of blood loss have mainly focused on increased suspectibility to infections and impairment of immunologic functions. Stephan et al(13) showed that the survival of mice subjected to sepsis three days after

hemorrhage was 0, while it was 42 percent in controls. In addition, they showed that despite adequate resuscitation 1 or 2 hours after bleeding, T-cell response to ConA was depressed for several days. Others have reported a diminished response to PHA(14) and disturbed macrophage functions after bleeding(15)(16).

LS-175 is a non-immunogenic tumor which makes it unlikely that modulation of T-cell function would affect tumor growth. On the other hand, as NK-cells recognize tumor cells in a rather non-specific way, changes in NK-cell activity by blood donation may be more important. Although the role of NK-cells on tumor growth is still not completely elucidated, there is accumulating evidence that they play a role in the control of metastatic spread of blood-born tumor cells. Recently, a specific monoclonal antibody against rat NK-cells has been developed(17). Selective depletion of NK-cells with this antibody resulted in decreased survival after intravenous injection of mammary adenocarcinoma cells, whereas it did not influence the growth of tumor cells injected subcutaneously(18). We observed a severe depression of the NK-cell activity, 24 hours after blood withdrawal at all studied lymphocyte-target cell ratios studied. Earlier (chapter 5) it was found that simple blood donation (± 10 percent of the blood volume) decreased the NK-cell activity both in healthy blood donors and in cancer patients. It is conceivable that a decreased NK-cell activity in cancer patients at the time of operation might influence the effective clearance of tumor cells released during manipulation of the tumor. In order to prevent the tumor-promoting effect of hemorrhage, we studied whether the induction of a normal Hb level after blood loss, or an immediate plasma transfusion might be effective procedures. Although r-HuEpo is a potent growth factor for red cell precursors, it did not affect tumor growth.

Pretreatment with r-HuEpo resulted in a normal hematocrit after blood loss, but this did not prevent acceleration of tumor growth by blood loss. The effect of blood taking could, however, be abolished completely with a plasma transfusion. Since plasma is replaced rapidly(19), the most likely explanation for the effect of plasma transfusion is not supplementation of plasma factors, but the immediate restoration of the arterial blood pressure.

The present study suggests that blood donation especially for patients at risk, may have to be followed by transfusion with plasma substitutes. However, the real risks and possible benefits of autologous blood donation in cancer patients will only be known in the near future, when the results of our ongoing blood transfusion trial will become available(20).

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PART III: ERYTHROPOIESIS

CHAPTER 7

RECOMBINANT HUMAN ERYTHROPOIETIN IN AUTOLOGOUS BLOOD DONATION PROGRAMS. A DOSE-EFFECT STUDY IN RATS

Summary

In pre-operative autologous blood donation programs a rapid increase of the erythropoiesis may be obtained by treatment with recombinant human Erythropoietin (r-HuEpo). The use of r-HuEpo treatment will be determined predominantly by its effectiveness and costs. Therefore, we studied the most optimal way to speed up erythropoiesis by using r-HuEpo.

Five days of r-HuEpo treatment resulted in increase of the hemoglobin level and hematocrit of almost 20 percent. There was a maximal effective dose of r-HuEpo (200 U/kg), and the best effects were obtained with daily administration of this dose. Two types of autologous blood donation programs were studied: 1) a daily blood donation program, and 2) a program in which blood was taken twice a week. In the first experiments ten percent of the blood volume was taken each time and r-HuEpo was administered daily. The yield of red blood cells in this aggressive donation program was only slightly higher in the rats treated with r-HuEpo. Apparently, the frequent blood donations had stimulated endogenous erythropoietin production to a level equally effective as when r-HuEpo was administered.

However, r-HuEpo treatment was effective in raising the number of red cells collected when it was given: 1) As pretreatment in a daily donation program; 75 percent more red-cells were harvested. 2) When it was given during a non-aggressive blood donation program; the hemoglobin level after four blood donations in a two weeks period was 192 g/L in a daily treated group and 120 g/L in the control group.

Introduction

Allogeneic blood transfusions are associated with risks of immunisation, transmission of viral infections and immune-suppression. The increased awareness of these risks necessitated a reassessment of transfusion practice and has rekindled the interest in autologous blood usage. The Council on Scientific Affairs(1) stated that when the guidelines established by the American Association of Blood Banks (AABB) are followed, autologous blood is the safest type of blood for transfusion. Autologous transfusion includes predeposit autologous blood donation, intra- and postoperative autotransfusion of shed blood, and hemodilution. The benefits of pre-operative blood donation have been well documented, but concern remains about limitations of the endogenous erythropoietic response to serial phlebotomy. Patients are often not able to donate the requested number of blood units(2).

Some authors(3) claim that this is because endogenous erythropoietin production will only be significantly elevated if the Hct drops below 30, and blood donations are only permitted with a Hct of 35 or higher. Therefore r-HuEpo, may have a therapeutic role in increasing the number of predeposited blood units.

Winearls(4)and Eschbach(5) were the first to show the potentialities of r-HuEpo in stimulating erythropoiesis in anaemic patients suffering from end-stage renal disease. Recent animal(6)- and human studies(7)(8) have focused on the possibilities of r-HuEpo to accelerate erythropoiesis during pre-operative blood donation programs. They showed that with r-HuEpo therapy both the number of collected units and the mean red cell content of the units increased. In two studies(6)(8) very high doses of r-HuEpo (600-1000 U/kg) were given in order to obtain an optimal erythropoiesis, the other study(7) however indicated that a much smaller dose (± 100 U/kg) could reduce the anemia induced by pre-operative donation as well.

The applicability of r-HuEpo treatment in surgery will be dictated by the costs and the complications of r-HuEpo treatment compared to blood transfusion treatment. Therefore, it is of economic importance to find the lowest active dose; the optimal dose schedule; and best route of r-HuEpo administration. The aim of the present study was to investigate in a rat model 1) the most effective dose of r-HuEpo and 2) the efficacy of r-HuEpo in aggressive and physiological blood donation programs.

Materials and methods

Animals and r-HuEpo treatment

Male rats of both the inbred Wag and Brown Norway (BN) strain were used; Wag rats have higher Hb levels than BN rats (mean ± SD, resp: 165 ± 4, 155 ± 5 g/L). The animals were bred under specific pathogen-free conditions, were 12-16 weeks old and weighed ± 250 grams.

R-HuEpo was obtained from Cilag (Brussel, Belgium). Different concentrations were made by diluting r-HuEpo (4000 U/mL) in 0.9 % saline to an injection volume of 0.5 mL. Doses are reported per rat, in general they should be multiplied by four to obtain the dose per kg. Injections were given intravenously (iv) or subcutaneously (sc). Iv injection and blood letting was done under ether anesthesia; rats were bled by tail incision before receiving treatment.

In order to investigate the most effective scheme, dose and way of r-HuEpo administration, five different protocols were studied (Table 1). In each of the experiments, 5 control rats received 0.5 ml of 0.9 % saline and 5 rats were used for each dose under study.

Table 1. Different protocols used for investigation of the dose-, dosage- and the way of administration of r-HuEpo.

Exp	Rat strain	Protocol
I	Wag	200 units r-HuEpo iv, daily for 5 successive days.
II	Wag	20 or 40 units r-HuEpo iv, daily for 5 successive days.
III	Wag	40 units r-HuEpo sc or iv, daily for 5 successive days.
IV	BN	20, 40 or 100 units r-HuEpo sc, daily for 5 successive days.
V	Wag/BN	A total dose of 200 units r-HuEpo sc; given from day 1 to 5 (40 U), on day 2 and 4 (100 U), or 200 U on day 3.

Autologous blood donation

In the blood donation programs 2 ml of blood (about 10 % of the blood volume) was taken by tail incision. Blood was taken either daily in an aggressive donation program or two times weekly in a less aggressive program. In the aggressive programs rats were treated with saline or 40 U r-HuEpo during the donation period or in addition

were pretreated for 3 days with 40 U r-HuEpo. In the final experiment blood was taken two times weekly, for two weeks. Rats were treated either with saline, or 120 U r-HuEpo after each donation, a third group received 40 U r-HuEpo for six days a week, so that the total dose of r-HuEpo given was also 480 U.

Measurements and statistical methods

Hemoglobin level (Hb), hematocrit (Hct), leukocyte differentiation, erythrocyte-, platelet- and leukocyte counts were counted electronically with a Sysmex microcellcounter and MCV-Hemato calculator and a TOA hemoglobin counter. The proportion of reticulocytes was counted after staining with new methylene-blue; and the absolute reticulocyte count was computed by multiplying the proportion of reticulocytes with the erythrocyte count. MCV, MCH and MCHC were calculated.

In experiments I-V, values were assessed at least two days after the last dose; in the other experiments every sample taken for blood donation was tested. The percentagewise decrease in Hb level per donation was calculated by the following formula: Percentagewise decrease due to donation = (Hb day x - Hb day x-1)/Hb day x. These percentagewise decreases in Hb level due to donation are depicted as numbers in the figures.

The effect of r-HuEpo treatment was assessed by comparing the hematologic data of the experimental rats with the control rats. When more than 2 treatment schedules were tested in one experiment the Student Newman Keuls tests (SNK-test) was used, otherwise an unpaired two-tailed Student' T test (T-test) was used. Statistical significance was accepted at a p value less than 0.05. Data will be presented as mean ± SD if not stated otherwise.

Results

Most effective dose, timing and way of administration of r-HuEpo

To determine the most effective daily dose, rats were treated with different doses of r-HuEpo (Table 1) for five successive days; hematologic parameters were assessed 2 days after stopping the treatment, and are given in Table 2. In Wag rats daily administration of 200 U r-HuEpo (exp I, Table 2) resulted in a 12 % increase in the mean number of erythrocytes. The mean Hb concentration on day 7 was 189 g/L and the Hct 58, compared to 161 g/L and 47 in untreated rats. However, similar Hb levels

were reached when rats were treated with 40 U during 5 days, while a dose of 20 U was only half as effective in stimulating the erythropoiesis (exp II, Table 2).

In the BN-rat similar dose-effect relations were seen with doses ranging from 20-100 U (exp IV, Table 3). The Hb level was not significantly different between rats treated with 40 or 100 U; the mean proportion of reticulocytes and the mean absolute reticulocyte count were significantly different between groups treated with 20 or 40 U and did not differ between the rats treated with 40 U and 100 U r-HuEpo. Thus in both rat strains 40 U seemed to be a maximal effective daily dose.

No difference was seen between sc or iv r-HuEpo administration (exp III, Table 2); therefore sc injections were given in the following experiments.

The importance of timing of r-HuEpo administration was studied by giving a total dose of 200 U r-HuEpo within a time period of 5 days in different dosages (exp V). In both rat strains, five daily doses of 40 U stimulated erythropoiesis significantly better than two injections of 100 U or 1 injection of 200 U. All groups had significantly different Hb levels on day 7; the proportion of reticulocytes and the absolute reticulocyte count were significantly higher in the group treated daily. Data of this experiment in the BN rat are given in Table 3.

Table 2. Effect of r-HuEpo treatment in the WAG-rat on Hb, Hct and erythrocyte counts in experiments I, II, III. Blood was samples at day 7, 2 days after the last treatment day.

	treatment	Hb-level (g/L)	Hct	erythrocytes (10^{12}/L)
I	Controls	161 ± 6.1[1]	47 ± 2[1]	8.1 ± .31[1]
	200 U iv	189 ± 7.3	58 ± 1	9.2 ± .45
II	Controls	172 ± 4.0[2]	45 ± 1[2]	8.1 ± .37[2]
	20 U iv	189 ± 4.8	51 ± 1	8.9 ± .10
	40 U iv	201 ± 5.0	55 ± 1	9.9 ± .44
III	40 U sc	200 ± 2.4	52 ± 5	9.9 ± .33
	40 U iv	200 ± 2.3	53 ± 2	9.8 ± .28

[1]: $p<0.001$ (T-test); [2]: $p<0.005$ (SNK-test), the three subgroups are all significantly different from each other ($p<0.05$).

Table 3. Effect of r-HuEpo treatment in the BN rat on Hb and reticulocytes in experiments IV and V. Blood was sampled on day 7.

	treatment	Hb-level (g/l)	reticulocytes (o/o)	reticulocyte (10^9/L)
IV	Controls	154 ± 2.8[1]	0.8 ± 0.14[1]	65 ± 12[1]
	20 U day 1 to 5	175 ± 4.7	2.8 ± 0.60	260 ± 37
	40 U day 1 to 5	180 ± 5.0	4.8 ± 0.67	444 ± 68
	100 U day 1 to 5	176 ± 2.5	5.6 ± 1.21	485 ± 72
V	Controls	156 ± 6.0[1]	0.8 ± 0.16[1]	70 ± 13[1]
	40 U day 1 to 5	186 ± 8.1	4.4 ± 0.52	453 ± 69
	100 U day 2 & 4	177 ± 9.4	1.3 ± 0.34	126 ± 29
	200 U day 3	166 ± 5.8	1.1 ± 0.27	105 ± 20

[1] $p<0.005$ (SNK-test)

The effect of r-HuEpo in daily blood donation

Firstly an aggressive blood donation program was studied, in which 10 % of the blood volume was taken. Rats were treated daily either with saline or with r-HuEpo. In this way, we were able to compare the potentialities of endogenous erythropoietin production with those of exogenous r-HuEpo therapy. The Hb levels during and after the blood donation period are depicted in Figure 1. From day 3 and further the mean Hb levels were significantly higher in the treated group, but the mean established difference of 16 g/L did not increase further during the next donations. The mean percentagewise Hb-decrease per donation (Figure 1) was significantly different between the two groups only after the second donation. The percentagewise Hb decrease is the sum of Hb decrease due to donation and Hb increase due to erythropoiesis; so erythropoiesis was equally stimulated in both groups after the third donation.

Pretreatment with r-HuEpo for three days (Figure 2) resulted in significantly higher Hb levels at the start of the donation program. As a result the mean percentagewise Hb decreases after the first two blood donations were significantly lower in the treated group compared to controls. The mean Hb level was 101 g/L in the untreated group and 130 g/L in the treated group after 4 donations.

Therefore,blood donation was only continued in the treatment group, leading to a mean Hb level of 118 g/L after 6 donations. Since both the number of donations and the mean red cell content of each donation were higher in rats treated with r-HuEpo, 75 % more red cells were harvested.

Recovery to normal Hb levels was the same in both groups, indicating that endogenous erythropoietin production was not suppressed by r-HuEpo administration. In both groups there were no signs of iron deficiency; the MCHC in both groups did not change during the donation course (data not shown).

Figure 1. Mean Hb levels (g/L) + or - SD and percentagewise Hb-decreases (numbers along the curve) during a daily blood donation program. From day 1 to 6, 2 ml blood was taken daily, and rats were treated with either saline (———) or 40 U of r-HuEpo (- - -). Hb levels were significantly different from day 3 to 10. Percentagewise Hb-decreases were significantly different only after the second blood donation.

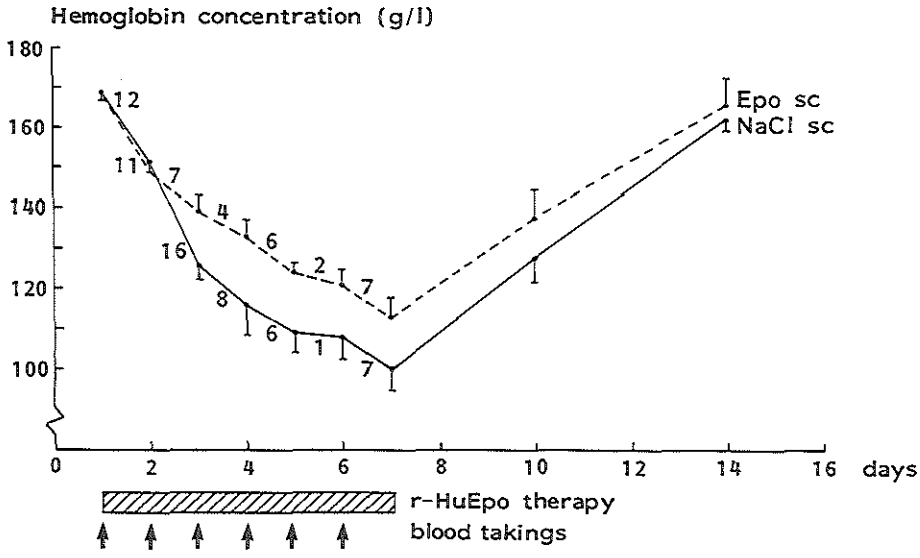
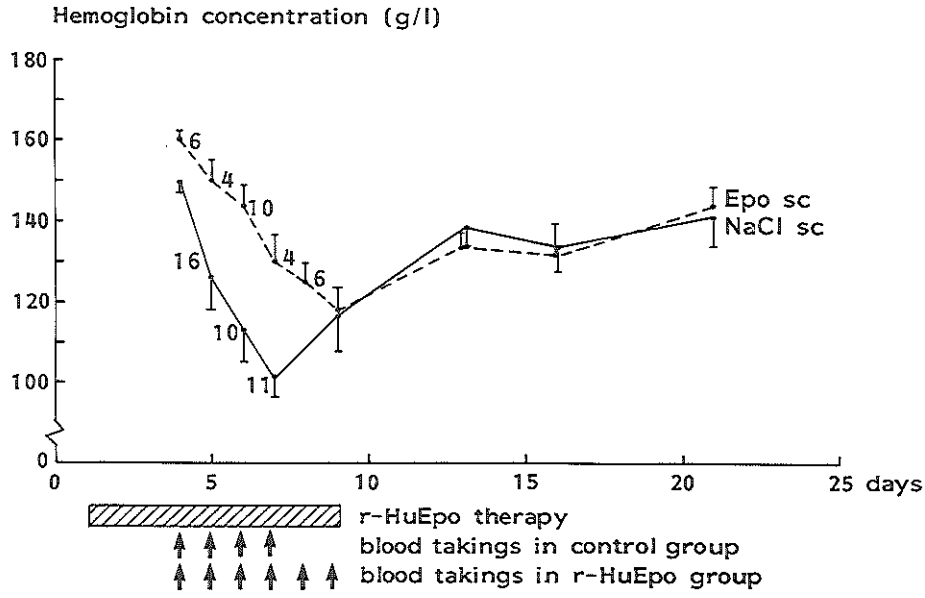


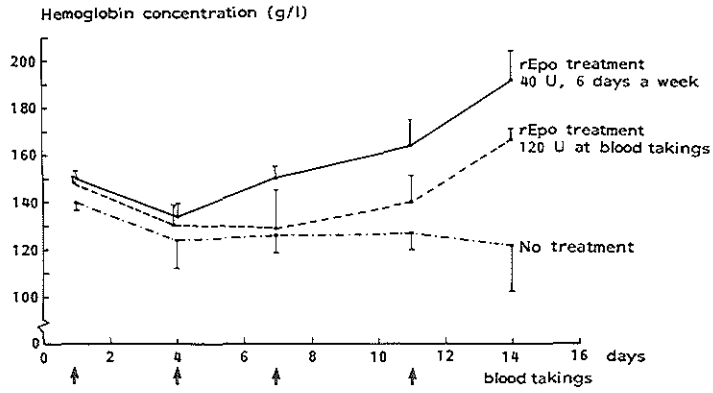
Figure 2. Mean Hb levels (g/l) + or - SD and percentagewise Hb-decreases (numbers along the curve) during a daily blood donation program with pretreatment from day 1 to 3. Rats were treated with either saline (————) or 40 U of r-HuEpo (- - -). In the control group blood was taken from day 4 to 7 (4 donations), in the r-HuEpo group from day 4 to 9 inclusive (6 donations). Hb levels were significantly different from day 4 to 7 inclusive. The % Hb-decreases were significantly different on day 4 and 5.



Effect of r-HuEpo in blood donation twice a week

A blood donation program, with 2 blood takings per week, resulted in a stabilized Hb level of 125 g/L in untreated rats. Apparently 2 ml blood was produced every three days. This was not a maximal stimulation of red cell production. Daily treatment with r-HuEpo resulted, namely, in significantly higher Hb levels from day 7 to 14 (SNK: $p<0.05$). The mean Hb level was 192 g/L at the end of the donation period. The same total dose of 480 U r-HuEpo was also given in another group of rats as doses of 120 U after each donation. The results were less impressive: the Hb levels were only significantly higher compared to controls at the end of the donation period.

Figure 3. Hb levels (g/L) during a blood donation program, in which blood was taken two times a week for two weeks. Rats received either saline (- · -) or 120 U r-HuEpo (- - -) after each donation, or 40 U r-HuEpo 6 days a week during the whole donation period (————). Hb levels were significantly different on days 7,11 and 14 (SNK-test).



Discussion

The purpose of peri-operative r-HuEpo therapy, or r-HuEpo treatment during autologous blood donations, is to accelerate erythropoiesis. To attain this goal most studies have used very high doses of r-HuEpo(6)(8). Although, we did not perform real dose response studies, our results suggest that in the rat the maximal effective dose of r-HuEpo is 160 U/kg. After 5 days of treatment with this dose the Hb concentration increased in both rat strains with 25 to 30 g/L. Clear dose-effect relations have been reported by others. Eder et al(9) reported a dose-effect response on the number of reticulocyte in rats after a single dose of r-HuEpo. However, their results also did indicate that with doses above 50 U/rat plateau levels were reached.

We showed that not the total dose of r-HuEpo, but rather the frequency of r-HuEpo treatment determined the effect on erythropoiesis. A total dose of 200 U/rat was far more effective when given in 5 days, than when given in 2 days or 1 day.

When blood was donated two times a week, 40 U of r-HuEpo each day was more effective than 120 U after each donation.

Sc and iv administration of r-HuEpo was found to be equally effective. Dialysis patients receiving r-HuEpo are always treated iv, but recent studies have indicated that there are advantages in sc administration(10)(11).

Daily r-HuEpo therapy during an aggressive blood donation program did lead to significantly higher Hb levels; though the maximal difference was merely 16 g/L. Therefore only a slight increase in harvested red cells in the treated rat group was achieved. It has been shown (12) that the number of erythropoietin producing cells in the kidney increases within some hours in an exponential manner as Hct decreases. Apparently, the anemia induced by the frequent blood donations had stimulated endogenous erythropoietin production to a level equally effective as when r-HuEpo was administered. Acceleration of the erythropoiesis in advance, with pretreatment for 3 days with r-HuEpo, was very successful: 75 % more red cells could be saved in the treated group. Such aggressive blood donation programs will, however, not be used in man. Therefore, the prospects of r-HuEpo therapy were also studied in a more physiological donation program. The rapid erythropoiesis as seen in the daily donation program, was not observed when blood was taken twice a week; the mean Hb level without treatment being given was only 125 g/L after 4 blood donations. At that moment, the Hb level in rats treated with daily doses of r-HuEpo, was 195 g/L, indicating that endogenous erythropoietin production was not sufficiently stimulated with this donation scheme. As a consequence r-HuEpo treatment was effective.

Autologous blood donors are often deferred for further blood donation because they become anaemic(2). Kirckler and Spivak(3) showed that in autologous blood donors erythropoietin levels are only slightly increased after blood donation. In patients suffering from simple anemia high erythropoietin levels are only found in patients with a Hb below 110 g/L (Hct:32)(13). Spivak and colleagues therefore concluded that as long as blood donations are only allowed with a Hct above 35, erythropoiesis will not be stimulated enough and consequently not enough blood can be donated.

Our results subscribe to this viewpoint, if more autologous blood units are needed, either more aggressive donation programs should be permitted (with the disadvantage that each unit contains a low number of red cells) or donors should be

treated with r-HuEpo. In subsequent studies, lower subcutaneously doses should be used and daily administrations and pretreatment should be considered; r-HuEpo therapy may both help to increase the number of blood donations and their mean red cell content.

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CHAPTER 8

THE EFFICACY OF RECOMBINANT ERYTHROPOIETIN TO STIMULATE ERYTHROPOIESIS AFTER BLOOD LOSS AND SURGERY

Summary

Peri-operative treatment with recombinant human Erythropoietine (r-HuEpo), may reduce the need for allogeneic blood transfusions by diminishing the time-lag between blood loss and erythropoiesis and by generating more adequate Epo levels.

We studied the efficacy of pre-, peri-, and post-operative r-HuEpo therapy in rats subjected to blood loss and surgery (ileum resection). It was found that erythropoiesis was less stimulated by r-HuEpo in operated rats than in controls; after 5 days of treatment with 200 U r-HuEpo/kg following surgery, the increase in hemoglobin (Hb) was 15.7 g/L in operated rats and 36.9 g/L in controls ($p<0.05$), indicating that surgery has an inhibitory effect on erythropoiesis.

If surgery was combined with blood loss (20% of the blood volume), a treatment course with 200 U r-HuEpo/kg/day during five days, starting either four or two days before surgery resulted in significantly higher post-operative Hb levels as compared to untreated controls. Such differences were not observed if treatment with rEpo was started on the day of surgery. Prolonged post-operative treatment with r-HuEpo for ten days only induced significantly elevated Hb and Hct levels from day 8 onwards. Our results indicate that r-HuEpo is a promising agent to obviate the need for blood transfusions at surgery provided the treatment is started before operation.

Introduction

Recombinant human erythropoietin (r-HuEpo) is widely used to treat anemia in patients with chronic renal disease(1),(2). Recent studies have focused on the possibilities of r-HuEpo treatment in other types of anemia such as malignancy-induced anemia(3), postpartum anemia(4), anemia in AIDS patients receiving zidovudine(5) and anemia in Rheumatoid Arthritis.

In general and oncological surgery, r-HuEpo therapy may be useful to reduce the need for allogeneic blood transfusions, which entail the risk of immunisation, transmission of viral infections and immunosuppression(6). Reduction of allogeneic blood can be achieved in two ways: firstly by increasing the feasibility of pre-operative autologous blood donation programs and secondly by accelerating peri-operative erythropoiesis. The first option has shown to be effective; both animal(7) and human studies(8)(9) have provided evidence that the number of blood units as well as the mean red cell content of the collected units does increase as a result of r-HuEpo treatment. The peri-operative use of r-HuEpo to accelerate erythropoiesis has been studied by Levine and colleagues(10)(11) in monkeys which were bled to a hemotocrit of 15%. They found that both pre- and post-bleeding treatment with r-HuEpo shortened the time of recovery to a normal Hct. The clinical relevance of this extreme acute blood loss model in which no real surgical stress was imposed is questionable, especially since it has been reported(12)(13) that patients may become unresponsive to r-HuEpo because of operation or infection. We therefore studied the efficacy of peri-operative r-HuEpo treatment on erythropoiesis in rats subjected to both abdominal surgery and a blood loss.

Materials and methods

Animals and r-HuEpo treatment

Male rats of the Brown Norway (BN) strain were used. The animals were bred under specific pathogen-free conditions, were 12-16 weeks old and weighed about 250 grams. Human recombinant erythropoietin (r-HuEpo) was provided by Cilag (Brussels, Belgium). Different concentrations were made by diluting r-HuEpo (4000 U/mL) in 0.9 % saline to an injection volume of 0.5 mL. If not stated otherwise rats received 200 U/kg r-HuEpo subcutaneously (sc) for 5 consecutive days, controls

received physiological saline. We showed earlier that this is the maximal effective dose for stimulating erythropoiesis in the rat (chapter 7). Five rats were used for each experimental group.

Abdominal surgery and blood taking

Both operated and control rats were anesthetized with an ethrane/oxygen/nitroxide inhalation narcosis. Surgery consisted of dissection of the ileum followed by end-to-end anastomoses using continuous suturing with 7/0 silk. Great care was taken to minimize blood loss during surgery. At the end of the surgical procedure 20% of the blood volume was taken by tail vein incision. The whole procedure lasted 25-30 minutes. Blood sampling for hematology was done under ether narcosis.

Table 1. Experimental design.

Exp.	pre-operative days								post-operative days (POD)				
	-7	-6	-5	-4	-3	-2	-1	0	1	2	3	4	5-9
I								BL					
	E^1	E^1	E^1	E^1	E^1			BL					
	E	E	E	E	E			BL					
II								C&E	E	E	E	E	
								O&E	E	E	E	E	
III								Bl&O					
						E	E	BL&O&E	E	E			
IVa								BL					
								BL&O					
								BL&O&E	E	E	E	E	
IVb								BL					
								BL&O					
								BL&O&E	E	E	E	E	E
V				E	E	E	E	BL&O&E					
						E	E	BL&O&E	E	E			
								BL&O&E	E	E	E	E	

E^1: r-HuEpo treatment (100 U kg); E: r-HuEpo treatment (200 U/kg); BL: Blood loss 20 % of the blood volume; O: Operation; C: Controls, not operated.

Experimental Design

The different protocols to study the effectiveness of pre- peri- and post-operative treatment with r-HuEpo are given in Table 1. In experiment I rats were treated with different doses of r-HuEpo to demonstrate the feasibility of this treatment in animals which were bled only. In experiment II the effect of r-HuEpo treatment was studied in operated rats from which no blood was taken. In experiment III rats undergoing surgery and blood loss were treated peri-operatively, from two days prior to operation until the second post-operative day (POD 2). In experiment IV the effect of post-operative r-HuEpo treatment was studied; one group received r-HuEpo post-operatively for 5 days (Exp IVa) a second group for 10 days in Exp IVb. Finally the three r-HuEpo schemes were compared in bled and operated rats (Exp V).

Measurements and statistical methods

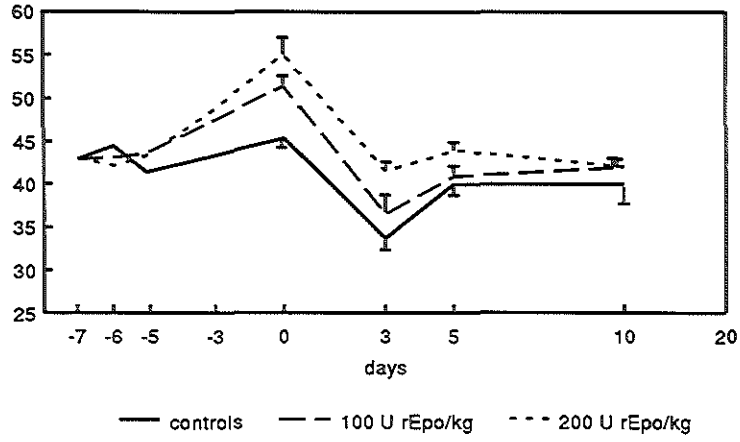
Hemoglobin level (Hb), hemotocrit (Hct), erythrocyte-, platelet- and leukocyte counts were counted electronically with a Sysmex microcellcounter and MCV-Hemato calculator and a TOA hemoglobin counter. The proportion of reticulocytes were counted after staining with new methylene-blue, and the absolute reticulocyte count was computed by multiplying the proportion of reticulocytes with the erythrocyte count. The effect of r-HuEpo treatment was assessed by comparing the hemotological data of the experimental rats with those of the control rats. If more than 2 treatment schedules were tested in one experiment the Student Newman Keuls test (SNK-test) was used, otherwise an unpaired two-tailed Student's test (T-test) was used. Increases within the group were studied by the paired two tailed Student's test. Statistical significance was accepted at a p value less than 0.05. Data will be given as mean ± SD.

Results

The efficacy of r-HuEpo treatment on erythropoiesis after blood loss

We showed in chapter 7 that treatment with r-HuEpo for 5 consecutive days stimulated erythropoiesis in rats dramatically. There appeared to be a dose-effect relation, with a maximal effective dose of 200 U/kg/day. In Figure 1 results are depicted of an experiment in which rats before blood taking were treated 5 times with either 100 U/kg or 200 U/kg of r-HuEpo are depicted (Exp I).

Figure 1. The Hct (mean + or - SD) during r-HuEpo therapy and blood loss (Exp I); rats were treated from day -7 to day -2 with saline (———), 100 U r-HuEpo/kg (—— ——) or 200 U r-HuEpo/kg (- - -).



R-HuEpo administration resulted in a significant rise in Hct and Hb concentration, and an increased number of reticulocytes and erythrocytes. Both before (day 0) and after blood loss (day 3) the Hct's were significantly different between the three groups ($p=0.0001$). The Hct in the group treated with the maximal effective dose (200 U/kg) was, despite blood taking, not significantly different from baseline level, indicating that by five days of treatment at least 20 % of the blood volume was produced.

Table 2. The effect of r-HuEpo treatment on hematologic values in operated (day 0) and non-operated rats (Exp II).

	Hb level		Hematocrit		Leukocyt count	
	Day 1	Day 7	Day 1	Day 7	Day 1	Day 7
Controls	150 ± 6.8	187 ± 8.3	39.7 ± 1.9	49.8 ± 3.9	12.1 ± 1.1	11.4 ± 0.6
Operated	153 ± 3.2	169 ± 10.5[1]	38.8 ± 1.5	45.8 ± 2.4[2]	8.0 ± 2.1[3]	11.4 ± 1.4

Signicficant differences: 1: $p=0.023$; 2: $p=0.066$; 3: $p=0.008$

The efficacy of r-HuEpo after surgery

Operated rats were treated for 5 days with r-HuEpo and compared with non-operated, r-HuEpo treated controls (Exp II). The results are given in Table 2. On day 1, Hb, Hct and the number of reticulocytes were similar in both groups; only the number of leukocytes was lower in the surgical group. On day 7 the Hb levels in the two groups were significantly increased compared to the levels on day 1. However, the mean increase (± SD) was 15.7 ± 8.3 g/l in operated rats and 36.9 ± 8.5 g/l in non-operated rats ($p=0.005$), indicating that surgery has an inhibitory effect on erythropoiesis.

The efficacy of peri-operative treatment with r-HuEpo in rats undergoing surgery and blood loss

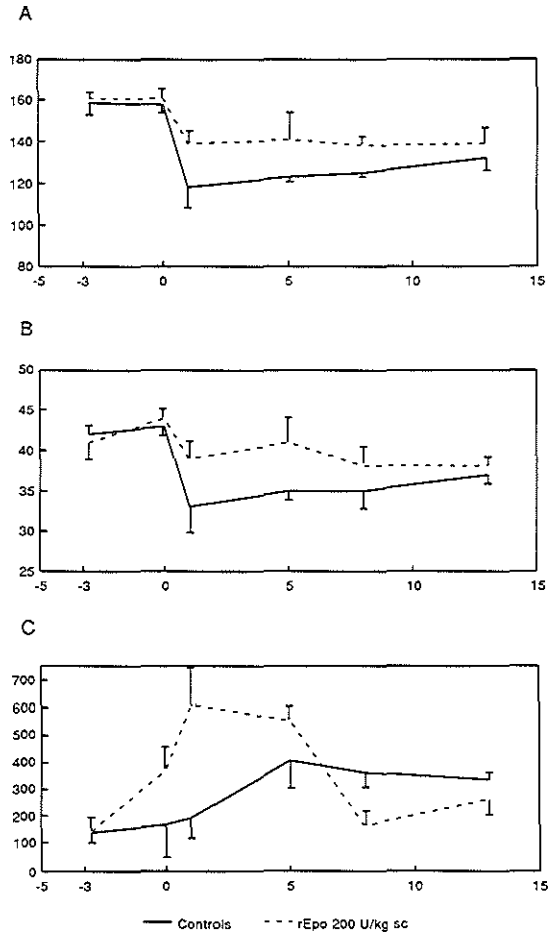
Peri-operative administration of r-HuEpo (Exp. III), from 2 days before until 2 days after operation, resulted in significantly higher Hb and Hct levels as compared to controls from post-operative day 1 to 8 (Figure 2). Already after 2 doses the absolute reticulocyte counts were significantly elevated compared to pretreatment values. Compared to controls the absolute reticulocyte counts were significantly different on days 1 and 8. The mean reticulocyte count increased in the control group from 191 $x10^9$/L on day 1 to 406 x 10^9/L on day 5, and was 552 x 10^9/L in the r-HuEpo group. On day 8 reticulocyte counts were higher in controls than in treated rats, and amounted to 360 x 10^9/L and 159 x 10^9/L respectively ($p=0.005$).

Table 3. The post-operative Hct (mean ± SD) in rats that were either bled (I) operated and bled (II), or operated, bled and treated with r-HuEpo (III) during the first 10 operative days (Exp IVb).

		Post-operative days		
		day 4	day 8	day 13
I	Bled	29.0 ± 1.4	39.0 ± 1.2	37.8 ± 1.8
II	Bled and operated	29.5 ± 2.5	36.7 ± 0.5	36.0 ± 1.6
III	Bled, operated & r-HuEpo therapy	33.0 ± 1.4	41.8 ± 2.3[1]	45.8 ± 2.5[2]

[1] SNK test $p=0.02$ (III significantly different from II)
[2] SNK test $p=0.001$ (III significantly different from I and II)

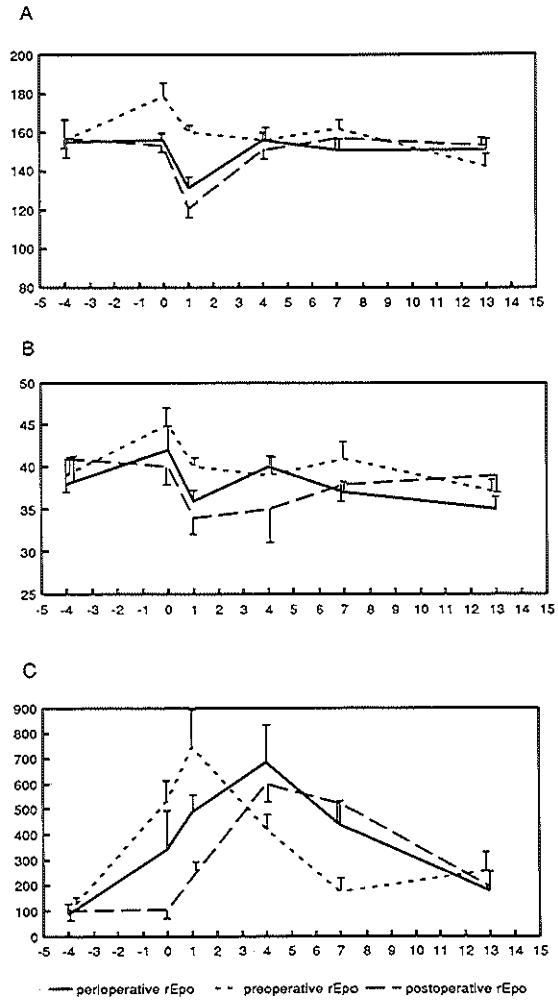
Figure 2. The Hb concentration (A: g/L), Hct (B) and absolute reticulocyte count (C: x 10^9/L) during peri-operative r-HuEpo therapy (Exp III); Rats were operated and blood was taken day 0, and they were treated with either saline (——), or with 200 U r-HuEpo/kg (- - -) from day -2 to day 2.



The efficacy of post-operative r-HuEpo treatment after surgery and blood loss

The post-operative Hct was not significantly higher in rats treated with r-HuEpo during the first five post-operative days, from day 1 until day 13 (Exp. IVa). The Hct (mean ± SD) was 34.2 ± 1.5 in no-treated operated rats, and 37.5 ± 1.7 in operated

Figure 3. Comparison of the effect of pre- peri- of post-operatively r-HuEpo therapy on Hb concentration (A g/L), Hct (B), and absolute reticulocyte count (C: x 10^9/L) (Exp V); Rats were operated at day 0 and were treated during 5 days with 200 U r-HuEpo/kg during 5 days, either pre-operatively (- - -); peri-operatively (———); or post-operatively (—— ——).



rats treated with r-HuEpo, on the fourth postoperative day. However, if the rats were treated during the first 10 post-operative days, the Hct became significantly different between the two groups on day 8 and day 13 (Exp. IVb: Table 3).

No significant differences were seen between the hematologic values of rats that were bled only, and rats that were operated in both experiment IVa and IVb. However, operated rats not treated with r-HuEpo experienced the lowest Hb and Hct levels in both experiments.

A comparison between pre- peri- or post-operative r-HuEpo treatment

The results of a comparative study between pre- peri- and post-operative r-HuEpo treatment (Exp. V) are given in Figure 3. Each experimental group received 5 doses of r-HuEpo; either given from day -4 to day 0; from day -2 to POD 2 or from day 0 to POD 4, respectively. The Hb-and Hct levels were significantly different between the three groups on the day of surgery and on day 1.

The Hct and Hb levels of the rats treated pre-operatively were already significantly elevated on the day of surgery; the Hb concentration had increased from 156 g/L to 179 g/L. On POD 1 the Hb en Hct levels were the highest in the group treated pre-operatively ($p=0.0001$ and $p=0.001$, respectively). On day 4 all groups had similar Hb and Hct levels. Before and immediately after operation the absolute reticulocyte counts were the highest in the groups treated pre- and peri-operatively; on POD 4 and 7 they were the highest in the groups treated peri- and post-operatively.

Discussion

Hypoxemia is the principal stimulus for Epo production by interstitial kidney cells(14). The number of cells producing Epo, and consequently Epo plasma concentration depends on the severity of induced anemia(15). In man elevated Epo levels following acute hypoxia can be found within one day(16). High Epo levels result in a rapid release of reticulocytes and stimulation of committed stem cells to proliferate and differentiate. It takes about five days before new red cells are produced and released from the bone marrow into the circulation(17). Pre-operative treatment with r-HuEpo might shorten the time-gap between blood loss and release of newly formed red cells.

Spivak and Hogans(18) showed that there exists an inverse exponential

relationship between Hb concentration and plasma Epo levels, provided the Hb levels are below 110 g/L. In clinical practice blood transfusions are often given at such Hb levels(19). Consequently post-operative Hb levels will not be low enough to generate a brisk erythropoietic response, therefore post-operative r-HuEpo therapy may be indicated to enhance erythropoiesis.

In the present experiments the production of new red cells in rats was found to be very fast, faster than in humans; already after 2 days of r-HuEpo treatment elevated absolute reticulocyte counts and Hct's were observed. Five days of maximal r-HuEpo therapy in rats resulted in the production of 20 % of the blood volume.

Both pre- and peri-operative r-HuEpo treatment were found to be very effective in enhancing erythropoiesis; this resulted in significantly higher post-operative Hb- and Hct levels as compared to controls. The effect of post-operative treatment with r-HuEpo was less impressive, no significant effect was seen if r-HuEpo was given for 5 days, while treatment during 10 days only resulted in significantly higher Hct's on post-operative day 8 and 10. Pre-operative r-HuEpo therapy did shorten the lag period between blood loss and the appearance of new reticulocytes in the circulation. On the other hand, post-operative r-HuEpo therapy did not result in more adequate erythropoiesis. Levine et al(10) studied the effect of post-operative treatment with 1000 U/kg r-HuEpo in baboons that were bled to a Hct of 15%. They found significantly higher Epo levels in the treated group as compared to controls from day 1 to day 18. However, the absolute reticulocyte counts were only significantly different from day 11 onwards, which indicates that a high Epo level is not necessarily associated with increased erythropoiesis. This is supported by our earlier findings that there is a maximal effective r-HuEpo dose (chapter 7).

In the present study prolonged post-operative administration of r-HuEpo resulted in significantly increased Hct's in the second post-operative week. Since at that time the Hct of the controls was within acceptable margins, we think that this is un undesirable overtreatment.

Following surgery erythropoiesis was found to be inhibited, despite adequate Epo levels. It has been reported earlier that hemodialysis patients may become unresponsive on prolonged r-HuEpo therapy following operation(12) or because of infection(13). A decreased erythropoietic response on r-HuEpo therapy might be due to surgical stress alone, or due to simultaneous infection. In our experiments surgical

stress is likely to have been the predominant inhibitory factor since we never observed any sign of infection in our experimental animals. Possible explanations for a suppressed erythropoiesis following surgery are: relative inability to use iron(20); production of lymphokines suppressing erythropoiesis(21); and release of endotoxin (22).

The aim of r-HuEpo therapy in surgery is reduction of allogeneic blood transfusions. It is clear from our experiments that the best results are to be expected by pre-operative treatment. A disadvantage of such a therapy is an elevated Hct during operation. This results in hyperviscocity of the blood which may hamper peripheral circulation. In addition, a high Hct leads to a relatively greater loss of red cells during operation. Both of these disadvantages can be overcome by blood donation directly prior to surgery, followed by hemodilution. At the end of the operation the autologous unit can than be returned to the patient without risk.

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CHAPTER 9

PERIOPERATIVE TREATMENT WITH RECOMBINANT HUMAN ERYTHROPOIETIN, RESULTS FROM AN OPEN RANDOMIZED STUDY

Summary

We studied the possibility of Recombinant Human Erythropoietin (r-HuEpo) to increase the post-operative erythropoiesis in a group of gastro-intestinal cancer patients. Thirty patients were randomized for either r-HuEpo treatment or no treatment. The treated patients received 200 U/kg r-HuEpo daily, from 5 days before surgery up to the third post-operative day.

This resulted in a clear-cut stimulation of the erythropoiesis in these patients; both the Hct and the number of reticulocytes was elevated significantly, 1 day before surgery. The highest reticulocyte counts were reached in the treated group 2 days after surgery, and in the control group only 8 days after surgery. The effect of the five pre-treatment days was beyond any doubt, however, the effect of the post-operative r-HuEpo administrations was less obvious. This might be due to the very low post-operative serum iron concentrations. The efficacy in reducing the need for blood transfusion was not established. Patient numbers were too small and the amount of blood loss ranged too great in the patient population studied. There was a tendency for fewer transfusions to be needed in the treated patients. Future studies are indicated.

Introduction

Recombinant human erythropoietin (r-HuEpo) has now been used in clinical trials for over 5 years. It has been found very effective in correcting the anemia of patients on hemodialysis(1) or patients with chronic renal failure not yet on dialysis(2). In addition, studies have been published indicating that the need for blood transfusion in patients with Rheumatoid Arthritis(3), cancer(4), multiple myeloma(5) and AIDS(6) is reduced. Treatment with r-HuEpo also increases the ability of individuals to donate blood for autotransfusion(7)(8)(9).

R-HuEpo treatment in the pre-operative and post-operative period, may be another potential treatment modality. While pre-operative treatment with r-HuEpo may shorten the lag period between blood loss and the appearance of new reticulocytes in the circulation, post-operative treatment may generate more adequate erythropoietin levels, leading to a stronger erythropoiesis. Experimental studies in baboons(10)(11), and dogs(12) have indicated that the post-operative anemic period is indeed shorter in animals treated peri-operatively.

We will present the data of an open randomized study in which 30 cancer patients were randomized for peri-operative treatment with either r-HuEpo or no treatment. This was studied in cancer patients, because there is considerable evidence that blood transfusions may affect the survival of cancer patients(13); r-HuEpo might provide a tool to omit the use of allogeneic blood thereby improving their clinical outcome.

The aim of this study was:

1) To assess the safety of r-HuEpo if administered in the peri-operative period.
2) To determine the efficacy of r-HuEpo on the stimulation of erythropoiesis if administered in the peri-operative period.
3) To determine whether r-HuEpo can diminish or prevent post-operative anemia.

Materials and methods

Patients

Patients with a gastro-intestinal malignancy who were scheduled for a surgical resection, with an expected blood loss of more than 500 ml, were asked to participate

in this study. Patients were eligible if they were, except for their malignancy, in good general condition. This was checked by medical history, physical examination, laboratory tests, X-ray of the chest and electrocardiography. In addition all patients were screened for operability of the tumor. Patients should have a hemoglobin (Hb) level >7 mmol/L (115 g/L) and no evidence of folate-, vitamine B12-, or iron deficiency. Patients were excluded if they had; 1) a history of any primary hematologic disease; 2) a history of seizures; 3) uncontrolled hypertension (i.e., diastolic blood pressure >110 mmHg); 4) cerebral metastases and; 5) any acute illness within 7 days of study entry. Patients were also excluded if they had received any therapy which could influence bone marrow activity (e.g., corticosteroid or other immunosuppressive therapy, chemo- or radiation therapy). Women of childbearing age were eligible only if they used adequate contraceptives and had a negative pregnancy test immediately prior to study entry.

Study design

The clinical trial was approved by the medical ethical committee of our hospital. Patients were, after informed consent was obtained, randomized for either r-HuEpo treatment or no treatment (no placebo was given).

R-HuEpo was supplied by CILAG N.V. (Brussels, Belgium) as a sterile buffered solution with a specific activity of 4000 U/ml. The intravenous dose was 200 U/kg and was given daily, from 5 days before surgery (day -5) up to the third post-operative day (day 3). Treatment was given between 8:00 and 11:00 AM, except for the day of surgery (day 0), when it was administered some hours after surgery. Patients received treatment either during admission, or at the outpatients' clinic. After each administration, temperature, respiration, pulse, and systolic and diastolic blood pressure were measured.

Both patients groups were treated daily, whenever possible from day -5 with oral administration of 200 mg elemental iron daily. Post-operatively enteral iron administration was started as soon as possible. Blood loss was carefully measured during surgery. Allogeneic blood transfusion were allowed to be given if blood loss exceeded 1000 ml or hemoglobin levels were below 6.0 mMol/L (± 100 g/L).

Measurements

The hematologic status (hemoglobin [Hb], Hematocrit [Hct], erythrocytes, the number of reticulocytes per 1000 erythrocytes, leukocyte differential, leukocyte counts and

number of platelets) was measured before study entry, on day -5, day -3, day -1, and then daily until day 5; thereafter it was measured every two days (day 6, day 8, day 10). Reticulocytes are given as number per 1000 erythrocytes, and the absolute reticulocyte count was computed by multiplying the proportion of reticulocytes with the erythrocyte count.

In addition serum chemistry, including serum Iron, Total Iron Binding Capacity (TIBC) and Ferritin was monitored on day -5, day -1, day 1, and day 5. All of these parameters were assessed with automatic analyzers, except for leukocyte differential and the reticulocyte counts, which were determined manually. Serum was collected and frozen for monitoring erythropoietin levels at baseline and 5 to 7 days after surgery. In some patients treated with r-HuEpo samples were taken during the pre-operative treatment period.

The immune-reactive erythropoietin was measured with a commercially available radio-immuno assay (INCSTAR EPO-Trac ^{125}I RIA), that uses radio-labelled r-HuEpo as the antigen and a single lot of polyvalent rabbit anti-r-HuEpo. With this assay, the range of normal values in adults without anemia is 4 to 26 U/l.

Statistics

Patients were excluded from analysis if blood loss was less than 500 ml, or if the treatment protocol was violated. Non-parametric tests were used to test for significant differences both between the groups and within the groups. Increases and decreases within the group were tested with the Wilcoxon matched-paired test. If comparisons between the two groups were made, the Mann Whitney-U test was used. All analysis were done with the SPSS-PC statistical program.

Table 1. Patient's characteristics (n=30).

	r-HuEpo	Control
male (n)	13	13
female (n)	2	2
mean age (yrs)	60.9	62.4
tumor site (n)		
-cardia	10	9
-pancreas	5	5
-sigmoid	0	1

Results

Patients and safety of r-HuEpo treatment

Patient's characteristics are given in Table 1, the two aselectively randomized groups did not differ, with regard to age, sex and tumor site. Four patients out of the 30 were excluded from further analysis. Two patients (controls) appeared to have liver metastases at the start of the surgery; no surgical resection was therefore done and hardly any blood was lost. Two other patients with a locally irresectable tumor were not excluded from this analysis, because their blood loss exceeded 500 ml. Two patients randomized for r-HuEpo were excluded because their surgery was postponed resulting in violation of the protocol. The first patient developed fever one day after starting of treatment, r-HuEpo was continued, but stopped after the 5th dose when surgery was postponed. Besides a pancreatic carcinoma this patient had colonic ulcers, which probably caused the fever. The second patient received his pretreatment according to protocol, but developed atrial fibrillations during the induction of anesthesia; surgery was postponed for one week, after which r-HuEpo treatment was continued again.

Table 2. Baseline hematologic values (mean ± SD) of 26 eligible patients.

	r-HuEpo	Control
Hb (g/L)	14.7 ± 1.0	14.6 ± 1.3
Hct	42.1 ± 3.7	42.4 ± 2.9
Erythrocytes (10^{12}/L)	4.61 ± 0.27	4.81 ± 0.46
Reticulocytes (o/oo)	10.8 ± 4.9	7.3 ± 2.8
Erythropoietin (U/L)	15.7 ± 8	18.9 ± 6

No adverse effects were seen in the 15 treated patients; especially no changes occurred in systolic or diastolic blood pressure, in clinical chemistry and in platelet numbers during the 5 pretreatment days. After surgery all these values were dictated by the effect of surgery. Patients did not report any side-effects.

Blood loss and blood transfusions

The amount of blood loss experienced during surgery varied from 550 ml to 3800 ml. With one patient a secondary hemorrhage occurred, the patient lost a total of 6500 ml blood. Blood transfusions were given in 7 patients treated with r-HuEpo and in 9 control patients. The total number of transfusions was 19 and 25 respectively. While only 2 r-HuEpo treated patients were transfused in the post-surgical period, 5 patients in the control group were transfused in this period (Table 3). R-HuEpo transfused patients received more often only 1 unit of blood, as can be seen in Figure 2. There was no evidence that patients were differently transfused; blood transfusion were in all cases only given if Hb < 100 g/L and blood loss at surgery exceeded 2000 ml. However, one should realize that the randomization was not blinded.

Table 3. Number of patients transfused during surgery, in the post-surgical period or in both situations.

	r-HuEpo	Control
Transfused during surgery	5	4
Transfused post-surgically	0	3
Transfused in both situations	2	2

Erythropoiesis

The hematologic baseline values of the 26 trial patients are given in Table 2, there were no significant differences between the two groups at baseline.

The Hct in the patients treated with r-HuEpo was significantly elevated in comparison with baseline values on day -1 ($p=0.0033$, Wx). However the Hct was at that moment, nor at any other moment before or after surgery, significantly different between the two groups (Figure 2). The same applied to the Hb concentration and number of erythrocytes. The Hct values reached their minimal value 3 days after surgery, from this day until 5 days later it increased significantly in both groups.

Figure 1. Number of patients receiving 0, 1, 2, 3, or >3 blood transfusions, during and after surgery.

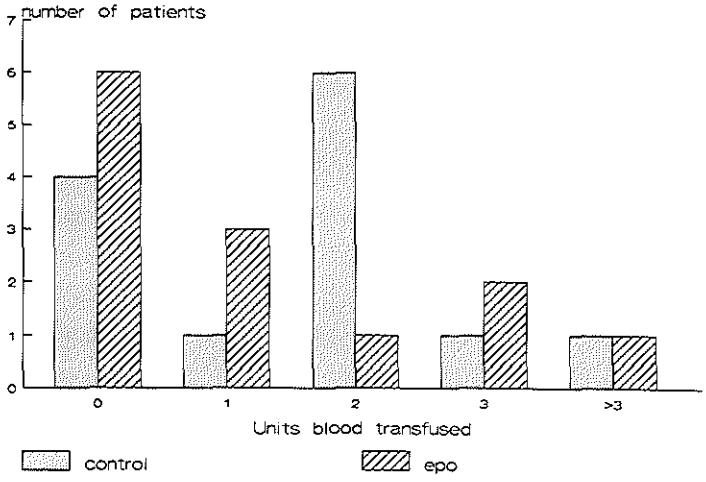


Figure 2. Pre- and post-surgical hematocrit values (mean ± SEM).

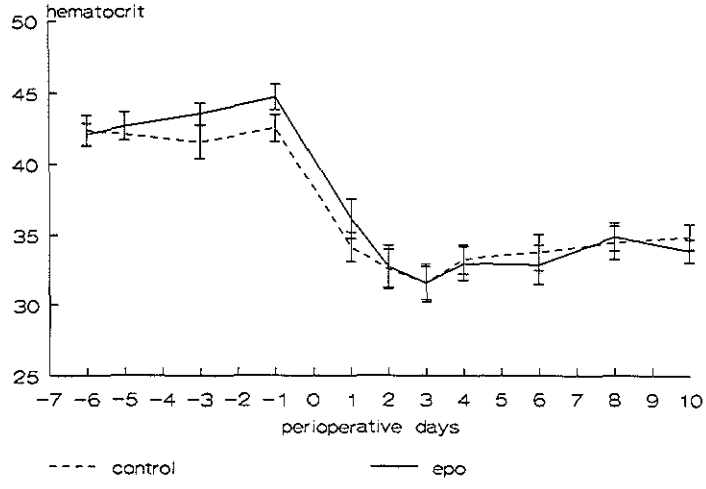
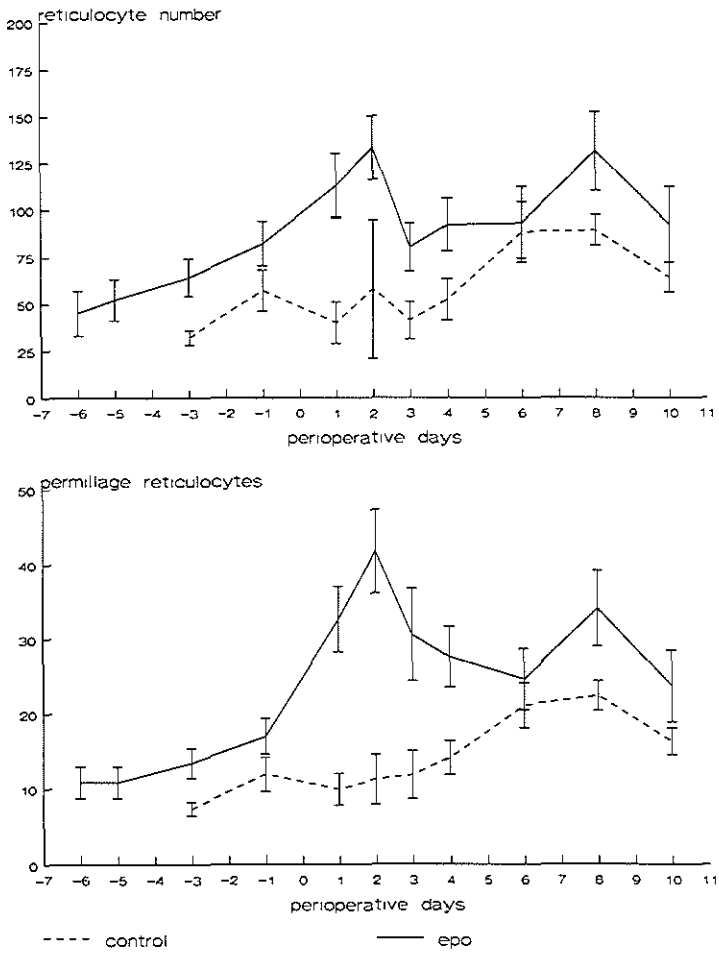


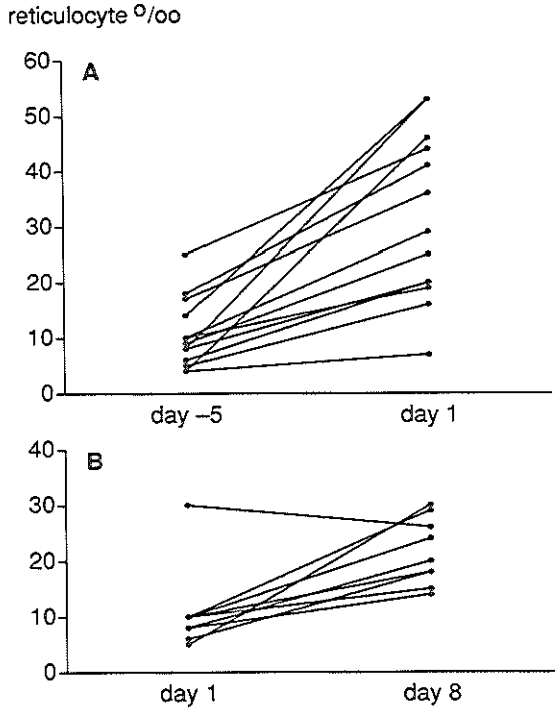
Figure 3. Pre- and post-surgical absolute reticulocyte counts and per 1000 erythrocytes.



Reticulocytes, expressed as number per 1000 erythrocytes, and the absolute reticulocyte counts are given for both patient groups in Figure 3. Significantly higher reticulocytes counts were found in treated patients on day -3 and from day 1 up to day 4.

Compared to baseline values, treatment with r-HuEpo resulted in an increased number of reticulocyte already after two days of treatment ($p=0.01$). Numbers increased further and the maximal value was reached on day 2. The number was lower on day 3 compared to day 2 ($p=0.046$), and did not increase significantly thereafter. Similar changes were seen in the absolute reticulocyte counts. Figure 4 shows that all treated patients experienced an increase in the number of reticulocytes if the values of day -5 was compared with day 1 ($p=0.005$).

Figure 4. Per patient increase in the reticulocyte counts per 1000 erythrocytes, (A) during r-HuEpo treatment (B) and post-operatively in control patients.



The same Figure shows that the number of reticulocytes per 1000 red cells in untreated patients was increased compared to the first post-surgical day on day 6 ($p=0.018$). The maximum value of reticulocytes in this group was reached on day 8 and it dropped again on day 10 ($p=0.012$)(Figure 3). The reticulocyte count as seen on day 1 and 2 in the r-HuEpo treated patients was significantly higher than the maximum value in the untreated group.

Erythropoietin levels

The mean baseline erythropoietin level of all patients was 18 U/l; only two patients had erythropoietin levels just above the normal range. Erythropoietin levels in treated patients ranged between 240 and 850 U/l. Erythropoietin levels were in both groups significantly elevated after surgery; the mean value 1 week after surgery was 50 U/l in the control group and 42 U/l in the treated group. Figure 5 shows that the pre-surgical and post-surgical erythropoietin levels of the control patients were significantly related to the Hb concentration (correlation coefficient was 0.76; $p=0.007$).

Table 4. Iron and Ferritin serum concentration and the Total Iron Binding Capacity (TIBC) before and after surgery in controls and in patients treated with r-HuEpo (mean ± SD).

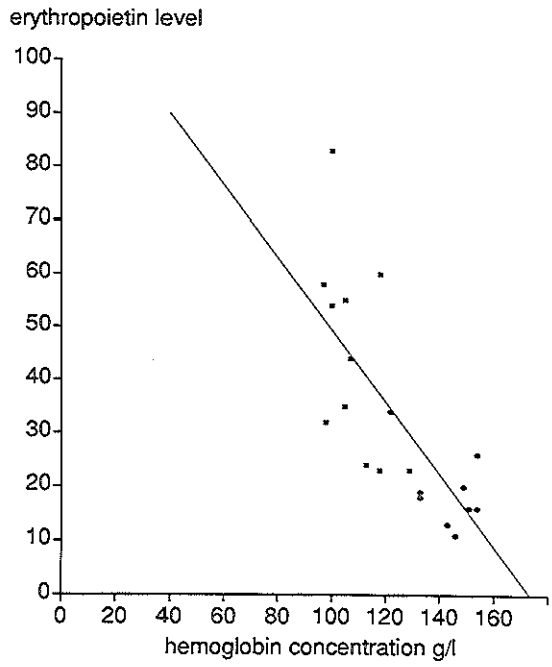
		r-HuEpo	Controls
Iron	day -5	14.7 ± 6.9	10.7 ± 3.0
	day -1	11.6 ± 6.4	16.5 ± 6.3
	day 1	3.2 ± 1.0	4.3 ± 1.2
	day 5	7.9 ± 3.0	6.3 ± 2.1
TIBC	day -5	75.5 ± 10.6	74.8 ± 12.9
	day -1	76.4 ± 12.2	77.7 ± 11.3
	day 1	45.1 ± 8.7	50.0 ± 10.2
	day 5	47.8 ± 12.5	55.0 ± 12.1
Ferritin	day -5	232 ± 279	137 ± 132
	day -1	136 ± 143	227 ± 224
	day 1	187 ± 177	1494 ± 4222
	day 5	306 ± 231	601 ± 1012

Iron

The iron, and ferritin- concentrations and the TIBC are given for both groups in Table 4. Iron concentration was not significantly different between the two randomized patient groups. Some patients had low normal iron concentrations at the start of the treatment. However, no difference in the reticulocyte response was seen between patients with high and low levels of serum iron (data not shown).

Both the serum iron-concentration and the TIBC dropped dramatically after surgery and still was depressed 5 days after surgery. Iron concentrations were, at that time, below the normal range in almost every patient. The serum ferritin levels ranged widely in this patient group, which was probably related to the underlying disease and the levels were not indicative for the iron store.

Figure 5. Correlation between Hb concentration (presurgical values . and post-surgical values x) and erythropoietin level in the control group.



Discussion

Walker(14) estimated that 20 % of all allogeneic blood transfusions result in some type of adverse effect. Blood transfusions administered to cancer patients during surgery may in addition affect their prognosis(13). These risks may be overcome by transfusing the patients with predeposited autologous blood. However, the number of collectable units of blood, in a period of two or four weeks before surgery, is often disappointing(15). Moreover, not all cancer patients will fulfil the criteria set for autologous blood donation.

The recent success of r-HuEpo to abolish the anemia in patients with different type of chronic diseases(16), and the increased harvest of autologous blood in r-HuEpo treated blood donors(7),(8),(9) have put forward the question whether peri-operative treatment with r-HuEpo would reduce the need for post-operative blood transfusions.

The idea to peri-operative treatment with r-HuEpo is that this may shorten the physiologic lag period between onset of the anemia and red cell production. In addition, higher erythropoietin levels may result in a stronger stimulation of the erythropoiesis.

The normal total marrow transit time of erythroid cells is 7 days, however, at high erythropoietin concentration the reticulocyte pool will be shifted to the peripheral blood within 1 day and newly formed red cells will appear in the circulation within 5 days(17). Therefore therapy was started 5 days before surgery and was continued up to the third post-operative day. Goodnough et al(7) treated autologous blood donors with a dose of 600 U/kg two times a week. We showed in our rat studies that daily administration is more effective than giving the same cumulative dose in fewer doses (chapter 7). Therefore patients were treated daily with 200 U r-HuEpo/kg. At that time clinical experience with subcutaneous administration was limited, therefore r-HuEpo was administered intravenously.

The number of reticulocytes was elevated compared to baseline values already after 2 treatment days. Highest values were reached in the first two post-operative days. In the control patients reticulocyte levels were significantly elevated from day 6 of; maximum values were reached 2 days later. The maximal counts were significantly lower in the non-treated patients compared to the patients treated with r-HuEpo. So indeed, both the lag period before erythropoiesis started after surgery was shortened

and higher reticulocyte counts were reached.

Compared to the pre-surgical values, erythropoietin levels were significantly higher 1 week after surgery in both groups. With a mean postoperative Hb level of 115 g/L, the erythropoietin serum level was 50 U/L in the control patients. Studies in patients with iron-deficiency(18) have shown similar erythropoietin levels with such an anemia; indicating that the erythropoietin level was appropriate for the anemia experienced. There was no evidence for a depression of the erythropoietin production in our patient group; others have reported low erythropoietin levels in cancer patients(19). The serum erythropoietin level was linearly and negatively correlated with Hb concentration (Figure 5). Suwati and colleagues measured serum levels in the first operative week in patients of different age groups, and reported a similar correlation between Hb concentration and serum erythropoietin level in the patients younger than 65 years(20).

The serum iron concentration and TIBC dropped dramatically after surgery. The values at day 1 were so low that maximal erythropoiesis can not be expected. Hillman and Henderson(21) showed that during severe anemia marrow production response correlates with serum iron levels. At serum iron values below 7 ug/l (± 14 uMol/l) erythropoiesis is only about 2.5 to 3.5 times normal; and increases 4 to 5 times with levels between 7.0 and 15.0 ug/l (14 to 30 uMol/L).

The number of reticulocyte per 1000 red cells, and the absolute reticulocyte count dropped significantly between the second and third post-operative day, despite continuation of r-HuEpo treatment. This drop may very well be explained by this low serum iron concentration. Which means that the lower maximal reticulocyte value that was reached in the control patients does not necessarily have to be due to lower erythropoietin levels but may be due to post-operative iron deficiency.

Both Hb levels and Hcts were at no time-point significantly different between the two groups, however, the overall number of transfusions seemed to be smaller in the r-HuEpo treated patient group, especially with regard to transfusions given in the post-operative period. Although there were no indications that transfusions were given differently in the two groups, one can not rule out such bias, since the current study was not placebo controlled.

In conclusion

Pretreatment with 200 U r-HuEpo/kg i.v. was successful in stimulating the

erythropoiesis. The time period before new erythrocytes entered the circulation was shifted from the sixth post-operative day towards the first post-operative day; and higher reticulocyte counts were reached during the first two post-operative days. An additional beneficial effect of post-operative treatment on the erythropoiesis was not found. Which may be due to the very low post-surgical serum iron levels, but can also mean that is hampered by itself after surgery, this has been reported incidentally(22). More recently cases of renal failure patients have been presented who became unresponsive to rHuEpo after surgery(23) or during an infectious period(24). Patients from our study all underwent a considerable surgical stress, whereas the published animal studies(10),(11),(12) used hemodilution as a model for surgery. These studies all reported that the "post-operative" anemia was much shorter in the r-HuEpo treated groups. Our studies in rats who underwent an ileum resection (chapter 8) indicated that 1) erythropoiesis is disturbed after surgery and 2) 5 days of treatment is more effective when given before surgery than after surgery.

Future studies should focus on pre-operative treatment. A combination of r-HuEpo pretreatment followed by blood donation just before surgery may be on of the most promising strategies. Because the Hct and the reticulocyte counts will be elevated after some pretreatment days, blood donation would prevent the loss of just newly produced erythrocytes.

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CHAPTER 10

FEASIBILITY OF A PREDEPOSIT AUTOLOGOUS BLOOD DONATION PROGRAM IN COLORECTAL CANCER PATIENTS: RESULTS FROM A RANDOMIZED CLINICAL STUDY

Summary

The hematologic and transfusion data of a multicenter randomized trial investigating the effect of blood transfusions on the 5 year survival, were used to study the feasibility of an autologous blood donation program in colorectal cancer patients. Three hundred ten patients were randomized for autologous blood transfusions (donating 2 units of blood) or allogeneic blood transfusions and transfusion rules were standardized.

The Hb level in the patients who donated blood decreased by 20 g/L pre-operatively and 5 g/L post-operatively, and in controls 4 g/L and 17 g/L (significantly different between the two groups, both pre-and post-operatively: $p<0.01$). Because blood loss and number of transfusions were similar in both groups, this indicated that post-operative erythropoiesis was stronger in patients who had donated blood.

Twenty-three percent of the autologous patients and 61% of the allogeneic patients were exposed to allogeneic blood. The effectiveness of the procedure differed per tumor localization. In patients with a right-sided colon carcinoma 22% of the control patients needed allogeneic blood, compared to 10% of the autologous patients. In patients with other colon carcinomas this was 52% and 16% respectively and in patients with a rectal carcinoma 85 and 41%.

We conclude that predeposition of 2 units of blood for colorectal cancer surgery is feasible and useful to prevent allogeneic blood usage in a significant number of patients with a left colon carcinoma or rectal carcinoma.

Introduction

Autologous blood obtained pre-operatively is the preferred form of blood replacement for elective surgery and its usage is endorsed by numerous organizations such as the American Medical Association(1), the AABB(2), and the American Red Cross(3). The benefits of autologous transfusions have been well documented, but a number of aspects are still open to discussion. First the procedure is still very much under-utilized, especially in general surgery(4)(5). Secondly, over-usage has been reported, due to inappropriate autologous donation before low-risk elective surgery(6). Thirdly, there is concern about limitations of the erythropoietic response to serial phlebotomy(7)(8).

We present the hematologic- and blood transfusion data of a prospective randomized multicenter study in which colorectal cancer patients scheduled for curative surgery were randomized for allogeneic or autologous blood transfusions.

The main objective of the study is to elucidate the effects of allogeneic blood transfusions on the 5-year survival of colorectal cancer patients(9). The randomization of the patients, however, also enables us to study the efficacy of pre-operative blood donation: Do blood donors need more blood due to their lower pre-operative hematocrit? Is pre-operative donation a useful method in colorectal cancer surgery?

Materials and methods

In a multicenter study 310 patients with colorectal cancer who were not suspected of having metastatic disease and met the AABB criteria for autologous blood donation (Table 1) were per center randomized for autologous- or allogeneic transfusions (autologous- and allogeneic patients). The study was reviewed and approved by the ethical committees of all participating hospitals. Autologous patients donated two units of blood, between 14 and 7 days before operation. The interval between the two donations was at least 3 days. Before the first donation autologous patients started with iron supplementation therapy (ferrous fumarate 3 times daily 200 mg). In all hospitals except one, patients donated their blood in the regional blood banks, where autologous and allogeneic blood was processed to erythrocyte concentrates (without buffy coat) and fresh frozen plasma (FFP).

Table 1. Patient selection criteria.

Inclusion criteria:
- Patients with a histologically proven colorectal carcinoma or a radiologically suspected lesion for malignancy.
Exclusion criteria:
- Previous malignancy
- History of severe cardiovascular or respiratory disease
- Convulsions after infancy
- Colitis ulcerosa or polyposis coli
- Preoperative irradiation
- Emergency operation
- Blood transfusion during pre-operative period
- Hb pre-operatively < 120 g/L and/or Hct <35%
- Evidence of metastatic disease

The intra-operative blood loss was estimated by weighing swabs and measuring the amount of blood in the suction apparatus. When blood loss was less than 500 ml, blood transfusions were only given when Hb concentration was repeatedly below 105 g/L. Blood loss greater than 500 ml had to be followed by administration of two units of blood (autologous or allogeneic) and depending on Hb concentration (Hb< 105 g/L) more allogeneic units were allowed to be given. Autologous units of FFP were available for the patients. All unused autologous units were discarded.

Surgical procedures were standard. The patients were subdivided into 5 groups according to their tumor localization: Group I; coecum, colon ascendens, flexura hepatica and colon transversum, group II; flexura lienalis and colon descendens, group III; sigmoid, group IV; recto-sigmoid, and group V rectum. Histopathological staging was performed according to the Dukes classification. Hb concentration and Hct were assessed at study entry (day -14), before operation (day -1), one day after operation (day +1) and at discharge (day ± 10).

Statistical methods

The drop in Hb levels per patient were calculated for both the pre-operative and post-operative period. The pre-operative Hb drop was calculated by subtracting the Hb level of day -1 from Hb level of day -14. The post-operative Hb drop was calculated by subtracting the Hb level of day 10 after operation from the Hb level of day -1 before operation. The Hb level of day 10 was used because sometimes transfusions were given in the first post-operative days. The same applied to Hct.

Correlation of various factors with baseline Hb and Hct and Hb decreases were analysed using multiple regression. Other statistical methods used are indicated in the text. In all cases analyses were performed by computer with the use of the SPSS package. Five percent (two-sided) was considered the limit of statistical significance.

Results

Patients

In the period between September 1986 to January 1988, ten participating centers entered 310 patients, 282 of whom were histopathological classified as having colorectal carcinoma. Of the 145 patients randomized for autologous blood, 14 ultimately did not donate blood for various reasons (refused by blood bank, patient refused delay of operation, emergency operation). The age of the remaining 131 autologous patients and 137 allogeneic patients (122 women and 146 men) ranged from 39 to 89 years and was not significantly different between the two groups, being (mean ± SD) 67 ± 10 years in allogeneic patients and 65 ± 10 years in autologous patients. The pre-operative Hct was exactly the same in both groups (43 ± 4) and the same applied for the pre-operative Hb level and tumor localization (Table 2 & 4).

Preoperative variation in Hb levels

Baseline Hb levels in both treatment groups combined were significantly higher in men than in women, respectively 146 ± 1.1 g/L and 138 ± 1.0 g/L (mean ± SEM)(Mann-Whitney test: $p<0.001$). Using multiple regression no relation with tumor localization, tumor status and age could be determined.

The mean Hb decreases in the pre-operative period are given in Table 2. The Hb levels of allogeneic patients dropped slightly (mean=4 g/L). The mean decrease in Hb level for autologous patients was significantly greater (20 g/L). The Hb

decrease in autologous patients correlated with baseline Hb concentration and sex. With increasing Hb level, the drop was greater ($p<0.001$). Taking baseline values into account, the Hb levels of females dropped 3 g/L more on the average as compared to males ($p<0.05$). No relation could be found between the drop in Hb levels and age, localization of the tumor and tumor status. Similar findings applied to Hct.

Effect of operation on Hb levels

With a median blood loss of 950 ml, Hb and Hct dropped significantly following operation and remained low during the post-operative period in both groups (Table 2). Compared to autologous patients, allogeneic patients more frequently needed either no blood at all or three units (Table 3). This in spite of equal transfusion rules according to protocol. The mean number of blood transfusions however did not differ significantly between the two groups (autologous patients: 2.1 ± 0.2 (mean ± SEM), allogeneic patients: 1.9 ± 0.2). The decrease in post-operative Hb concentration was significantly different between both treatment groups (Mann-Whitney test:$p<0.001$). The mean drop in autologous patients was 5 g/L, in allogeneic patients 17 g/L (Table 2). As a result of this, autologous patients had significantly lower Hb levels on days -1 and +1 than allogeneic patients (MW-test $p<0.001$ respectively 0.004), but 10 days post-operatively both Hb and Hct levels were similar in the two groups (Table 2).

Table 2. Hb levels baseline (day -14), pre-operative (day -1), post-operative (day +1) and at discharge (day ± 10). Mean calculated Hb decrease per patient, pre- and post-operatively (mean ± SEM).

	Hb levels (g/L)			
	day -14	day -1	day +1	day ±10
Allogeneic pts	142 ± 1.2 (132)	138 ± 1.6 (90)	125 ± 1.5 (132)	124 ± 1.3 (113)
Autologous pts	142 ± 1.1 (128)	122 ± 1.4[1](108)	119 ± 1.4[1](128)	122 ± 1.2[1](114)

	Hb decrease (g/L) per patient	
	pre-operatively	post-operatively
Allogeneic patients	3.7 ± 1.1 (87)	16.5 ± 1.9 (87)
Autologous patients	20.1 ± 1.3[1] (104)	4.5 ± 1.8[1] (106)

[1] Significantly different from allogeneic patients; () Numbers of patients.

Table 3. Number of patients receiving 0, 1, 2, 3 or >3 transfusions.

Number of Transfusions	Allogeneic patients	Autologous patients
0	53 (39%)	35 (27%)[1]
1	4 (3%)	8 (6%)
2	43 (31%)	58 (44%)[1]
3	11 (8%)	1 (1%)[1]
>3	26 (19%)	29 (22%)

[1] Significantly different (Fisher exact-test: $p<0.05$) from allogeneic patients

Effectiveness of pre-operative blood donation in colorectal surgery

The 137 allogeneic patients received a total of 264 blood units. The 131 autologous patients received a total of 283 blood units of which 183 were autologous and 100 allogeneic. This is a reduction of 61% in the number of allogeneic units given. Twenty-seven percent of the autologous patients did not need their own blood but 23% needed additional allogeneic blood.

Blood loss ranged from 0.1 to 11.5 L. Blood loss, and consequently the median number of blood transfusions, was significantly different for the 5 demarcated tumor localizations. Table 4 presents the median blood loss and the percentage of patients who had received either autologous or allogeneic blood transfusions, classified for the different tumor localizations.

In tumor localization group I (coecum, colon ascendens, flexura hepatica, colon transversum) 50% of the autologous patients received their own blood with 10% needing additional allogeneic blood. Because only 22% of the allogeneic patients received blood, pre-operative donation had led to a mere increase of 12% of patients not exposed to allogeneic blood (Table 4).

For patients with a carcinoma of the flexura lienalis or colon descendens (group II) 87% of the autologous patients received autologous blood with 25% needing additional allogeneic blood. Of the allogeneic patients 55% needed blood. Therefore 75% of the autologous patients and 45% of the allogeneic patients were not exposed to allogeneic blood.

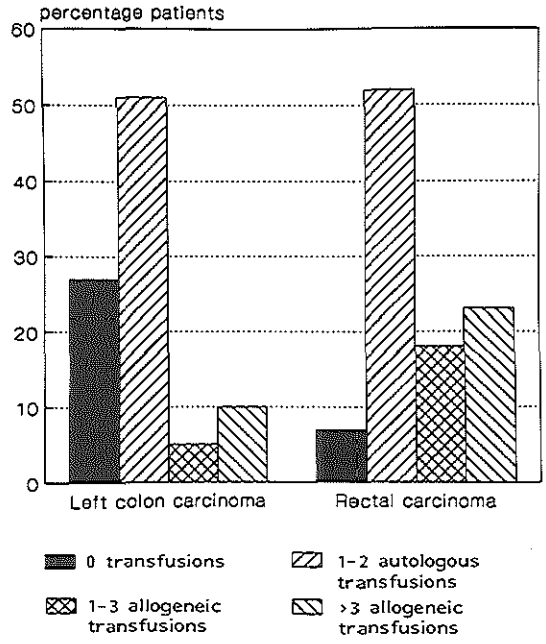
Table 4. Percentage of patients receiving autologous blood transfusions or allogeneic blood transfusions in relation to tumor localization (see text).

Randomi-zation		number of pts	blood loss (median ml)	Patients % transfused with Autologous blood	Homologous blood
I	autologous	16	375	50 %	10 %
	allogeneic	14	400	-	22 %
II	autologous	8	900	87 %	25 %
	allogeneic	11	500	-	55 %
III	autologous	31	400	52 %	10 %
	allogeneic	33	500	-	37 %
IV	autologous	32	710	78 %	19 %
	allogeneic	25	1000	-	68 %
V	autologous	44	1340	93 %	41 %
	allogeneic	54	1500	-	85 %
all	autologous	131	850	63 %	23 %
	allogeneic	137	775	-	61 %

For patients in group III (sigmoid carcinoma) 10% of the autologous patients and 37% of the allogeneic patients were exposed to allogeneic blood. For patients with a recto-sigmoid carcinoma (group IV) this was 19 and 68% respectively. For group V (rectal carcinoma) the figures were 41% of the autologous patients and 85% of the allogeneic patiens who received a allogeneic transfusion.

In Figure 1 the percentage of patients needing 0, 1-2, 2-5 or over 5 transfusions are shown for patients with a left-sided colon carcinoma (group II,III,IV) and for patients with a rectal carcinoma. Autologous patients with a left-sided colon carcinoma had donated sufficient blood: only 15% needed additional allogeneic blood (Figure 1). However, of the patients with a rectal carcinoma (group V) 41% needed additional allogeneic blood, 18% needed 1 to 3 allogeneic units of blood and 23% even needed more than 3 allogeneic units (Figure 1).

Figure 1. Percentage of autologous randomized patients with a left colon carcinoma or a rectal carcinoma receiving 0, 1-2 autologous blood transfusions, 1-3 allogeneic blood transfusions or >3 allogeneic transfusions.



Discussion

Routine implementation of predeposited autologous blood donations in elective surgery is still poor(4)(5). In an American National Multicenter Study(5) concerning 5000 patients, only 5% of the eligible patients had predeposited blood. Of the 162 eligible orthopedic patients 13% had predeposited blood , no one from the 93 eligible patients for general surgery had donated blood. This under-usage of predeposit programs especially in general surgery also becomes evident in the literature concerning predeposit autologous blood donation programs; almost without exception the studies are in orthopedic(10)(11) and cardiovascular surgery(12).

The indication for autologous blood is simple and straightforward: any patient facing elective surgery who may or will require blood should be considered for such transfusion. In colorectal cancer surgery blood is often needed: Blumberg and Heal(13), in a meta-analysis of 14 retrospective studies concerning the detrimental

effect of blood transfusion in cancer patients, reported that 45 to 84% of the patients had received blood transfusions. Due to the multicenter nature of our study the percentage of colorectal cancer patients who were eligible and cooperating is unknown. In our own center, which entered 14% of the patients, 35% of all colorectal cancer patients were eligible according to protocol. It is therefore possible that the results of the study may be useful to the care of a fraction of the patients undergoing this type of surgery.

As in other studies(14)(15), the mean drop in Hb level after donation of two units of blood was 20 g/L; the Hb decrease due to donation depended on sex and baseline Hb level. With higher baseline Hb levels the drop due to donation was greater. It may be that the Hb concentration has to drop below a certain threshold before erythropoiesis is stimulated. Spivak and Hogans(16) showed that Epo concentrations were only elevated in patients with Hb values below 110 g/L.

Taking baseline values into account, the Hb levels of females dropped 3 g/L more on the average as compared to males. Goodnough and Brittenham(8) demonstrated in a prospective study that more female than male blood donors are deferred because of anemia. This could be ascribed to lower circulating red cell volumes and lower storage iron pools.

The 18 patients in our study who appeared to have advanced disease during operation did not experience a more severe Hb drop due to donation. Swanson and colleagues(17) reported in 1983 a successful blood donation program in 25 patients with bladder cancer undergoing irradiation and radical cystectomy. In his study half of the patients donated 4 units of blood in the radiation period without problems and without affecting the effect of radiotherapy.

There has been concern that the lower Hct often seen in autologous donors may necessitate the transfusion of larger amounts of blood(18). In some controlled studies the efficacy of pre-operative blood donation was investigated (10-12). They are retrospective, however, and included patients who were deferred for donation. Moreover blood transfusion rules were different for controls and autologous blood donors(12). Because patients are randomized in our study and similarly transfused, we could compare the hematologic and transfusion data of the two groups and study whether donation led to a higher transfusion need. The Hb level in the autologous randomized patients dropped pre-operatively, whereas in the allogeneic patients it

dropped post-operatively (Table 2); this resulted in equal Hb concentrations 10 days post-operatively. Although there were some qualitative differences in transfusion therapy between the groups (Table 3), other factors have to play a role because autologous patients only received an average of 0.2 blood units more. The most obvious explanation is that autologous patients at the moment of operation have a stimulated erythropoiesis which prevents Hb from falling, whereas erythropoietin production and development of erythroid precursors will take at least one week in the allogeneic patients. Levine and others(19) showed recently in baboons that blood donation before surgery stimulates post-operative erythropoiesis: more intense reticulocytosis and earlier recovery to normal Hct was seen in baboons who had undergone an aggressive donation program.

Autologous donations are only indicated when blood would ordinarily be ordered for type and cross-match(20). It has been suggested(6)(18) that the predeposition of autologous blood should not be encouraged in a procedure in which 90% or more of patients do not require transfusion and / or if the blood use routinely averages less than 1 unit per patient per case. Risks from the autologous donation, no matter how small, would be greater than potential benefits and over-collection is costly and time consuming. Efforts have been made to create a schedule of optimal pre-operative collection of autologous blood (SOPCAB)(20) The SOPCAB differs from the maximal blood order schedule (MSBOS)(21) because both intra, and post-operative blood requirements are considered.

In our study the above mentioned criteria were met: 61% of the allogeneic patients needed blood transfusion with a mean number of 1.9 unit. Of the patients who had donated blood, 27% did not need their autologous donated blood and 77% did not need allogeneic blood (Table 3). Since blood loss and consequently the number of blood transfusions differs with the different tumor localizations, the effectiveness of the program was further analyzed per tumor localization, since the tumor localization is known prior to operation (Table 4). In patients with a right-sided colon carcinoma donation is questionable, since the above-mentioned criteria were only met partly: 0.5 units of blood were transfused in the allogeneic patients, 22% of the allogeneic patients needed blood. In patients with a left colon carcinoma donation had led to a substantial increase of patients not exposed to allogeneic blood, while only 15% of the autologous patients needed additional allogeneic blood transfusions (Figure 1). Patients with a rectal carcinoma often needed additional

allogeneic blood, but additional donation of a further 2 units of blood would at the utmost be sufficient for 18% of the patients (Figure 1) while 59% of the patients would be overdonating and have an extra delay of two weeks before surgery. In our opinion this is not justified in oncologic patients as long as the immune-suppressive effects of transfusions have not been clarified.

Studies on pre-operative autologous blood donation have seldomly been conducted in general surgery. Our study indicates that it is feasible in colon cancer patients and that it is an effective procedure. There has been concern that lower Hct as seen in autologous donors necessitates the transfusion of larger amounts of blood. Due to the randomized nature of this study we were able to show that this is not true, it seems that post-operative erythropoiesis is stronger in the patients who had donated blood.

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GENERAL DISCUSSION

The immune-modulatory effects of blood transfusions have been well established in the last two decades. While the immune-suppressive effect is therapeutic in allograft recipients(1), it may be detrimental in other situations. There is, for example, considerable evidence that blood transfusions given during surgery increase the risk for post-operative infections(2), and blood transfusions administered in the peri-operative period may decrease survival in cancer patients(3). Blumberg and Heal(4) recently published a meta-analysis of the retrospective studies done in the last 10 years, including 14 retrospective studies in colorectal cancer patients. Taken together the 5-year recurrence rates in colorectal cancer patients were 31 % in the non-transfused and 52 % in the transfused patients, and transfusion was an independent significant factor on 5-year survival in four of nine studies.

Nevertheless, many physicians remain sceptical about the existence of this so-called "blood transfusion effect" in cancer patients. While it is accepted that blood transfusions modulate the immune response, it is not accepted that such an immune-depression may result in accelerated tumor growth, because spontaneous tumors are not antigenic and do not evoke an immune-response.

Some authors have suggested that a blood transfusion effect should not necessarily have to be caused by immunologic mechanisms. Lawrance et al(5) showed that in stored full blood, growth factors derived from degranulating platelets are present. These include epidermal growth factor, platelet derived growth factor and Beta-transforming growth factor. Extracts from platelets have been shown to enhance the growth of experimental tumors in vivo by a direct stimulatory effect rather than by immune-suppression of the host(6). Some retrospective studies showed the role of plasma in the blood transfusion phenomenon. Blumberg et al(7)(8) found that, in general, patients receiving < 4 units of blood that included only red blood cells had recurrence and survival rates identical to those in patients receiving no transfusions, while those receiving 1 unit of whole blood had significantly worse outcomes than those receiving 1 unit of red blood cells. Recently, Marsh and colleagues(9) also concluded from a retrospective study that plasma protein, rather than the cellular component of whole blood mediates the accelerated

tumor recurrence. Since most retrospective studies date from the time that blood transfusions were given as full blood, and at present most transfusions are given as packed red cells, the question arises as to whether a blood transfusion effect still will exist. However, experimental studies performed at our laboratory showed a tumor promoting effect of allogeneic red cells and not of plasma(10).

Thus, both retrospective studies and experimental studies provide evidence for the existence of a tumor promoting effect of blood transfusions. Although some retrospective studies have demonstrated an independent effect of blood transfusions, the basic question is whether transfusion is not a surrogate marker for one of more clinical factors predisposing to tumor recurrence. At the moment, the evidence is strong enough to eradicate the administration of blood transfusions that are not absolutely necessary(11). There are no indications that wound healing is improved, or infections prevented in post-operative patients with normal hemoglobin levels. Czer and Shoemaker(12) showed that a hematocrit of 30 is acceptable for critically ill patients in the post-operative period.

However, the administration of blood transfusions can not always be avoided. Therefore, prospective trials are being conducted in order to study whether the prognosis of patients can be improved by administration of other types of transfusions. Both in New York and in Leiden, prospective studies in colorectal cancer patients have been conducted, in which patients were randomized for leukocyte free blood transfusions or normal leukocyte poor transfusions (buffy-coat free red cells). We randomized patients between allogeneic blood transfusions (given as packed cells) and predeposited autologous blood transfusions. Although the implementation of such a protocol is more complicated when compared to leukocyte free blood transfusions, we feel that at present, there is not enough evidence that leukocytes play the key role in the immune-suppressive effect of blood transfusions.

Autologous blood: A solution?

The Council on Scientific Affairs of the American Medical Association(13) stated that when the guidelines established by the American Association of Blood Banks (AABB) are followed, autologous blood is the safest type of blood for transfusion.
We were able to show that in a group of elderly colorectal cancer patients, 77 percent of the patients did not require allogeneic transfusions after having donated 2 units of

blood. These patients were not exposed to the risks of immunization, transmission of infections and/or immune-suppression. The possible disadvantages of autologous blood donation as a strategy to avert the tumor promoting effect of blood transfusions are:

1) Not all cancer patients will be able to donate blood. We excluded in our study patients who were not to be curatively resected, who required urgent surgery, who were anemic, and who did not fulfil the criteria for autologous blood donation. In the Dijkzigt hospital approximately 30 % of all admitted patients with the diagnosis of a colorectal cancer were eligible to be entered into the trial.

2) Surgery may be delayed for 1 or 2 weeks due to blood donation. However, with the knowledge that it takes years to develop colorectal cancer, it is not plausible to conclude that a delay of 2 weeks will affect the prognosis.

3) Donation of 2 units will not be enough to prevent all allogeneic blood transfusions. Twenty three percent of the patients who donated blood still required allogeneic blood transfusions as well as their autologous units.

4) Blood donation itself may have unintended side-effects. We found that NK-cell acitivity was temporary depressed after blood donation, both in healthy blood donors and in colorectal cancer patients (chapter 5). At present, the clinical significance of a transient depression of the NK-cell activity is unknown. Because NK-cells are thought to play a role in the first line of defence against blood borne metastases(14)(15), a depression of the NK-cell activity just before surgery may affect the effective clearance of circulating tumor cells released during surgery(16) and especially during manipulation(17) of the primary tumor.

   A similar depression of the NK-cell activity has been seen in rats that were bled 24 hours before testing (chapter 6). In addition, bleeding 1 day before tumor-inoculation stimulated the outgrowth of metastases. This could not be ascribed to a low hemoglobin levels, but was due to hypovolemia. Immediate plasma transfusion did abolish the tumor promoting effect, while pretreatment with r-HuEpo did not.

In conclusion, we can say that blood donation itself may affect the survival of colorectal cancer patients. The 5-year survival of the patients of the "autologous blood transfusion trial" should give the final answer on the question as to whether the risks of blood donations outweigh the benefits of autologous blood transfusions. Therefore, the 5-year survival rates will be analyzed with an "intention to treat" analysis. Colorectal cancer patients should be advised to donate their own blood in the future, if the whole group of autologous patients has a significantly better survival than the allogeneic patient group.

Treatment with r-HuEpo?

Because not all cancer patients are able to donate their own blood, we studied in additon the possibility of r-HuEpo therapy. So far, all clinical studies with r-HuEpo have been conducted in anemic patients suffering from chronic diseases (18)(19)(20)(21)(22). The concept that r-HuEpo therapy also might be useful in acute anemia, either during autologous blood donations or after surgery, derives from the knowledge that:

1) Erythropoiesis is a slow process; after an hypoxic stimulus, new red cells will not appear in the circulation before 5 to 7 days(23).

2) Only with severe anemia are sufficient high enough erythropoietin levels reached to guarantee a strong erythropoiesis(24).

R-HuEpo therapy in autologous blood donation programs

The endogenous erythropoietic response to serial phlebotomy is limited; patients are often not able to donate the requested number of blood units(25), probably because endogenous erythropoietin production will only be significantly elevated when the Hct drops below 30(26). Blood donations are only allowed with a Hct above 35.

Our rat studies (chapter 7) confirm this. While r-HuEpo treatment resulted in much higher hematocrits (Hct) in treated rats compared to controls in a two-weekly donation program, erythropoiesis was equally strong in the control group as in the group treated with R-HuEpo after daily blood taking. Obviously, the two weekly

program stimulated erythropoiesis less than a daily donation program. Therefore, r-HuEpo treatment was effective.

Other investigators(27)(28) who have tested the effect of r-HuEpo in autologous blood donation programs used very high doses of rHuEpo. We showed in chapter 7 that there is a maximal effective dose of r-HuEpo; daily administration of this dose was the most effective dose in stimulating erythropoiesis.

Peri-operative r-HuEpo therapy

In order to reduce the need for post-operative blood transfusions, it is necessary that newly formed red cells come into the circulation immediately post-operatively. It is evident that this can only be achieved by pretreatment with r-HuEpo. Post-operative treatment may decrease the post-operative anemic period, only.

Pretreatment with r-HuEpo was both the most effective way to stimulate erythropoiesis in the rat studies and in the clinical study. In the rat studies it resulted in both an increased harvest of autologous blood (chapter 7) and prevented post-operative anemia (chapter 8). In man, pretreatment for five consecutive days resulted in a significant elevation of the reticulocyte counts and hematocrit even one day before surgery.

The contribution of post-operative treatment with r-HuEpo to the erythropoiesis was less obvious. In rats r-HuEpo therapy did not reduce the post-operative anemic period significantly. Moreover, erythropoiesis appeared to be depressed after surgery. The response to r-HuEpo was less strong in operated rats than in controls. A similar phenomenon was seen in cancer patients; despite continued r-HuEpo therapy the number of reticulocytes dropped after the second post-operative day. While this could be attributed to the very low post-surgical serum iron levels and iron binding capacity in the patients, it does not explain the depression seen in rats. It has been reported that patients with renal failure become temporarily unresponsive to r-HuEpo therapy after surgery(29) or during an infectious period(30). This can be due to an inability to use iron(31), production of lymphokines suppressing erythropoiesis(32), and endotoxin production(33).

Conclusions on r-HuEpo therapy

Pre-operative r-HuEpo treatment is more promising than post-operative treatment; if blood transfusions are to be prevented an immediate post-operative release of red cells is required. A disadvantage of this strategy is that part of the newly formed red

cells will be lost during surgery. Therefore, pretreatment should ideally be combined with blood donation just prior to surgery, and hemodilution. Post-operatively, patients should be treated with i.v. iron-solutions to compensate for the loss of iron (red cells) during surgery.

Closing remarks

In contrast to the countries surrounding the Netherlands, there are almost no autologous blood donation programs set up by the Dutch blood banks. There is indeed a very good blood banking system with voluntary blood donors, which has prevented large numbers of patients from becoming infected with hepatitis NANB(34) and HIV(35) after a blood transfusion. There are, however, many other side-effects contraindicate blood transfusions(36).

Some orthopedic clinics in Germany report that 90 % of their blood usage is covered with autologous blood. This may be due to the fact that in Germany anesthetists are often responsible for the hospital blood bank. Every study focusing on autologous blood has indicated that the success of a program depends principally on the enthusiasm of the physicians. Our experience with more than 40 patients from the Dijkzigt hospital, indicated that even elderly patients were very eager to donate their own blood. Therefore, my personal opinion is that every physician who would prefer his or her own blood during surgery, should give his or her patients the chance to donate blood, simply by informing them about this possibility.

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SUMMARY

For most malignancies surgery is the only curative treatment modality. However, many patients with colorectal cancer develop a local recurrence or distant metastases in the years after surgery. Apparently, metastases had already occurred before or during surgery. The immune system is probably capable of recognizing and eliminating tumor cells circulating in the bloodstream, and may also eliminate small numbers of tumor cells in the tissues. Therefore, it is important to note that anesthesia, surgery and allogeneic blood transfusion all depress the immune system temporarily. This may hinder the effective clearance of tumor cells, thus negatively influencing the survival of cancer patients.

The central theme of the studies presented in this dissertation is: prevention of blood transfusions in oncologic surgery and the consequences of such therapy. The first 3 Chapters of this dissertation, are an introduction to the two experimental parts. **Chapter 1** summarizes the risks associated with blood transfusions, with emphasis on their immune-suppressive action and **Chapter 2** discusses the process of erythropoiesis after acute blood loss. The purpose and design of the two clinical studies is expounded in **Chapter 3**. In the "Autologous Blood Transfusion Trial", patients were randomized for autologous or allogeneic blood transfusion therapy during surgery. The patients randomized for autologous blood transfusions donated 2 units of blood, 14 and 10 days before surgery. In the "EPO trial" patients were randomized for peri-operative treatment with recombinant Human Erythropoietin (r-HuEpo) or no treatment. In vitro immunologic studies were conducted in both patients groups, in patients with a metastazised carcinoma and in healthy blood donors. The results are of these immunologic studies are given in the **first experimental** part (Chapters 4-6), which deal with the effect of cancer, blood donation, surgery and blood transfusion upon the immune system and upon tumor growth. In the **second experimental** part (Chapter 7-10) the results of two techniques to prevent blood transfusions in cancer patients are discussed: 1) pre-operative autologous blood donation, and 2) treatment with r-HuEpo.

Chapter 4 compares the immune status of cancer patients with a local tumor with those of patients with distant metastases, and of healthy blood donors. While the T-cell response was depressed in both groups of cancer patients, compared to healthy blood donors, the NK-cell activity was depressed only in patients with distant metastases. We were not able to show that T-cell responses or NK-cell activity measured before study entry were of prognostic value. Patients who developed metastases or died within a period of two years had no different immunologic baseline values than surviving patients.

Chapter 5 describes the effect of blood donation, surgery and blood transfusion on NK-cell activity, mitogenic T-cell responses and lymphocyte subsets. A distinct effect of blood donation on the NK-cell activity was found; five days after donation of 1 unit of blood, the NK-cell activity of healthy blood donors was 70 % of the baseline value, but was normal again 1 week later. Colorectal cancer patients donated 2 units of blood, 14 and 10 days before surgery. Yet, their NK-cell activity was found to be significantly reduced 8 to 10 days after the second blood donation. As a consequence, the NK-cell activity of patients randomized for autologous blood at the time of surgery was lower than for patients randomized for allogeneic blood. However, since the post-operative NK-cell activity was depressed in patients who had received allogeneic transfusions, the net result was that the post-operative values were not significantly different.

Surgery had a distinctly negative effect on the T-cell response, but no significant differences were found between the two patient groups.

Chapter 6 reports the results of rat experiments on the effect of withdrawal of 20 percent of the blood volume on tumor growth. If rats were bled one day before tumor inoculation, significantly more lung metastases developed. This tumor promoting effect could be abolished with immediate i.v. infusion of plasma, whereas pretreatment with r-HuEpo, resulting in normal hemoglobine levels after blood taking, produced no such effect.

In addition, blood withdrawal caused a significant depression of the NK-cell activity in rats.

The following 4 Chapters deal with methods to avoid blood transfusions. The experimental results are given in **Chapter 7 and 8**, while the clinical results are

presented in the last two Chapters.

Five days of r-HuEpo treatment resulted in a 20 % increase of the hemoglobin level and hematocrit. There appeared to be a maximal effective dose of r-HuEpo in rats, and best effects were obtained with daily administration.

Two types of autologous blood donation programs were studied: 1) a daily blood donation program, and 2) a program in which blood was taken twice a week. Ten percent of the blood volume was taken each time and r-HuEpo was administered daily. The yield of red blood cells in the aggressive donation program was only slightly higher in the rats treated with r-HuEpo. Apparently, the frequent blood donations had stimulated endogenous erythropoietin production to a level equally effective as when r-HuEpo was administered.

R-HuEpo treatment did raise the number of red cells collected, either when it was given as a three day pretreatment in the daily donation program, or when it was given daily during a non-aggressive blood donation program. The hemoglobin level after four blood donations in a two weeks-period was 192 g/l in the treated group and 120 g/l in the control group.

The possibility of preventing post-operative anemia with peri-operative r-HuEpo treatment is discussed in **Chapter 8**. It appeared that erythropoiesis is hampered post-surgically; the effect of r-HuEpo therapy was less effective in operated rats than in control rats. In addition, post-operative treatment with r-HuEpo for five days in operated and bled rats did not result in a faster recovery from anemia.

Pre-operative treatment with r-HuEpo for five days (day -4 until day of surgery, day 0) was compared with peri- (day -2 to day 2) and post-operative treatment (day 0 to day 5). Pre-operative treatment was the most effective therapy in preventing post-operative anemia. We conclude from these studies that the effect of r-HuEpo is not the result of more effective post-operative erythropoietin levels, but is due to the fact that erythropoiesis is stimulated in advance.

Chapter 9 reports the clinical results of peri-operative r-HuEpo treatment in a group of patients who underwent surgery for gastro-intestinal cancer. Thirty patients were randomized for r-HuEpo treatment or no treatment. The treated patients received 200 U/kg r-HuEpo daily, from five days before surgery up to the third post-operative day. This resulted in a clear-cut stimulation of the erythropoiesis; both the hematocrit and the number of reticulocytes were increased already one day before surgery. The

highest reticulocyte counts in the treated group were reached two days after surgery, while erythropoiesis in the control group started six days after surgery.

Whereas the effect of pretreatment was clearly proved, the effect of post-operative treatment was less obvious. This may have been due to post-operative iron deficiency.

The efficacy in reducing the need for blood transfusions was not established. Patient numbers were too small and the amount of blood loss ranged too great in the patient population studied. There was a tendency for fewer transfusions to be needed in the treated patients. Future studies are indicated.

Chapter 10 discusses the feasibility of an autologous blood donation program. We were in the unique situation that in our autologous blood transfusion study, patients were randomized and both groups were subjected to the same blood transfusion rules. The patients who had donated two units of blood had a significantly lower hemoglobin level the day before surgery, compared to the patients randomized for allogeneic blood. While blood loss and number of blood transfusions were similar in both groups, the hemoglobin drop experienced during and after surgery was significantly lower in the autologous randomized patients, probably indicating that erythropoiesis was already stimulated in the blood donors.

The pre-operative autologous blood donation program was effective in the patient population under study; only 23 % of the patients who had donated blood, needed allogeneic blood in addition, while 61 % of the patients randomized for allogeneic blood were transfused. The necessity for blood transfusions depended on tumor localization. In patients with a right-sided colon tumor, however, the usefulness of blood donation was doubtful. Only 22 % of the allogeneic patients in this group required a blood transfusion.

The **General discussion** focuses on two ways to avoid blood transfusions: autologous blood donation and r-HuEpo therapy. The possibilities and disadvantages of both strategies are discussed.

SAMENVATTING

Voor de meeste maligniteiten is chirurgie de enige curatieve behandelingsmogelijkheid. Desondanks ontwikkelt een aanzienlijk deel van de patienten met darmkanker, in de jaren na operatie, een lokaal recidief of metastasen op afstand. Metastasering heeft klaarblijkelijk al voor of tijdens de operatie plaats gevonden. Het immuunsysteem van de mens is mogelijk in staat om tumorcellen in de bloedbaan te herkennen en te attaqueren en is kan daarnaast wellicht kleine hoeveelheden tumorcellen die in de weefsels zijn vastgelopen elimineren. Het is daarom verontrustend te weten dat zowel narcose, operatie als bloedtranfusie de werking van het afweersysteem tijdelijk kunnen onderdrukken. Dit kan een effectieve verwijdering van tumorcellen in de circulatie verhinderen, en zodoende een negatief effect op de overleving van kankerpatienten hebben.

Het centrale thema van de studies die in dit proefschrift gepresenteerd worden is: het voorkomen van bloedtransfusies binnen de oncologische chirurgie en de consequenties van zo'n strategie. De eerste drie hoofdstukken van dit proefschrift vormen de inleiding tot de twee experimentele delen. **Hoofdstuk 1** vat de risico's van bloedtransfusies samen, waarbij de nadruk ligt op de immunosuppressive werking van bloedtransfusies. In **hoofdstuk 2** wordt de erythropoiese (aanmaak van rode bloedcellen) na acuut bloedverlies besproken, terwijl **hoofdstuk 3** een nadere toelichting geeft op het doel en de opzet van de twee patienten-studies. In de zogenaamde " Autologe Bloed Transfusie Trial" worden patienten gerandomiseerd voor het krijgen van autologe of allogene bloedtransfusies tijdens de operatie. De "autologe" patienten doneerden 2 eenheden bloed, 14 en 10 dagen voor de operatie. In de "EPO Trial" werden patienten gerandomiseerd voor het al dan niet krijgen van recombinant Humaan Erythropoietine (r-HuEpo) in de periode rond de operatie. Bij de patienten uit beide studies, als ook bij patienten met een gemetastaseerd carcinoom en bij gezonde bloeddonoren werden enkele *in vitro* immunologische bepalingen gedaan. De resultaten van deze immunologische studies worden in het **eerste experimentele deel** beschreven; dit deel handelt over het effect van kanker, bloeddonatie, operatie en bloedtransfusie op de immuunstatus en op de tumorgroei.

In het **tweede experimentele deel** worden de resultaten besproken van 2 methoden om bloedtransfusies bij kankerpatienten te voorkomen: 1) pre-operatieve autologe bloeddonatie en 2) behandeling met recombinant humaan Erythropoietine (r-HuEpo).

Hoofdstuk 4 vergelijkt de immuunstatus van kankerpatienten met een lokale tumor met die van patienten met metastasen op afstand en met die van gezonde bloeddonoren. Terwijl de T-cel respons in beide groepen kankerpatienten in vergelijke met de groep bloeddonoren verlaagd is, is de NK-cel activiteit alleen verlaagd bij patienten met metastasen op afstand. De T-cel response en de NK-cel activiteit zoals deze bij aanvang van de studie gemeten werden, bleken niet van prognostische betekenis voor de patient. Patienten die gedurende een follow-up van 2 jaar metastasen ontwikkelden, of overleden, hadden geen andere uitgangswaarden dan patienten die overleefden.

Hoofdstuk 5 beschrijft het effect van bloeddonatie, chirurgie en bloedtransfusie op de NK-celactiviteit, mitogene T-cel response en de verschillende lymphocyten-subklassen. Bloeddonatie had een duidelijk effect op de NK-celactiviteit; na donatie van 1 eenheid bloed was de NK-celactiviteit bij gezonde donoren 5 dagen na donatie 70 % van de uitgangswaarde, maar was weer hersteld op dag 12. Darmcarcinoom-patienten doneerden 2 eenheden bloed 14 en 10 dagen voor de operatie; 8-10 dagen na de tweede donatie was de NK-celactiviteit nog steeds significant verlaagd. Hierdoor werden autoloog gerandomiseerde patienten geopereerd op een moment dat de NK-celactiviteit significant lager was dan die van de allogeen gerandomiseerde patienten. Daartegenover stond dat de NK-celactiviteit verlaagd was in allogeen getransfundeerde patienten; het netto resultaat was dat er postoperatief geen verschillen in de NK-cel activiteit was tussen de 2 groepen.

Operatie had een duidelijk negatief effect op de T-cel respons, maar er werden geen significante verschillen tussen de beide patientengroepen gevonden.

Hoofdstuk 6 bespreekt de resultaten van de experimentele studies naar het effect van bloedafname (20 % van het bloedvolume) op de tumorgroei in ratten. Wanneer er 1 dag voordat tumorcellen iv ingespoten werden, bloed afgenomen was, dan ontwikkelden er zich significant meer longmetastases. Het effect van bloedafname op de tumorgroei kon teniet gedaan worden door onmiddellijk na bloedafname plasma terug te geven, terwijl voorbehandeling met r-HuEpo (resulterend in een goed

Hemoglobine (Hb) gehalte na bloedafname) geen effect had.

Ook bij ratten werd 1 dag na bloedafname, een verlaagde NK-celactiviteit gevonden.

De volgende 4 hoofdstukken gaan over methodes om bloedtransfusies te voorkomen. In **hoofdstuk 7 en 8** worden de experimentele resultaten en in de laatste 2 hoofdstukken de klinische resultaten besproken.

Vijf dagen r-HuEpo behandeling resulteerde in een toename van het Hb en Hematocriet (Ht) met ongeveer 20 %. Er bleek een maximale effectieve dosis van r-HuEpo te zijn en de beste resultaten werden bereikt door r-HuEpo dagelijks toe te dienen.

Twee experimentele autologe bloed donatie programma's werden bestudeerd 1) een programma waarin dagelijks bloed werd afgenomen en 2) een twee wekelijks donatie programma. Elke keer werd 10 % van het bloedvolume afgenomen, en werden de ratten al dan niet met r-HuEpo behandelt. De opbrengst aan rode bloedcellen in het agressieve programma was nauwelijks verhoogd in de behandelde ratten. Klaarblijkelijk was met het frequente doneren ook de endogene erythropoietine produktie gestimuleerd tot een vergelijkbaar niveau als in de behandelde groep bereikt werd.

Een behandeling met r-HuEpo resulteerde wel in een hogere opbrengst aan afgenomen rode bloedcellen, wanneer het dagelijkse bloeddonatie-programma voorafgegaan werd door drie dagen van voorbehandeling met r-HuEpo, en eveneens wanneer het dagelijks tijdens een niet-agressief bloeddonatie-programma gegeven werd. Het Hb gehalte was na 4 bloeddonaties, in een tijdsperiode van twee weken, 192 g/L in de behandelde groep en slechts 120 g/L in de controle groep.

De mogelijkheid om postoperatieve anemie te bestrijden met peri-operatieve r-HuEpo behandeling wordt besproken in **hoofdstuk 8**. Allereerst bleek dat de erythropoiese na chirurgie gestoord is: een behandeling met r-HuEpo had minder effect op het Ht en Hb gehalte in geopereerde ratten dan in controle ratten. Daar kwam nog bij, dat postoperatieve behandeling met r-HuEpo gedurende 5 dagen, in een geopereerde groep ratten (met aanzienlijk bloedverlies) niet resulteerde in een versneld herstel van de anemie.

Pre-operatieve behandeling gedurende 5 dagen met r-HuEpo (dag -4 tot de OK dag; dag 0) werd vergeleken met peri-operatieve (dag -2 tot +2) of postoperatieve

behandeling (dag 0 tot 5). Pre-operative behandeling was het meest effectief om postoperatieve anemie te voorkomen.

Uit deze studies trekken we de conclusie dat het effect van r-HuEpo therapie nauwelijks lijkt te komen van meer effectieve postoperatieve erythropoietine-spiegels, maar doordat de erythropoiese al voor de operatie op gang komt.

Hoofdstuk 9 rapporteert de klinische resultaten van peri-operative behandeling met r-HuEpo bij een groep patienten die aan een gastro-intestinale tumor geopereerd werden. Dertig patienten werden hiertoe gerandomiseerd voor het al dan niet krijgen van r-HuEpo. De behandelde patienten kregen vanaf 5 dagen voor de operatie tot en met 3 dagen na de operatie 1 maal daags intraveneus 200 U r-HuEpo/kg. Onder invloed hiervan werd de erythropoiese duidelijk gestimuleerd; zowel het Ht als het aantal reticulocyten was 1 dag voor de operatie gestegen. Het hoogste promillage reticulocyten werd 2 dagen na de operatie gevonden; bij de controlegroep kwam de erythropoiese 6 dagen na de operatie op gang en werd het hoogste promillage op dag 8 bereikt. In tegenstelling tot het duidelijke effect van de voorbehandeling met r-HuEpo op de erythropoiese, was het additionele effect van de postoperatieve behandeling minder eenduidig. Dit is mogelijk toe te schrijven aan de zeer lage ijzergehaltes direct na de operatie.

In de door ons onderzochte groep van patienten liep het bloedverlies bij de operatie erg uiteen, en daarnaast was het aantal onderzochte patienten te klein, om een uitspraak over de effectiviteit te doen. Er was een duidelijke tendens dat minder transfusies nodig waren in de behandelde patienten. Aanbevelingen werden gedaan voor verdere studies.

Hoofdstuk 10 bespreekt de haalbaarheid van preoperatieve autologe bloeddonatie bij darmcarcinoom-patienten. We zaten in de unieke situatie dat in de autologe bloedtransfusie trial patienten aselectief werden gerandomiseerd en beide groepen volgens dezelfde bloedtransfusie-regels werden behandeld. In de groep patienten die bloed hadden gedoneerd was het preoperatieve Hb gehalte lager dan in de andere groep. Alhoewel het bloedverlies en het aantal transfusies in beide groepen gelijk was, was de Hb daling tijdens en na de operatie, geringer in de autoloog gerandomiseerde patienten. Kennelijk was de erythropoiese al voor de operatie, door de bloeddonaties gestimuleerd.

Het pre-operatieve autologe bloeddonatie programma was effectief in de

onderzochte patienten-populatie; slecht 23 % van de autoloog gerandomiseerde patienten had allogeen bloed nodig in vergelijke met 61 % van de allogeen gerandomiseerde patienten. De behoefte aan bloed was afhankelijk van de tumorlokalisatie, alleen bij patienten met een rechtszijdige tumor, kon het nut van een autoloog programma in twijfel getrokken worden, daar slechts 22 % van de allogeen gerandomiseerde patienten bloed nodig had.

De **algemene discussie** gaat in op de twee mogelijkheden om bloedtransfusies te voorkomen: autologe bloeddonatie en r-HuEpo therapie. De mogelijkheden en nadelen van beide strategien worden besproken.

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Submitted publication or pubications in preparation:

- Hoynck van Papendrecht MAW, Busch ORC, Jeekel J, Marquet RL. The efficacy of recombinant human erythropoietin to stimulate erythropoiesis after blood loss and surgery. Submitted for publication.
- Hoynck van Papendrecht MAW, vd Walle J, Jeekel J, Marquet RL. The effect of blood donation on NK-cell activity.
- Hoynck van Papendrecht MAW, Busch ORC, Marquet RL, Jeekel J. Perioperative treatment with recombinant human erythropoietin, results from an open randomized study.

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NAWOORD

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