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Ontogeny-related Changes in Proliferative Potential of Human Hematopoietic Cells

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Summary

Blood cells originate from hematopoietic stem cells that are located at different sites during ontogeny. Production of human stem cells and their progeny in culture is expected to have important implications for experimental therapeutic strategies involving gene transfer and transplantation. Here we report striking differences between primitive hematopoietic cells purified from adult bone marrow, umbilical cord blood, and fetal liver in cytokine-supplemented, serum-free cultures. In such cultures both the fraction of responding cells and their ability to produce $CD34^+$ progenitor cells decreased markedly with the age of the cell donor. These results document extensive, ontogeny-related functional differences between primitive hematopoietic cells.

The cells that circulate in the blood are derived from hematopoietic precursors that in the normal adult are located in the bone marrow (1). During mammalian ontogeny hematopoietic precursor cells are first found in the yolk sac and then in the fetal liver (2). Human umbilical cord blood is a rich source of hematopoietic precursor cells (3), which most likely represent a developmental stage intermediate between fetal liver and adult bone marrow. The proliferation of hematopoietic cells is dependent on the presence of hematopoietic cytokines (4). The recognition of the gene product of the Steel locus of the mouse as a potent synergistic growth factor for multipotent hematopoietic cells (5) has resulted in a number of studies aimed at the cultivation of primitive hematopoietic cells in vitro (6–12). In all these studies impressive proliferative effects of Steel factor on (purified) hematopoietic cells were observed, especially in combination with factors such as IL-1, IL-3, IL-6, and IL-11. The effects of Steel factor on the production of the most primitive hematopoietic cells have been less dramatic, suggesting that other growth factors or culture conditions that will allow "self-renewal" of stem cells await to be discovered (13, 14). An alternative explanation is that the most primitive hematopoietic cells in adult bone marrow are part of a large pool of quiescent (previously generated and long-lived) cells that is either maintained in size or slowly depleted throughout the life span of an individual. Such a notion is supported by a large body of experimental data in the mouse (reviewed in reference 15) that, however, has to be reconciled with data in support of extensive proliferation of individual stem cells in vivo (16, 17) and in vitro (18).

In recent studies we found that primitive human hematopoietic cells expressing CD34 (19) that were purified from adult bone marrow could sustain long-term in vitro hematopoiesis, apparently without decreasing the size of the precursor cell population (20). Although limited production of $CD34^+$ cells was partially responsible for maintaining the number of $CD34^+$ cells in these cultures (21), these results suggested that the actual production of more primitive hematopoietic cells in humans could be restricted to early stages of development (e.g., fetal life). To further study this possibility, we compared the in vitro production of $CD34^+$ colony-forming cells from $CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$ stem cell "candidates" (SCC)[1] that were purified from adult bone marrow, umbilical cord blood, and fetal liver.

Materials and Methods

Bone marrow cells were retrieved from vertebral bodies of cadaveric organ donors (20). Cord blood samples were obtained from clamped umbilical cords at the time of birth of normal full-term pregnancies. Fetal liver cells were obtained from elective, therapeutic abortions in the 10–16th wk of gestation. The use of human material in this study was approved by local Institutional Review Boards as well as the Ethical Screening Committee of the University of British Columbia. All cells were previously frozen, separated using Ficoll-Hypaque, and processed for flow cytometry cell sorting as described previously (20, 21).

Results and Discussion

$CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$ cells purified from various tissues were cultured in serum-free liquid cultures (20, 21) supplemented with IL-3 (20 ng/ml), IL-6 (10 ng/ml), Steel factor

[1] *Abbreviation used in this paper:* SCC, stem cell candidates.

Published September 1, 1993

(50 ng/ml), and erythropoietin (3 U/ml). In all cultures large increases in the total number of cells over a period of 7–12 d were observed that were repeated over a 5-wk culture period when $CD34^+$ cells were sorted from expanded cultures and used to reinitiate (sub)cultures (Fig. 1). In agreement with previous studies (20, 21), the total number of $CD34^+$ cells in cultures initiated with purified bone marrow cells remained more or less constant. In contrast, the total number of $CD34^+$ cells in cultures initiated with purified cord blood and fetal liver progenitors increased 31–250-fold and 490–3,200-fold, respectively, over the same culture period (results from three separate experiments). $CD34^+$ cells that were recovered from the cultures had a cloning efficiency in methylcellulose medium that ranged from 14 to 46%, and the production of colony-forming cells corresponded to the production of $CD34^+$ cells (Table 1). Interestingly, a clear shift towards the production of myeloid colony-forming cells was observed with time, similar to that described by others (11, 22). These results indicate that the culture conditions used do not support true self-renewal of fetal-derived stem cell "candidates" or, alternatively, that the latter were increasingly diluted by $CD34^+$ cells with a more limited proliferative potential.

The large and striking differences in culture between stem cell "candidates" purified from adult bone marrow, fetal liver, and cord blood are further illustrated in Fig. 2. In this representative experiment purified candidate stem cells were labeled with the fluorescent tracking dye PKH26 (23) and analyzed for CD34 expression and PKH26 fluorescence before and after 7 d of culture. Note that most $CD34^+$ cells from bone marrow remained brightly labeled with PKH26 (indicating no or single-cell divisions), whereas most $CD34^+$ cells from cord blood and especially those from fetal liver had decreased PKH26 fluorescence (indicative of multiple divisions) after 7 d of culture. Importantly, loss of PKH26 fluorescence of adult bone marrow cells coincided with loss of CD34 expression, whereas large numbers of $CD34^+$ $PKH26^-$ cells were recovered from cultures initiated with fetal liver or umbilical cord blood cells. These results indicate that the differences between stem cell "candidates" purified from different sources were not restricted to the fraction of responding cells, but included qualitative differences in the ability to generate $CD34^+$ daughter cells as well.

Taken together, these observations indicate large functional differences between phenotypically similar hematopoietic cell populations derived from blood-forming tissues at different stages in ontogeny. Differences in the developmental potential between purified fetal and adult hematopoietic cells were previously described for murine cells with respect to thymic maturation potential (24). An increased replating potential of human $CD34^+$ cells from umbilical cord blood compared with $CD34^+$ cells from bone marrow was reported in a recent study (22). Our findings are in agreement with those previous studies and suggest that the proliferation and differentiation potential of primitive hematopoietic cells may change extensively during ontogeny. In this respect, it will be of interest to determine if the common precursor for the hematopoietic microenvironment and hematopoietic stem cells that was recently isolated from fetal bone marrow (25) can also be found in adult tissue. In view of the ontogeny-related differences in the functional properties of purified primitive hematopoietic cells reported here, a search for the genes that are differentially expressed in such cells is indicated (26, 27). A possible link between the limited proliferative potential of adult hematopoietic cells and the length of their telomeres (28) also warrants further study.

From a practical point of view it appears of interest to pursue numerical expansion of both cord blood and fetal liver $CD34^+$ progenitor cells for potential clinical use, as has been suggested for cord blood cells by others (29, 30). The number of nucleated (Ficolled) cells that can be obtained from a fetal

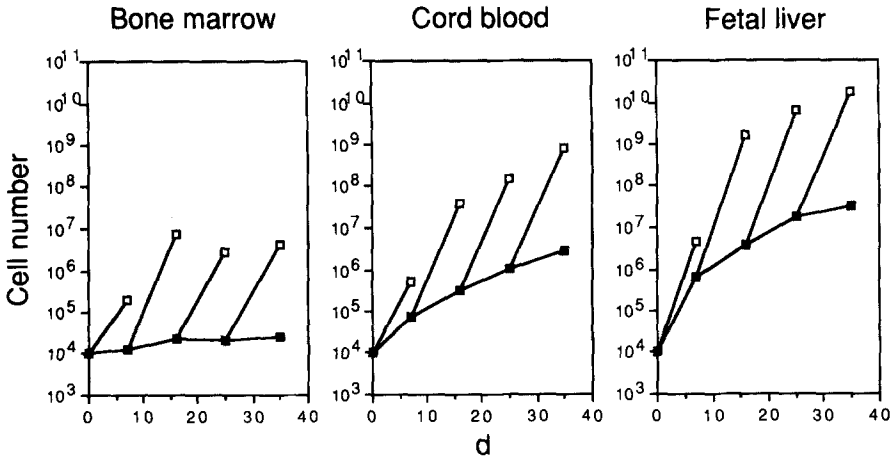

Figure 1. Ontogeny-related differences in production of $CD34^+$ cells in culture. $CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$ cells were purified from bone marrow (20), umbilical cord blood, and fetal liver, and cultured in serum-free culture medium supplemented with IL-6, IL-3, Steel factor (mast cell growth factor [MGF]), and Epo as described previously (20). Cultures (1 ml each) were initiated with 10^4 sorted cells. At the indicated time interval the total number of nucleated cells (□) and $CD34^+$ cells (■) present in the cultures was calculated from the cell counts and the percentage of viable $CD34^+$ cells measured by flow cytometry. All $CD34^+$ cells from bone marrow cultures and fractions of the $CD34^+$ cells from cord blood and fetal liver cultures were sorted and used for continuation of the cultures and measurements of clonogenic cells (Table 1). Only $CD34^+$ cells with the same (high) CD34 fluorescence as the cells sorted from primary tissues were sorted for replating. Plotted data points of a representative experiment (n = 3) were corrected for loss of cells during labeling and sorting as well as the fraction of sorted cells that was replated. In this particular experiment, $CD34^+$ cells represented 14.1, 1.7, and 10.7% of all viable low-density cells from, respectively, organ donor bone marrow, umbilical cord blood, and fetal liver (13–15 wk of gestation). The corresponding fraction of $CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$ cells among $CD34^+$ cells from these tissues was, respectively 30, 35, and 10%.

Published September 1, 1993

Table 1. *Ontogeny-related Differences in Production of Clonogenic Cells*

Cell type	Day	Cloning efficiency	CFU-c	BFU-e	CFU-mix
		%			
BM SCC	0	13	494	494	312
	7	26	2,602	575	203
	16	15	2,967	103	379
	25	14	2,604	0	196
CB SCC	0	25	1,050	550	900
	7	30	20,250	1,580	675
	16	22	69,960	0	0
	25	17	187,000	0	0
FL SCC	0	46	1,472	1,288	1,840
	7	33	187,968	6,336	16,896
	16	25	878,600	38,200	38,200
	25	17	3,128,000	0	0

SCC from bone marrow (BM), umbilical cord blood (CB), and fetal liver (FL) were purified and CD34+ cells recovered from the liquid cultures as described in the legend to Fig. 1. Sorted CD34+ cells were plated in methylcellulose medium for measurements of clonogenic cells (20). Colonies were scored 14–21 d later and the results of a representative experiment are shown. The absolute number of myeloid- (CFU-c; including CFU-G, CFU-M, and CFU-GM), erythroid- (burst forming unit-erythroid, BFU-e), and mixed myeloid-erythroid- (CFU-mix) colony-forming cells present at each time point was calculated from the fraction of CD34+ cells plated and the calculated number of CD34+ cells present in the cultures (see also legend to Fig. 1). Note the differences in clonogenic cell production between SCC from BM, CB, and FL and the shift towards production of myeloid colony-forming cells upon prolonged culture.

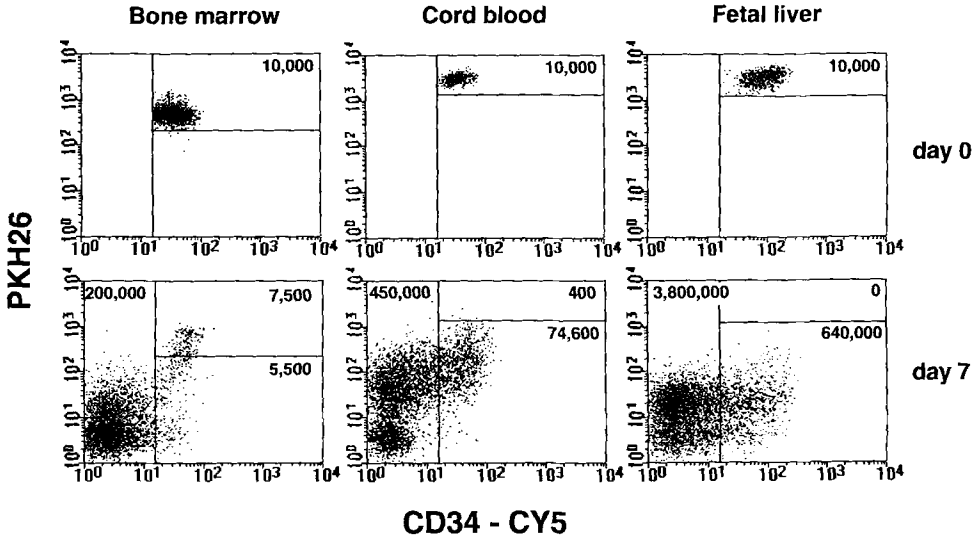

Figure 2. Ontogeny-related differences in response to a mixture of growth factors by highly purified hematopoietic cells. Fetal liver, cord blood, and adult bone marrow SCC with a CD34+ CD45RA^{lo} CD71^{lo} phenotype were purified by FACS® labeled with the membrane label PKH26 (21), and cultured in serum-free medium supplemented with IL-6, IL-3, MGF, and Epo. PKH26 is a bright fluorescent dye that is stably incorporated into the lipid bilayer of cell membranes and diluted among daughter cells upon division of the parental cells (21). In this representative experiment (n = 4), PKH26 was incorporated to different degrees in the three target cell populations resulting in differences in PKH26 fluorescence at day 0 (*top*). PKH26 fluorescence was measured again at day 7 (*bottom*) and plotted against CD34 fluorescence. Note that the majority of adult bone marrow CD34+ cells maintained their PKH26 fluorescence, indicating little turnover during the 7 d of culture and a weak response to the growth factor mixture. In contrast, practically all CD34+ cells from umbilical cord and fetal liver showed decreased levels of PKH26 fluorescence at day 7, indicating an active proliferative response that included production of CD34+ cells. Note that fetal liver CD34+ cells had a decreased PKH26 fluorescence compared with cord blood CD34+ cells, indicating a higher proliferative rate of the fetal cells. The numbers in the boxed areas represent the number of cells used to initiate the experiment (*top*) or the number of cells with the indicated phenotype that were recovered at day 7. This number was calculated from total cell counts and phenotypic analysis.

liver (8–16 wk of gestation) ranges from 10^7 to 10^8 cells, of which 1–4% are $CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$ (10^6 SSC/liver). For cord blood, minimal estimates (29) of these figures are 50–200 × 10^6 cells total, of which 0.5–1% are $CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$ (also ~10^6 SCC/cord blood sample). Upon culture for 25 d with the combination of growth factors used in this study, purified SCC from fetal liver and cord blood would be expected to yield, respectively, 10^9 and 10^8 $CD34^+$ cells, assuming that all $CD34^+$ cells could be recovered from the cultures and logistics of large scale cultures were established. These numbers are comparable to the number of $CD34^+$ cells in a typical allogeneic bone marrow graft (1–2 × 10^{10} total nucleated cells, of which 1–2% are $CD34^+$, for a total of ~2 × 10^8 $CD34^+$ cells/allograft) and are also comparable to the calculated number of $CD34^+$ cells that could possibly be obtained by culture of peripheral blood $CD34^+$ cells (11). Based on these numbers, further studies of the hematolymphoid function of the $CD34^+$ cells produced in cultures (i.e., in the SCID-hu mouse [31]) in relation to different cytokine combinations seem justified. The rapid turnover of fetal and cord blood $CD34^+$ cells in culture is likely to facilitate retroviral-mediated gene transfer (32), and this could represent another feature favoring the use of such cells. Taken together, it appears that hematopoietic cells derived from fetal tissue or umbilical cord blood may have significant advantages over hematopoietic cells derived from adults for the development of novel gene therapy and transplantation protocols for patients with a variety of currently fatal disorders. The extensive functional differences between primitive hematopoietic cells purified from tissues at various stages of development that are reported here should be considered in the planning of such clinical strategies.

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