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Cell-Cell Contact and Anatomical Compatibility in Stromal Cell-Mediated HSC Support During Development

KIRSTY HARVEY, ELAINE DZIERZAK

Department of Cell Biology and Genetics, Erasmus University Medical Center, Rotterdam, The Netherlands

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ABSTRACT

Hematopoietic stem cells (HSCs) are able to generate the wide variety of blood cells found in the adult and are maintained in the bone marrow (BM) stromal microenvironment. In the aorta-gonads-mesonephros (AGM), which autonomously generates the first HSCs, the stromal microenvironment is largely uncharacterized. We have previously made an extensive panel of stromal clones from AGM subregions and have found that clones from the urogenital ridges (UG) provide the most potent support for adult BM HSCs. However, it is unknown to what extent the stroma from this developmentally and anatomically distinct microenvironment can support HSCs from other regions of the embryo, such as yolk sac. Moreover, it is unknown whether cell-cell contact is necessary in this microenvironment. Here, we show that the HSCs from the embryonic aorta are the most potently supported HSCs in UG stromal clone co-cultures and that contact is required for the maintenance and expansion of embryo-derived HSCs. ***Stem Cells*** *2004;22:253-258*

INTRODUCTION

In the adult, mature blood cells are derived from hematopoietic stem cells (HSCs) through a complex cell differentiation hierarchy. HSCs are defined by the ability to self-renew and differentiate to all blood lineages, as demonstrated by clonal marking and complete repopulation of hematopoietic ablated adult recipient mice [1]. The potential of HSCs is maintained primarily by the microenvironment, which consists of stromal cells (SCs). The experiments of *Dexter* originally showed the importance of SCs, with colony-forming unit spleen (CFU-S) ability being lost in primary bone marrow (BM) cultures, when no SCs were present [2]. Since then other studies have shown the close association of HSCs and SCs in vitro with embryonic cells and in vivo within niches of the adult BM [3-6] and also the importance of cell-cell contact in long-term culture-initiating cells (LTC-IC) and cobblestone area-forming cell ex vivo cultures [7, 8].

Although HSCs are harbored in the BM microenvironment of the adult mouse, during ontogeny HSCs are found in different anatomical sites. These sites include first the aorta-gonads-mesonephros (AGM) region and then the yolk sac (YS) and fetal liver (FL) [9]. It is thought that these microenvironments differ in their capacity to support hematopoietic progenitors and/or HSCs and also that ontogenically early HSCs may possess different characteristics allowing them to be harbored and active in the various embryonic hematopoietic microenvironments. Indeed, it has

Correspondence: Elaine Dzierzak, Ph.D., Erasmus University Medical Center, Dept. of Cell Biology and Genetics, P. O. Box 1738, 3000 DR Rotterdam, The Netherlands. Telephone: 31-10-408-7172; Fax: 31-10-408-9468; e-mail: e.dzierzak@erasmusmc.nl *Received September 30, 2003; accepted for publication November 7, 2003.*

been shown that while FL hematopoietic progenitors (BFU-E) are not supported in vitro in a BM stromal microenvironment [5], both adult BM and FL HSCs can interact effectively in vitro with the FL stromal microenvironment [6] and in vivo by yielding complete hematopoietic repopulation in irradiated adult recipients. Since the first HSCs emerge during mouse midgestation in the AGM region [10, 11] and AGM explant cultures show large increases in HSC numbers, this microenvironment and the HSCs within it are especially interesting for our understanding of hematopoietic regulation.

To study the microenvironment, SC lines from the mouse AGM region [12-14] and its component subregions, aorta (Ao) and urogenital ridges (UG), have been generated [15, 16]. AGM-derived stromal clones have been shown to maintain HSCs from different developmental, anatomical, and species sources such as human umbilical cord blood (UCB) [14, 17], mouse FL [13], mouse BM, and sorted $CD34^+$ $c\text{-}kit^+$ populations of embryonic day (E)11 YS/AGM [15]. However, none of these studies have directly compared the quality of HSC support provided by AGM SCs to midgestation aortic, UG or YS HSCs, the three most closely related HSC populations during this stage of development [9]. Moreover, since these embryonic cells are tightly organized and undergoing proliferation and differentiation in the context of local signaling factors, it is postulated that cell-cell contact between HSCs and stroma is important in the AGM.

Previous studies by several laboratories have shown that physical contact between human hematopoietic cells from fetal/adult sources and mouse SCs is important for providing the efficient maintenance of human progenitors and HSCs [8, 12, 18]. The mouse stromal line (MS5) provides better support of human progenitors than human BM stroma, suggesting that the match between species is not important for adult hematopoietic cells. Others have found efficient maintenance of human hematopoietic progenitors/HSCs in non-contact cultures or in the complete absence of SCs over periods of 2 to 5 weeks [19, 20]. While the conditions necessary for the support of human hematopoietic cells remain controversial, to date little is known about the stromal contact requirements for mouse HSCs from the midgestational hematopoietic sites. Studies with FL hematopoietic progenitors suggest that contact with AGM stromal lines is essential [13]. However, these studies did not address whether contact is advantageous for specific HSCs from the same or different developmental/anatomical site(s), particularly the AGM region. Thus, we studied the cell-cell contact requirements of HSCs from three different developmentally early anatomical hematopoietic sites in UG stromal clone co-cultures. We show here the highest support of E11 Ao HSCs in such co-cultures as compared to HSCs from the E11 UG and YS and that contact is required. These results indicate differences in the interactions between HSCs from different sources and the stroma, and suggest an important role for AGM microenvironment in the maintenance and expansion of the earliest Ao-derived HSCs.

Materials and Methods

Animals

Mice were bred at the Erasmus Animal Facility and adult (C57BL/10 × CBA) F1 mice were used as transplant recipients. Transgenic mice lines Ln7 human β-globin [21] and Ly-6A green fluorescent protein (GFP) [22, 23] were used as donor cells for transplantation. For embryo generation, timed matings were set up between the transgenic males and (C57BL/10 × CBA) F1 females. The day of vaginal plug was counted as day 0. Ly-6A GFP transgenic embryos were typed by fluorescence microscopy (LX70 fluorescent microscope, Olympus; Albertslund, Denmark; http://www.olympus.com).

Tissue and Cell Preparation

Pregnant mice were sacrificed by cervical dislocation and the embryos were isolated from the uterus. Aos, UGs or YSs were (sub)dissected as previously described [23], and a single cell suspension was obtained by incubation with collagenase (Sigma; Zwijndrecht, NL; http://www.sigma aldrich.com) at a final concentration of 0.12% volume/volume.

Contact and Non-Contact Co-Cultures

Stromal clones were grown to confluence in 0.1% gelatin-coated 24-well plates in the presence or absence of transwell inserts (0.4 μm pore; Costar; Corning/Costar; Schipol, NL; http://www.corning.com) for non-contact cultures and contact cultures, respectively. A single cell suspension obtained after collagenase treatment of tissues was plated at 1 embryo equivalent (ee) of cells on each UG 26 stromal clone (irradiated at 30 Gy) for contact cultures or inside the transwell for non-contact cultures. Cultures were for 5 days at 33°C in LTC medium plus hydrocortisone (M5300, StemCell Technologies, Inc; Vancouver, BC, Canada; http://www.stemcell.com). After culture for both non-contact and contact cultures, adherent and non-adherent fractions were pooled and tested in hematopoietic assays.

Hematopoietic Progenitor Assay

Cells were plated in methylcellulose (MC) (M3434, StemCell Technologies) according to manufacturer's instructions. After 7 days at 37°C the colonies (erythroid, myeloid, granulocytic and mixed colonies) were counted.

HSC Transplantation Assay

Cells from (sub)dissections were either immediately injected or were injected after culture into irradiated (split

dose of 9 Gy) recipient mice. A limiting radioprotective dose of 2×10^5 (C57BL/10 × CBA) F1 spleen cells was co-injected into each recipient. At 4 months post-transplantation, peripheral blood DNA was obtained and β-globin or GFP specific polymerase chain reaction (PCR) was performed in a semi-quantitative manner with known standards (Image-quant software; Imagequant-Amersham Biosciences; Rosendaal, NL). Only recipients showing >10% donor-derived engraftment were considered reconstituted.

Results

Three SC lines previously isolated from the UGs of E11 AGMs were chosen for these studies: UG 26.1B6 (1B6) because it is the best supporter for mouse BM HSCs in long-term co-cultures [15], UG 26.3D4 (3D4) since it most highly supports human UCB hematopoietic progenitors in long-term co-cultures [16, 17], and UG 26.3B5 (3B5) as a non-supportive stromal control, since it has been shown to be unsupportive of mouse BM LTC-ICs, resulting in 35-fold fewer CFU-cultures (CFU-C) as compared to 1B6 and 3D4 [15].

UG Stromal Co-Cultures Support Midgestation Aortic Hematopoietic Progenitors More Efficiently in Contact Cultures

To determine if the three stromal clones 1B6, 3D4, and 3B5 could support E11 aortic hematopoietic progenitors, conventional co-cultures were established in which contact could occur between the hematopoietic cells and the SCs. After 5 days, Ao cells were tested for hematopoietic progenitor activity. As shown in Figure 1, most CFU-Cs were found in the adherent fractions (300 to 500 CFU-Cs/E11 Ao) and fewer in the non-adherent fractions (40 to 300 CFU-Cs/E11 Ao) of the co-cultures. CFU-Cs varied in number between the stromal clones, but not in size or morphology. Indeed, in all cases CFU-granulocyte macrophage, BFU-E, and CFU-mix were found. No CFU-Cs were found in the absence of stroma. The 1B6 and 3D4 stromal clones provided similarly good support for midgestation Ao hematopoietic progenitors and expanded input numbers by 9- to 14-fold. However, while 3B5 did not support progenitors in the non-adherent fraction, it did unexpectedly support hematopoietic progenitors in the adherent fraction, increasing input progenitor numbers by 10-fold. Thus, 3B5 can no longer be considered as a non-supportive stromal clone. Nonetheless, it has very different supportive properties than 1B6 and 3D4. The differences most likely are inherent in signaling interactions and/or adhesive properties.

Next we compared the support of CFU-Cs in conventional stromal co-cultures with the support provided by transwell co-cultures (non-contact). In such transwell co-cultures the SCs are physically separated from the hematopoietic cells by a filter, which is permeable to growth factors/signaling molecules. A clear decrease in CFU-C numbers is observed in non-contact co-cultures of all three stromal clones. The cumulative numbers of CFU-Cs were decreased by factors of 6-, 5-, and 5-fold for 1B6, 3D4, and 3B5, respectively, in non-contact cultures, as compared to contact cultures. Finally, while the CFU-C numbers in contact co-cultures are consistent with a 9- to 14-fold expansion of the input number of E11 Ao progenitor cells (5.2 ± 1.6), the non-contact cultures showed only a 1.5- to 3-fold expansion. These data demonstrate that all three E11 UG-derived stromal clones can maintain E11 Ao CFU-Cs independent of cell-cell interactions, but that direct contact is required for efficient expansion of CFU-Cs.

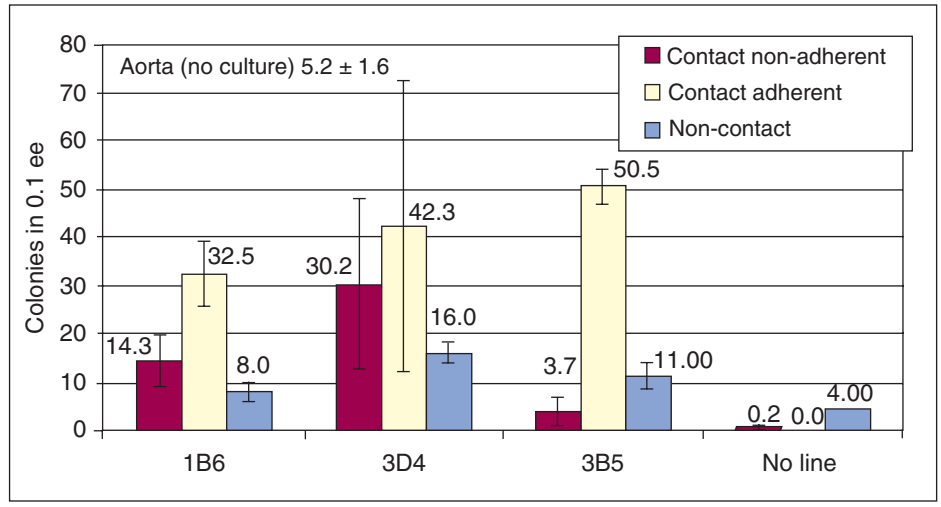

Figure 1. Hematopoietic progenitors are maintained in contact and non-contact cultures with midgestation UG stromal clones. *A pool of E11 Ao cells was obtained. The input number of aortic hematopoietic progenitors was measured by MC CFU assay and found to be 5.2 ± 1.6 per 0.1 (ee) of tissue. For contact cultures, a single cell suspension of Ao cells was plated at 1 ee of cells on each of the UG 26 SC lines (irradiated at 30 Gy) and cultured for 5 days at 33°C. The UG 26 SC lines are as indicated: 1B6, 3D4, and 3B5. No line indicates the results of the control culture of Ao cells in which no SC line was used. For non-contact cultures, a single cell suspension of Ao cells was cultured in the transwell above the SCs indicated. All cells were harvested from the transwell of the non-contact cultures. From the contact cultures, the non-adherent and adherent cell fractions (containing the stromal line) were harvested. Cells were plated in MC 37°C for 7 days and hematopoietic colonies counted (CFU-GM, BFU-E, CFU-mix). The mean of triplicate samples of four to six experiments (except for contact adherent data, n = 2) is indicated above each bar along with the standard error.*

Contact with UG Stromal Clones is Essential to Maintain and Expand Midgestation Aortic HSCs

We next examined the effects of cell-cell contact on the maintenance and expansion of HSCs from E11 Aos, UGs, and YSs in co-cultures with the three UG stromal clones. After contact or non-contact co-culture of genetically marked embryonic cells with the stromal lines, cells were transplanted into irradiated adult recipients and tested for HSC repopulation 4 months post-transplantation. Also, 1 ee of these cells was injected directly into irradiated recipients as a control for input HSCs in the co-cultures. In the long-term in vivo repopulation assay, we found that all three stromal lines behaved similarly in their support of HSCs. As shown in Table 1, 4/9, 5/9, and 2/5 recipients were high level, multilineage repopulated with a limiting dilution (0.3 ee) of Ao cells co-cultured with 1B6, 3D4, and 3B5 respectively. Thus, these contact co-cultures support a 3-fold increase in Ao HSCs as compared to input HSC number. Moreover, despite injection of 1 ee of cultured Ao cells, no Ao HSCs were found in non-contact stromal co-cultures (0/4, 0/3, and 0/5 recipients repopulated/transplanted for 1B6, 3D4, and 3B5, respectively, or 0/12 when data are pooled). Hence, E11 Ao-derived HSCs require SC contact for maintenance and expansion ex vivo.

To directly compare support of HSCs from other E11 hematopoietic tissues to Ao HSCs, and to determine if SC contact is required for the support of these HSCs, contact and non-contact co-cultures of 1B6, 3D4 and 3B5 were established with UG, YS and FL cells. Injection of a limiting dilution (0.3 ee) of UG cells from contact co-cultures with 3D4 revealed the presence of UG HSCs in one out of six recipients, while injection of 1 ee of uncultured UG cells gave no repopulation in five recipients. This increase in UG HSC activity requires stromal contact, since no UG HSCs were found after 1 ee of such cells was transplanted. In contrast to the increase found for Ao and UG HSCs in contact co-cultures, no recipients were found repopulated after injection of 0.3 ee of contact co-cultured YS cells. YS HSC activity was also absent in the non-contact cultures as determined by transplantation of 1 ee of cells. In studies with FL cells, support of HSCs was found in contact cultures but not in non-contact cultures (data not shown). Hence, UG SC lines expand and maintain Ao and UG HSCs, but do not support YS HSCs. These results suggest that HSCs and SCs are most functionally compatible when derived from closely related embryonic tissue. Moreover, contact with UG stromal clones is essential for the support/expansion of these midgestation HSCs.

Discussion

In our studies of the support provided by the three midgestation UG stromal clones for midgestation hematopoietic progenitors and HSCs, we observed several differences between the stromal clones. Although the stromal clones are closely related and derived from the same starting long-term culture of UG cells, distinct properties emerged. In non-contact cultures, 3D4 supports two times more CFU-C than 1B6. Since the transwell culture system allows the diffusion of molecules across the membrane, this result suggests that 3D4 may be producing soluble factors responsible for an expansion of progenitors. Previously, we have examined the transcription profiles of 1B6 and 3D4 SCs by semi-quantitative RT-PCR for growth factor gene expression and found that interleukin-11 (IL-11) is expressed more highly by 3D4 [16]. While this expression profiling was not exhaustive and other factors are also likely to be involved, it would be interesting in future experiments to test if addition of IL-11 improves progenitor support in co-cultures.

Table 1. Support of HSCs from different embryonic sources in contact and non-contact cultures with midgestation UG stromal clones

	***n* repopulated mice /*n* transplanted mice (% repopulated)**					
Co-culture	**None**	**Contact**				**Non-contact**
Stroma	**–**	**1B6**	**3D4**	**3B5**	**Total**	**Total**
ee injected	**1.0**	**0.3**	**0.3**	**0.3**	**0.3**	**1.0**
Ao	5/11 (45)	4/9 (44)	5/9 (55)	2/5 (40)	11/23 (48)	0/12 (0)
UG	0/5 (0)	0/3 (0)	1/6 (17)	0/3 (0)	1/12 (8)	0/10 (0)
YS	2/6 (33)	0/3 (0)	0/4 (0)	0/3 (0)	0/10 (0)	0/15 (0)

A single cell suspension obtained from aorta (Ao), urogenital ridge (UG), and yolk sac (YS) tissues was co-cultured for 5 days at 33°C in contact or non-contact with UG 26.1B6, 3D4, and 3B5 stromal clones. After culture, cells were isolated (combined adherent and non-adherent fractions) and injected (0.3 ee of input cells) into irradiated adult recipients. For the non-contact cultures, 1 ee of input cells together with the SCs underneath the transwell barrier were injected. This eliminates any effect the stromal component might have on repopulation as compared to contact cultures. As a control to determine HSC input, the single cell suspension of 1 ee of each indicated tissue was directly injected into irradiated adult recipients. For the contact culture experiments $n = 4$ and for the non-contact culture experiments and direct transplantation controls $n = 3$.

In the support of midgestation (aortic and UG) HSCs, again 3D4 appears to be slightly better than 1B6. Although this is a relatively small increase in HSC activity, further comparisons of the 3D4 stromal clone with 1B6 may reveal important factors involved in HSC regulation and expansion by comparative expression profiling. However, since HSC maintenance and expansion requires cell-cell contact between the HSCs and 3D4, instead of looking for differences in the expression of soluble growth factors, the important molecules would most likely be signaling and/or adhesion molecules [24].

Surprisingly, we found that the 3B5 stromal clone, which we previously considered a non-supporter cell line (based on LTC-IC experiments performed with low density adult BM cells [15]), did provide potent support for hematopoietic progenitors and HSCs from the midgestation Ao. This difference in support reflects the importance of tissue and ontogenic compatibility between hematopoietic cells and the stromal microenvironment. While only 58% and 69% of CFU-Cs in 3D4 and 1B6 contact co-cultures are found in the adherent fraction, 94% of the CFU-Cs are adherent in 3B5 co-cultures. As it appears that contact with 3B5 is essential for the survival of CFU-Cs (none are found in the non-adherent fraction), the stromal clones most likely possess different adhesive properties for aortic cells. Thus, profiling of the molecules responsible for the subtle functional differences between these clones should provide greater insight into the optimal culture conditions for hematopoietic progenitor and HSC maintenance and expansion.

Finally, since UG cells show no HSC activity prior to contact co-culture, our results suggest that co-culture on the 3D4 stromal clone induces the generation and/or expansion of UG HSCs. Indeed, previous studies show no HSC activity in uncultured E11 UGs (even when four to five ee(s) of this tissue are transplanted) but following 3 days of explant (non-dissociated tissue) culture, large numbers of HSCs are found in the UGs [25]. It is thus far unknown if this is due to de novo HSC generation in the UGs or the expansion of a limited number of Ao-derived HSCs colonizing the UGs. We suggest that following the generation of HSCs in the Ao, HSCs could be harbored temporarily (and perhaps expanded) in the UG microenvironment between E11 and E13. Thereafter these HSCs would colonize the FL where the microenvironment is highly supportive and promotes the expansion of HSCs, as shown by *Takeuchi et al*. [26]. In these studies E11 AGM cells were co-cultured on E14.5 FL non-hematopoietic cell monolayers, and the FL microenvironment was found to amplify AGM hematopoietic progenitors and HSCs. Irrespective of whether HSCs are de novo generated in or colonize the UGs, 3B4 co-cultures can be used to identify the direct precursor cells to HSCs and the molecules and signaling cascades necessary for generation of HSCs. Future comparisons of the E11 UG (and particularly 3D4) and E14 FL stromal microenvironments should provide insight into how these early HSCs may be amplified. Taken together, these data show that direct cell-cell contact between HSCs and UG-derived stromal clones is required for ex vivo survival/maintenance and expansion of Ao and UG HSCs, and that anatomically matched embryonic HSCs and SCs are the most compatible for such supportive interactions ex vivo. Future studies will focus on the identification of tissue-specific and stage-specific molecules that through cell-cell contact facilitate the important communication and crosstalk between HSCs and SCs and may thereby provide novel tools for ex vivo clinical HSC manipulation.

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