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Effect of Age on the Frequency, Cell Cycle, and Lineage Maturation of Rhesus Monkey (*Macaca mulatta*) CD34$^+$ and Hematopoietic Progenitor Cells

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ABSTRACT

The effects of maturation and aging on hematopoietic progenitor cells, blood and bone marrow from second- and third-trimester fetal, newborn, infant, adult, and aged rhesus monkeys (*Macaca mulatta*) were analyzed. CD34$^+$ cells were immunoselected and stained with propidium iodide for cell cycle analysis. Blood and bone marrow mononuclear cells were plated in methylcellulose, and erythroid and myeloid progenitors were grown and counted. A higher frequency of circulating CD34$^+$CD38$^-$ and CD34$^+$DR$^-$ cells was observed in second-trimester fetuses compared with the other age groups. The frequency of bone marrow CD34$^+$CD38$^-$ and CD34$^+$DR$^-$ cells declined in adult and aged animals when compared with the younger age groups. Cell-cycle analysis showed 4.5% second-trimester fetal bone marrow CD34$^+$ cells entering the G_2/M phase, compared with 1.7% CD34$^+$ cells in aged animals. More than 95% of circulating CD34$^+$ cells remained quiescent for most age groups, except for second-trimester fetuses. Adult marrow myeloid progenitors were found in a lower quantity when compared with third-trimester fetuses, whereas erythroid progenitors were greatest in early-gestation fetuses and adults. The results of these studies suggest that *1*) the greatest quantity of CD34$^+$CD38$^-$ and CD34$^+$DR$^-$ cells was found in fetal and infant bone marrow, *2*) the frequency of cycling CD34$^+$ cells declines with maturation and aging, and *3*) an age-dependent difference in lineage commitment occurs. (***Pediatr Res* 58: 315–322, 2005**)

Abbreviations

BFU-E, burst-forming unit–erythroid
CBC, complete blood count
CFU, colony-forming unit
HSC, hematopoietic stem cell
MNC, mononuclear cell

Somatic cells possess a limited ability for self-renewal, as described in the study of cellular aging by Hayflick (1). This intrinsic capability or "Hayflick limit" of cell division, also described as self-renewal potential and proliferation of somatic cells, is believed to decline in chronologically older cells (2). Unlike somatic cells, CD34$^+$ hematopoietic stem cells (HSCs) are a long-lived population that can sustain blood cell production for the lifetime of an individual. A balance among self-renewal, proliferation, differentiation, and migration of pluripotent HSCs is the hallmark of hematopoiesis. For example, Andrews *et al.* (3) showed that the CD34$^+$ population, which contains HSCs, is responsible for hematopoietic reconstitution of lethally irradiated baboons, whereas others have shown that a small number of HSCs from the bone marrow of C57BL/6 mice can repopulate lymphohematopoietic tissues of irradiated animals (4,5). However, in mouse studies, a greater quantity of HSCs was required for reconstitution when donor HSCs were obtained from older *versus* younger mice. In similar studies, HSCs that were obtained from fetal liver have shown greater long-term repopulating capability when compared with HSCs from adult marrow. HSCs from DBA/2, BALB/c, and CBA/CaH-T6 mice indicate a significant functional decline with advancing age (6–11), and transplant studies suggest that murine HSCs from a single donor can be serially transplanted only to five to seven recipients (12–14). These observations suggest that HSCs may possess finite self-renewal capabilities and are subject to the Hayflick limit.

A recent stochastic study on the replication, apoptosis, and differentiation of HSCs has revealed that mouse, cat, and nonhuman primate HSCs are biologically different in their intrinsic capabilities and that findings in the mouse may not represent *in vivo* behavior of HSCs in large animals or humans (15). Other studies in baboons and cats concur with this observation (6,16–18). Studies with CD34$^+$ HSCs from large-animal models have shown that a large fraction of these cells remain quiescent during steady-state conditions, whereas more

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rapid cycling of HSCs has been observed in mice (15,19,20). Therefore, insights into the behavior of nonhuman primate HSCs and progenitor cells can contribute to a better understanding of human hematopoietic cells and their potential uses for cell- and gene-based therapies.

The goal of this study was to determine whether the quantity and the quality of $CD34^+$ and hematopoietic progenitor cells changes with maturation and aging in rhesus monkeys. The results of these investigations suggest that the greatest relative quantity of $CD34^+CD38^-$ and $CD34^+DR^-$ cells is found in fetal and infant bone marrow and that the frequency of cycling $CD34^+$ cells and differentiation toward a myeloid lineage declines with advancing age.

METHODS

Animals. All animal procedures conformed to the requirements of the Animal Welfare Act, and protocols were approved before implementation by the Institutional Animal Care and Use Committee at the University of California at Davis. A total of 25 animals were included in this study, ranging from second- and third-trimester fetuses (90, 120, and 140 d gestation; term 165 ± 10 d; $n = 9$), newborns and infants ($n = 8$), and adult and aged animals ($n = 8$; Table 1). All gravid adults ($n = 17$) that were included to obtain fetuses, newborns, and infants as noted above were examined sonographically during gestation to confirm normal fetal growth and development (21). Second- (90 d gestation) and third-trimester (120 and 140 d gestation) fetuses were delivered by hysterotomy, and newborns were delivered by cesarean-section at term then raised in the nonhuman primate nursery, using established techniques (22).

Sample collection and immunostaining. Blood samples (4–10 mL) were collected for complete blood counts (CBCs; 250 μL), flow cytometry (1 mL), immunoselection (2–5 mL), and hematopoietic progenitor assays (1 mL) by fetal cardiocentesis, from the umbilical cord at the time of delivery of newborns, or from a peripheral vessel (infants, adults, and aged animals), as previously described (23). Fetal bone marrow was collected from the long bones (humerus, femur, tibia/fibula, and radius/ulna) by flushing with heparinized RPMI at tissue harvest, using established techniques (23). Collected cells were filtered through Falcon 40-μm Nylon cell strainers (Fisher Scientific, Pittsburgh, PA). Bone marrow (2–5 mL) was aspirated under aseptic conditions from newborn, infant, adult, and aged monkeys under ketamine (10 mg/kg) and local lidocaine, as previously described (22). A total of 80 μL of whole blood and bone marrow was stained with CD34 MAb (clone 563-PE; BD PharMingen, San Diego, CA) and lineage-specific markers, including CD3 (clone SP34-2-PerCP; BD PharMingen), CD14 (clone MφP9-FITC; BD Immunocytometry Systems, San Jose, CA), CD16 (clone 3G8-FITC; BD PharMingen), CD20 (clone L27-FITC; BD Immunocytometry Systems), CD38 (clone OKT10-FITC; American Type Culture Collection, Manassas, VA), CD45 (clone Tü116; BD PharMingen), and HLA-DR (clone G46-6; BD PharMingen), for 20 min at room temperature. Red blood cells were lysed using the Coulter Q-Prep (Beckman Coulter, Fullerton, CA). A FACSCalibur flow cytometry system (Becton-Dickinson, San Jose, CA) was used for these studies. Isotype control IgG was used as a negative control (Exalpha Corp., Boston, MA). Mononuclear cells (MNCs) were gated by their forward- and side-scatter characteristics to exclude contaminating red blood cells, granulocytes, and monocytes. A minimum of 1×10^4 cells were analyzed for each sample. All $CD34^+$ cells that were analyzed were also positive for CD45, confirming hematopoietic origin (data not shown).

Immunomagnetic selection of CD34⁺ cells. $CD34^+$ cells from blood and bone marrow were selected using the mini-MACS immunomagnetic separation system (Miltenyi Biotec, Bergisch Gladbach, Germany). MNCs (1×10^7) were suspended in selection buffer (0.5% BSA and 2 mM EDTA in PBS) and incubated with anti-CD32 nonspecific blocking immunoglobulin to FcRIIA for 10 min at 4°C, followed by a 20-min incubation with CD34 MAb (clone 563; StemCell Technologies, Vancouver, BC, Canada) at 4°C. Cells were washed with 5 mL of selection buffer and centrifuged at $400 \times g$ for 5 min. The pellet was resuspended in selection buffer that contained magnetic colloids (StemCell Technologies) and incubated for 30 min at 4°C. Cells were washed and resuspended in 500 μL of selection buffer, then applied to separation columns. Separation columns were washed three times, and retained cells were eluted with 1 mL of selection buffer. A second round of separation was performed on the eluted cells. Purity of selected $CD34^+$ cells was 97.01 ± 1.32%.

Cell-cycle analysis. Cell-cycle characteristics of immunoselected $CD34^+$ cells from blood and bone marrow were determined by propidium iodide DNA staining. Cells were washed, and 4.5 mL of 70% ethanol then was added to each pellet. Cells were stored at 0–4°C for at least 2 h before staining, then centrifuged for 5 min; the ethanol was decanted; and the cell pellet was resuspended in 5 mL of PBS and centrifuged again after 1 min. The cell pellet was resuspended in propidium iodide [Sigma Chemical Co., St. Louis, MO; 0.5 mg/mL PBS with 0.1 sodium azide, (pH 7.4); 20 units/mL RNaseA; and 0.1% wt/vol Triton X-100]. Stained $CD34^+$ cells then were incubated at 37°C for 15 min, and cell fluorescence was examined by flow cytometry. A minimum of 2×10^4 cells were analyzed from each sample, and the percentage of cells within the G_0/G_1, S, and G_2/M phases was determined using the ModFit software program (Verity Software, Topsham, ME).

Hematopoietic progenitor assay. Hematopoietic progenitor assays were performed on MNCs, as previously described (22). Briefly, MNCs from blood and bone marrow were isolated with density gradient centrifugation using Histopaque (Sigma Chemical Co.). The cells were resuspended in RPMI, then washed. A total of 5×10^4 cells/plate (six plates, 3×10^5 MNC) from blood and 2×10^4 cells/plate (four plates, 8×10^4 MNC) from bone marrow were plated in 1 mL of MethoCult GF+ H4435 that contained human erythropoietin, granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, stem cell factor, IL-3, and IL-6 (StemCell Technologies) in fibroblast-free 35-mm culture plates. Erythroid and myeloid progenitor colonies were counted after a standardized 10-d incubation period (22).

Statistical analysis. Results that were obtained from experiments were reported as the mean ± SEM and calculated using Apple Macintosh systems with statistical software (StatView 5.0.1; Brainpower, Calabasas, CA). Statistical significance ($p < 0.05$) was determined by ANOVA or two-sided t test.

Table 1. *Overview of rhesus monkeys (Macaca mulatta) included in the study*

Group (N)	Age	Sex	Body weight
Fetal (9)	90 d (3)*	2 F, 1 M	94.84 ± 3.03 g
	120 d (3)*	3 M	257.72 ± 14.40 g
	140 d (3)*	1 F, 2 M	395.58 ± 12.66 g
Newborn (4)	Birth	1 F, 3 M	408.23 ± 11.06 g
Infant (4)	6 m	2 F, 2 M	1.52 ± 0.06 kg
Adult (4)	7–14 y	4 F‡	7.93 ± 0.86 kg
Aged (4)	23–26 y	4 F§	10.21 ± 0.54 kg

* Gestational age. 90 d = second trimester and 120 and 140 d = third trimester; term 165 ± 10 d. F = female; M = male; no significant differences were observed between females and males, therefore all data were grouped by age.

‡ All animals displayed normal menstrual cycles.

§ 1 of 3 with irregular menstrual cycles, and 2 of 3 were post-menopausal.

RESULTS

In this study, we measured the relative frequency of $CD34^+CD38^-$ and $CD34^+DR^-$ cells, lineage-committed precursors, and colony-forming units (CFU) in blood and bone marrow and assessed the cell cycle to investigate the effects of maturation and aging on hematopoiesis in healthy rhesus monkeys from fetal through adult life.

CBCs. CBCs all were within normal limits for each respective age group when compared with historical controls ($n = 150$; data not shown).

$CD34^+$ cells. Blood and bone marrow from second- and third-trimester fetuses, newborns, infants, adults, and aged monkeys were immunostained with CD34 MAb, and the relative frequency of $CD34^+$ cells was measured using flow cytometry (Fig. 1). A higher frequency of circulating $CD34^+$ cells was observed in second-trimester fetuses (4.99 ± 0.16%) when compared with third-trimester fetuses (2.55 ± 0.09%; 120 d; $p < 0.05$) and newborns (0.79 ± 0.08%). The quantity of $CD34^+$ cells then remained relatively stable in infants (0.98 ± 0.07%), adults (0.82 ± 0.07%), and aged animals (0.59 ± 0.05%; $p > 0.05$). In contrast to blood, a steady increase in

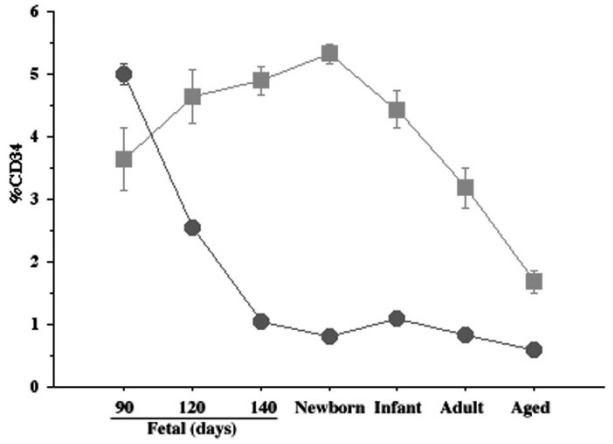

Figure 1. Frequency of $CD34^+$ cells. MNCs from blood and bone marrow were stained with CD34 IgG_1-PE, and $CD34^+$ expression was analyzed using flow cytometry. A significantly greater number of circulating $CD34^+$ cells were observed in second-trimester fetuses when compared with all other age groups ($p < 0.05$). A gradual increase in $CD34^+$ cells was seen in second- and third-trimester fetal bone marrow, followed by a decline in adult and aged bone marrow —●— Blood; —■— Marrow. ($p < 0.05$).

bone marrow $CD34^+$ cells was observed from the second trimester to birth (3.65 ± 0.49%, 90 d; 5.33 ± 0.15%, birth; $p < 0.05$) followed by a decline in infants (4.43 ± 0.29%), adults (3.19 ± 0.32%), and aged animals (1.68 ± 0.18%; $p < 0.05$). Overall, a significantly higher number ($p < 0.05$) of $CD34^+$ cells were observed in bone marrow when compared with blood.

Primitive hematopoietic progenitor cells. We also analyzed the frequency of $CD34^+CD38^-$ and $CD34^+DR^-$ cells in blood and bone marrow from second- and third-trimester fetuses, newborns, infants, adults, and aged monkeys (Fig. 2). A greater quantity of $CD34^+CD38^-$ cells was observed in fetal blood (90 and 120 d gestation; 1.64 ± 0.09%) when compared with the other age groups ($p < 0.05$; Fig. 3). In contrast to blood, the highest quantity of $CD34^+CD38^-$ cells was observed in newborn bone marrow (3.98 ± 0.02%). Approximately 1.5% of MNCs were $CD34^+CD38^-$ in adult and aged marrow. In addition, a significantly higher frequency of $CD34^+DR^-$ cells (2.94 ± 0.16%) was observed in second-trimester fetal blood (90 d gestation) when compared with $CD34^+CD38^-$ cells ($p < 0.05$; Fig. 3). The quantity of circulating $CD34^+DR^-$ and $CD34^+CD38^-$ cells was similar for the other age groups. A greater quantity of bone marrow $CD34^+DR^-$ cells was observed in fetuses (3.67 ± 0.23%) and infants (2.58 ± 0.43%) when compared with adults (1.32 ± 0.26%; $p < 0.05$). However, fewer $CD34^+DR^-$ cells were observed in aged bone marrow (0.25 ± 0.14%) when compared with adults ($p < 0.05$). Overall, the relative frequency of $CD34^+CD38^-$ and $CD34^+DR^-$ cells in bone marrow remained relatively stable in developing monkeys, then declined in adult and aged animals, whereas circulating $CD34^+CD38^-$ and $CD34^+DR^-$ cells declined during the third trimester and were stable thereafter.

Lineage markers. We assessed the proportion of $CD3^+$ (T lymphoid precursors), $CD14^+$ (monocytic precursors), and $CD20^+$ (B lymphoid precursors) cells within the $CD34^+$ immunoselected population to determine the frequency of lineage-committed hematopoietic progenitor cells (Fig. 4). A significantly lower proportion of $CD34^+CD3^+$ cells in second-trimester fetal blood were found (18.66 ± 5.10%) when compared with newborns (63.22 ± 7.61%) and adults (61.76 ± 10.48%; $p < 0.05$). However, the proportion of $CD34^+CD3^+$ cells was significantly lower in aged animals (46.40 ± 4.57%) when compared with infants (57.89 ± 4.64%; $p < 0.05$). Similarly, the proportion of circulating $CD34^+CD14^+$ cells in second-trimester fetuses (19.01 ± 1.34%) was significantly lower than that found in third-trimester fetuses (46.61 ± 6.02%) and adults (44.18 ± 10.07%; $p < 0.05$). A significant decline in the frequency of $CD34^+CD14^+$ cells was observed in aged animals (24.21 ± 5.14%; $p < 0.05$). A greater quantity of circulating $CD34^+CD20^+$ cells were found in infants (14.58 ± 2.62%) and adults (11.74 ± 0.38%) when compared with fetuses (2.84 ± 0.38%; $p < 0.05$). The proportion of $CD3^+$, $CD14^+$, and $CD20^+$ cells within the $CD34^+$ populations in

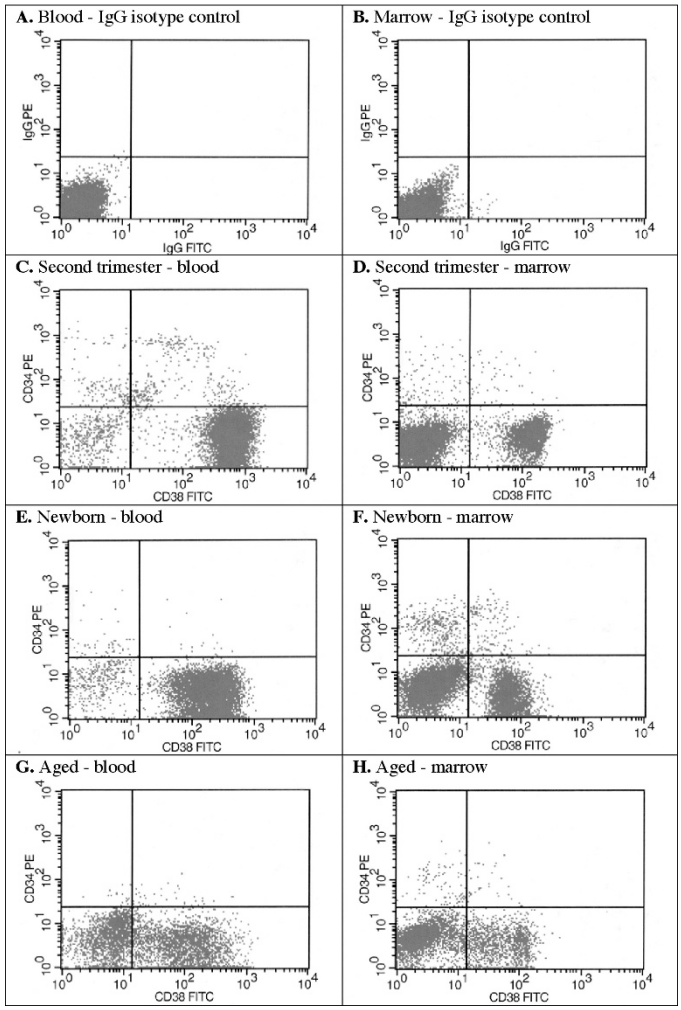

Figure 2. Flow cytometric analysis of $CD34^+CD38^-$ and $CD34^+CD38^+$ cells. MNCs from blood and bone marrow were stained with CD34-PE and CD38-FITC. A higher frequency ($p < 0.05$) of $CD34^+CD38^-$ cells was observed in second-trimester (90 d gestation) fetal blood (*C*) when compared with newborns (*E*) or aged animals (*G*). In bone marrow, a greater quantity of cells was observed in newborns (*F*) when compared with second-trimester fetuses (*D*) or aged animals (*H*). Blood (*A*) and bone marrow (*B*) were also stained with appropriate isotype controls.

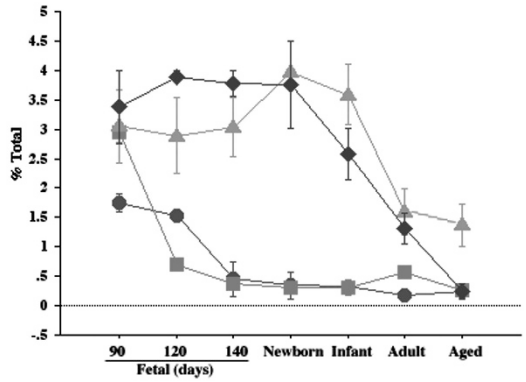

Figure 3. $CD34+CD38^-$ and $CD34^+DR^-$ cells in blood and marrow. $CD34^+CD38^-$ and $CD34^+DR^-$ cells were analyzed by flow cytometry. The frequency of $CD34^+CD38^-$ cells decreased in adult and aged bone marrow when compared with younger animals. A significantly higher quantity of $CD34^+CD38^-$ cells were observed in fetal blood at 90 and 120 d gestation when compared with the other age groups ($p < 0.05$). A higher frequency of $CD34^+DR^-$ cells were observed in fetal blood at 90 d gestation compared with older animals. The frequency of $CD34^+DR^-$ cells was also higher in bone marrow from fetuses and newborns when compared with the other age groups. —●— Blood, $CD34+CD38^-$; —■— Blood, $CD34+DR^-$; —▲— Marrow, $CD34+CD38^-$; —◆— Marrow, $CD38+DR^-$.

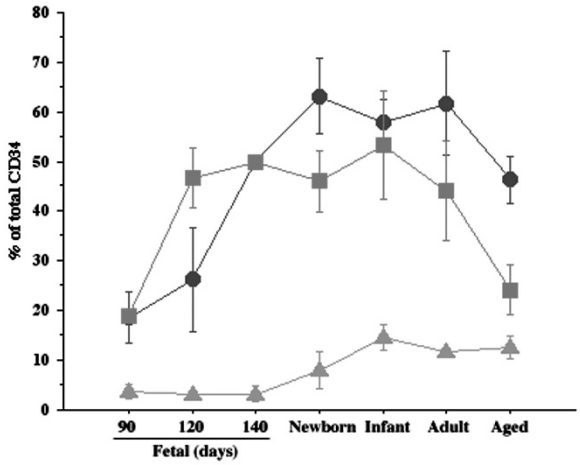

Figure 4. Lineage markers. $CD34^+CD3^+$ (T lymphoid precursors; circles), $CD34^+CD14^+$ (monocytic precursors; squares), and $CD34^+CD20^+$ (B lymphoid precursors; triangles) cells were analyzed by flow cytometry. The frequencies of $CD34^+CD3^+$ and $CD34^+CD14^+$ cells increased significantly during the third trimester ($p < 0.05$). However, a significantly lower quantity of $CD34^+CD3^+$ and $CD34^+CD14^+$ cells were observed in blood from aged animals when compared with newborns and infants ($p < 0.05$). A significantly higher number of circulating $CD34^+CD20^+$ cells were observed in infants, adults, and aged animals when compared with fetuses ($p < 0.05$).

bone marrow of adults was significantly higher when compared with younger animals ($p < 0.05$; data not shown).

Cell cycle. Immunoselected $CD34^+$ cells from blood and bone marrow were stained with propidium iodide, and DNA content was analyzed using ModFit software. Cellular debris and aggregates were excluded from the analysis by gating (Fig. 5). These studies revealed that ≥94% of circulating $CD34^+$ cells were quiescent in third-trimester fetuses (120 and 140 d gestation), newborns, infants, adult, and aged animals (Table 2). The frequency of 90-d fetal blood $CD34^+$ cells entering the G_2/M phase of the cell cycle was significantly greater than cells from newborn, infant, adult, and aged animals ($p < 0.05$). In contrast, ≥20% of bone marrow $CD34^+$ cells from most age groups were observed at the S/G_2/M phase. The quantity of marrow $CD34^+$ cells that was obtained from 90-d fetuses in the S phase (28.81 ± 3.86%) was significantly higher when compared with aged animals (14.45 ± 0.34%; $p < 0.05$). Overall, the frequency of cycling $CD34^+$ cells gradually declined with maturation and aging (Table 2).

Hematopoietic progenitors. To assess the effects of aging on the quantity of hematopoietic progenitors, MNCs from blood and bone marrow from fetal (90, 120, 140 d gestation), newborn, infant, adult, and aged rhesus monkeys were plated in complete methylcellulose medium, and hematopoietic progenitor colonies were counted after a standard incubation period (22). In second-trimester fetal blood, 12.00 ± 4.00 burst-forming unit–erythroid (BFU-E) per 3×10^5 total MNCs were observed in the peripheral circulation (Fig. 6*A*), whereas very few colonies were found in newborn, infant, adult, or aged animals. Significantly higher numbers of erythroid and myeloid progenitors were observed in bone marrow when compared with blood. Analysis of second-trimester fetal bone marrow revealed 32.67 ± 2.19 BFU-E per 8×10^4 MNCs, whereas 17.01 ± 5.01 and 7.50 ± 4.98 BFU-E were observed in 120- and 140-d fetal bone marrow, respectively. A significant increase in the frequency of erythroid progenitors was observed in aged bone marrow (39.01 ± 7.37) when compared with third-trimester fetal bone marrow ($p < 0.05$). In contrast, a greater quantity of myeloid progenitors was observed in second-trimester fetal bone marrow (199.00 ± 84.34) when compared with aged bone marrow (17.00 ± 1.53; Fig. 6*B*). In the peripheral circulation, a significantly higher quantity of myeloid progenitors was observed in second-trimester fetal blood (134.00 ± 24.58) when compared with infants (4.40 ± 1.09; $p < 0.05$). Very few colonies were observed in culture when grown from blood samples that were obtained from adult or aged animals.

DISCUSSION

We have demonstrated a decline in the frequency of bone marrow $CD34^+CD38^-$ and $CD34^+DR^-$ cells with advancing age in rhesus monkeys. The relative quantity of myeloid progenitors was also found to decrease with aging, although the relative quantity of erythroid progenitors increased in older animals. A significantly higher number of $CD34^+$ cells in the G_2/M phase were found in bone marrow when compared with the peripheral circulation in all age groups ($p = 0.001$).

The transition from fetal to adult hematopoiesis occurs in fetal liver and bone marrow, where a large and expanding pool of multipotent hematopoietic cells emerge during middle to late gestation (24,25). The migration and homing of HSCs from fetal liver to bone marrow during the second and early third trimesters of pregnancy have been proposed to be controlled by adhesion molecules, such as integrins (25,26). Studies have assessed migrating or newly resident cells in hematopoietic organs both quantitatively and qualitatively using immunophenotypic and CFU assays (27), and human fetal blood has been shown to contain high quantities of hemato-

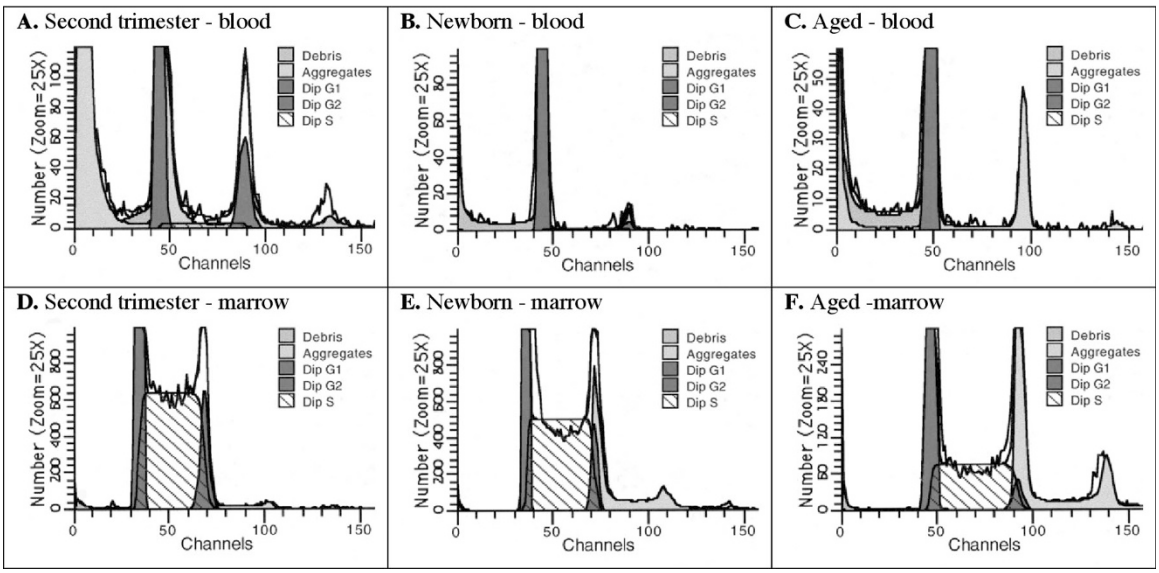

Figure 5. Cell cycle. Double-immunoselected $CD34^+$ cells from blood and bone marrow were stained with propidium iodide. DNA content was measured by flow cytometry. The frequency of cells within the G_0/G_1, S, and G_2/M phases was determined using ModFit software. Both cellular debris and aggregates were excluded from the analysis by gating. The frequency of $CD34^+$ cells entering the $S/G_2/M$ phase was higher in second-trimester fetuses (*A*) when compared with $CD34^+$ cells from newborn (*B*) or aged (*C*) animals. Fewer numbers of cycling $CD34^+$ cells were observed in newborn (*E*) or aged (*F*) bone marrow when compared with second-trimester fetuses (*D*).

Table 2. *$CD34^+$ cell cycle analysis*

Age	Sample	G_0/G_1	S	G_2/M
90 d*	Blood	89.63 ± 1.94	5.20 ± 1.10	5.17 ± 2.29
	Marrow	66.85 ± 3.91	28.81 ± 3.86	4.34 ± 0.46
120 d*	Blood	94.45 ± 2.58	1.44 ± 0.46	4.11 ± 2.13
	Marrow	73.52 ± 0.45	23.54 ± 0.45	2.95 ± 0.07
140 d*	Blood	95.22 ± 3.89	1.70 ± 1.06	3.01 ± 2.82
	Marrow	72.80 ± 2.75	23.48 ± 1.83	3.72 ± 1.49
Newborn	Blood†	97.28 ± 0.81	1.13 ± 0.39	1.44 ± 0.88
	Marrow	75.75 ± 2.19	20.16 ± 2.85	4.09 ± 0.92
Infant	Blood	96.14 ± 0.37	3.32 ± 0.39	0.54 ± 0.17
	Marrow	75.38 ± 2.10	20.68 ± 2.93	4.26 ± 0.54
Adult	Blood	95.24 ± 1.65	4.25 ± 1.85	0.51 ± 0.23
	Marrow	77.03 ± 1.76	20.02 ± 1.52	2.95 ± 0.48
Aged	Blood	99.42 ± 0.22	0.40 ± 0.11	0.18 ± 0.16
	Marrow	82.97 ± 1.22	14.45 ± 0.34‡	1.69 ± 0.34

* Gestational age.

† Umbilical cord blood was analyzed.

‡ $p < 0.05$ compared to the other age groups.

poietic progenitors (28). Data in rhesus monkeys suggest that the greatest quantity of hematopoietic progenitor and $CD34^+$ cells can be found in the peripheral circulation during fetal development, whereas a significant decline in the number of these cells occurs in the third trimester, when bone marrow becomes the major site of hematopoiesis (29). Human cord blood has been shown to contain HSCs in sufficient quantity for hematopoietic reconstitution, although the number of MNCs in cord blood is only one tenth of that observed in adult bone marrow (30–33). It has been estimated that the relative proliferative potential of MNCs from cord blood to reconstitute the hematopoietic system is 6-fold higher than cells that are obtained from adult bone marrow and, thus, provides an attractive source of hematopoietic cells for transplant purposes (33,34). However, consistent with reports in human and baboon fetuses (34,35), data in rhesus monkeys suggest that the highest frequency of $CD34^+$ cells is found in fetal blood, and these cells decline significantly toward the end of gestation.

Nonspecific T cell–like immunologic reactivity has been demonstrated in yolk sac, liver, and bone marrow of developing fetuses, although the establishment of mature T cells depends on the normal growth and development of the thymus (36,37). Cortical and medullary populations of human T cells in the thymus can be distinguished by 12 wk gestation, and these cells are found in the fetal circulation by 14 wk gestation (36). At term, T cells can proliferate in response to mitogens at levels comparable to adults (38); we have observed a similar outcome in rhesus monkeys (unpublished observations). In this study, we have shown that the quantity of $CD34^+CD3^+$ T lymphoid precursors increases during the second and third trimesters and reaches adult levels at birth. Although the ability to synthesize antibodies is acquired early in gestation, the

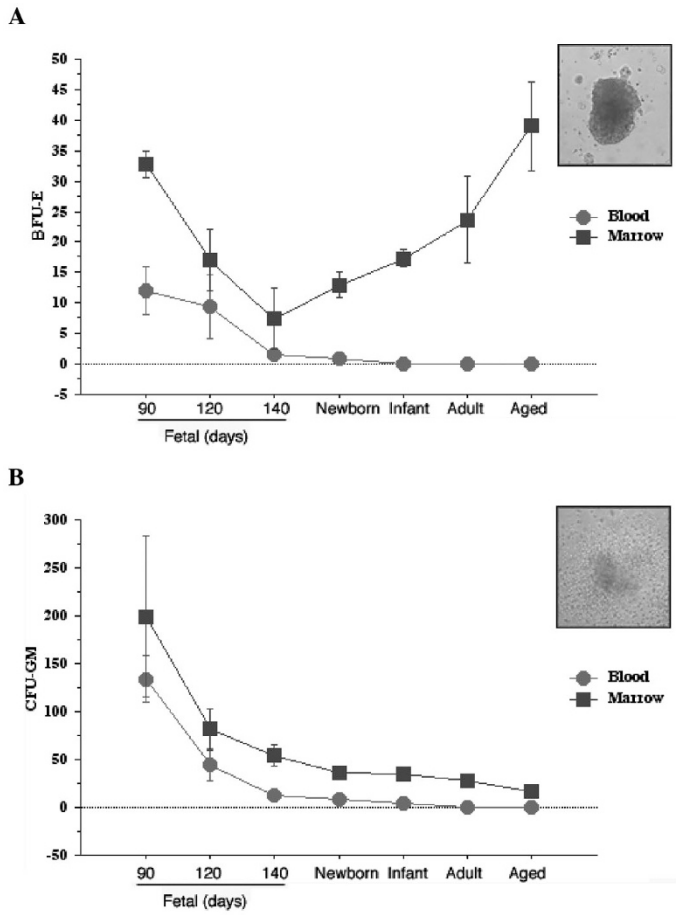

Figure 6. Hematopoietic progenitors. MNCs from blood and bone marrow from the different age groups were plated in methylcellulose medium and incubated for a standard time period (see text). The quantity of blood BFU-E decreased over time, whereas marrow erythroid progenitors decreased during gestation then increased significantly as the animals matured (*A*). In contrast, the frequency of CFU–granulocyte-macrophage (CFU-GM) decreased in both blood and bone marrow with advancing age (*B*).

frequency of $CD20^+$ B cells does not reach adult levels until 2–3 mo after birth (39,40). A delay in the production of IL-7 by bone marrow stroma, which is critical in the development of B cells, may play a role in the late onset of B lymphopoiesis (41). In contrast to lymphoid precursors, the frequency of $CD34^+CD14^+$ monocytic precursors reaches adult levels at 120 d gestation in fetal monkeys, similar to human fetuses, suggesting that innate immunity develops much earlier than humoral or cell-mediated immune responses (42).

The majority of circulating $CD34^+$ cells was noted in the G_0/G_1 phase of the cell cycle. These observations are very similar to findings in humans, in whom $<0.5\%$ of $CD34^+$ cells have been reported in the peripheral circulation and 1–4% in bone marrow (33,43–45). Our observations also agree with findings in humans, in whom cord blood $CD34^+$ cells have been shown to possess different characteristics when compared with cells that are obtained from fetal or adult bone marrow (33–35,46). During steady-state hematopoiesis, peripheral blood $CD34^+$ cells have been hypothesized to migrate between bone marrow and organs differentially expressing many functional proteins (47,48). Down-regulation of proteins related to the cell cycle has been shown in peripheral blood, whereas these proteins are abundantly expressed in bone marrow $CD34^+$ cells. $CD34^+$ cells from bone marrow have been reported to express nine cell cycle–driving genes and 11 genes that are required for DNA synthesis at distinctly higher levels than $CD34^+$ cells in the peripheral circulation (49). We also observed 30 erythroid and 35 myeloid colonies per 1×10^5 MNCs in adult bone marrow, whereas very few progenitors were observed in the peripheral circulation for all age groups. These observations suggest that blood $CD34^+$ cells are typically quiescent and may require bone marrow stroma for entrance into the cell cycle, which is necessary for hematopoietic proliferation and differentiation (50,51).

Aging of human HSCs has been characterized by the loss of telomeric DNA, which has been proposed to be closely related to the self-renewal potential and senescence of these cells (52,53). Consistent with this finding, Lansdorp *et al.* (54) reported a marked decrease in the ability to produce $CD34^+$ progenitor cells in blood, bone marrow, and fetal liver cultures with advancing donor age. In contrast to findings in humans, HSCs in mice have been reported to accumulate with age, accompanied by reduced lymphoid differentiation potential (55,56). In the rhesus monkey studies described here, the frequency of $CD34^+CD38^-$ and $CD34^+DR^-$ cells declined with advancing age. CD38 is a 46-kDa type II glycoprotein that is strongly expressed on the cell surface of lymphoid progenitor cells and also weakly expressed on mature T and B lymphocytes, monocytes, granulocytes, natural killer cells, and plasma cells (57–61). HLA-DR is also a transmembrane glycoprotein expressed primarily on antigen-presenting cells (B cells, monocytes, macrophages, and thymic epithelial cells) and activated T cells (62). Both CD38 and HLA-DR have been used to distinguish primitive hematopoietic progenitor cells from more mature cells within the $CD34^+$ population. However, $CD34^+DR^-$ cells that were obtained from mobilized peripheral blood and fetal liver have been shown to contain both erythroid and myeloid progenitor cells, whereas the $CD34^+CD38^-$ population contains only myeloid progenitor cells. These data suggest that the $CD34^+DR^-$ population may represent more primitive hematopoietic progenitor cells, although a definitive functional assay would be required to elucidate any differences (63,64). A significant decline in marrow $CD34^+DR^-$ cells in aged animals was found when compared with $CD34^+CD38^-$ cells ($p < 0.05$) and may indicate a further decline in the frequency of more primitive hematopoietic progenitor cells with advancing age.

Studies also revealed that the frequency of $CD34^+$ cells entering the cell cycle decreases with advancing age, whereas most $CD34^+$ cells in the peripheral circulation remained at the G_0/G_1 phase for all ages evaluated. These results suggest that the self-renewal capabilities of $CD34^+$ cells may diminish with aging in rhesus monkeys. An accumulation of intracellular damage leading to apoptosis and decreased efficiency in the marrow microenvironment are hypothesized to explain the age-related changes in HSCs in humans (65). Our findings also suggest that fewer myeloid progenitors are observed in older animals when compared with younger animals. Consistent with this observation, the frequency of $CD34^+CD3^+$ T lymphoid

and $CD34^+CD14^+$ monocytic precursors also decline with advancing age and is accompanied by a decrease of cells in the G_2/M phase of the cell cycle, as noted above. These findings are consistent with studies that have described the age-dependent decline in immune competence and host response to infectious and inflammatory stimuli in both humans and animals (66–68). Although the findings in this study indicate that the concentration of $CD34^+CD20^+$ B lymphoid precursors are shown to be maintained at a similar level in aged animals when compared with younger individuals, further functional assays are necessary to demonstrate the effects of aging on the immune system. However, greater quantities of erythroid progenitors were found in adult and aged animals, which may be explained by a compensatory response to the increased removal of erythrocytes observed in older individuals (69,70). This compensatory response may be crucial in maintaining a sufficient quantity of erythrocytes in the peripheral circulation with advancing age, which is more similar to findings in early-, rather than late-, gestation fetuses (34).

CONCLUSION

In summary, these studies have shown that the greatest quantity of primitive hematopoietic progenitor cells can be found in third-trimester fetal and infant rhesus monkey bone marrow and that entry of $CD34^+$ cells into the cell cycle gradually declines with aging. Similar to findings in humans and baboons (34,35,71,72), these results suggest that bone marrow $CD34^+$ cells from young rhesus monkeys retain higher self-renewal and proliferative potential than those obtained from older animals. The rhesus monkey model will be crucial for assessing differences in stem cell biology with advancing age and for exploring unique needs that may arise in the use of cell- and gene-based therapies in older compared with younger individuals.

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