Early Ontogeny of the Human Marrow From Long Bones: An Immunohistochemical Study of Hematopoiesis and Its Microenvironment

By Pierre Charbord, Manuela Tavian, Laurent Humeau, and Bruno Péalut

We examined long bones from 42 human embryos and fetuses whose gestational ages ranged from 6 to 28 weeks. Bone rudiment sections were stained using a panel of monoclonal antibodies directed against proteins expressed by hematopoietic cells, endothelial cells, smooth muscle cells, fibroblasts, and stromal cells, to describe the events preceding and accompanying the onset of hematopoiesis in the diaphyseal region. Five distinct stages were identified. Stage I (6.6 to 8.5 gestational weeks [gw]) was characterized by the presence of hematopoietic cells. At stage II (8.5-9 gw) chondrolysis was actively proceeding; numerous CD68+ cells were observed, interspersed within the marrow cavity. Stage III (9 to 10.5 gw) was characterized by the development of the vascular bed in the absence of detectable hematopoiesis. At mid-diaphyseal, specific structures that we named primary logettes were discernible; they consisted of small chambers of connective tissue, framed by a loose network of CD45-negative cells organized around an arteriole and limited from the surrounding sinus by a clearcut lining of CD34+ endothelial cells and flattened endothelial-like (although consistently CD34-negative), aligned cells limiting small capillary lumina. Stage IV (10.5-15 gw) was characterized by the onset of hematopoiesis. Hematopoietic cells were found exclusively in the primary logettes that had considerably increased in size. Logettes filled with hematopoietic cells were immersed within large and almost empty vascular sinuses. Logettes were attached by a short pedicle to connective tissue adjacent to bone/cartilage remaining formations; this tissue contained very rare hematopoietic cells. Logettes were few, usually less than 10 per long bone, and found solely in the diaphyseal area. Most hematopoietic cells found inside logettes were CD15+ myeloblasts; rarely seen were glycoprotein A+ immature erythroblasts and CD34+ nonhematopoietic cells. Hematopoietic cells within the logettes were in contact with aSM actin+ myoid cells and flattened endothelial-like (although consistently CD34-negative), aligned cells limiting small capillary lumina. Stage V (16 gw onward) was that of final organization of the long bones with areas of fully calcified bone and areas of dense hematopoiesis where logettes were no longer visible. This study shows three major features of incipient long bone hematopoiesis: 1) absence of CD34+ hematopoietic precursors before the onset of hematopoiesis; 2) extreme rarity of those in the emerging blood-forming marrow; 2) predominance of granulopoiesis, and 3) exclusive development in specific structures organized by vascular cells. This study also suggests that CD68+ cells are instrumental in the chondrolysis process while vascular cells (endothelial and myoid cells) may be the critical microenvironment at the onset of hematopoiesis.

Hematopoiesis in mammals occurs in strictly defined sites. In normal human adults hematopoietic stem cells, progenitors, and precursors for all lineages but T lymphocytes are found exclusively in the marrow of flat bones and vertebrae. Under pathologic conditions only, a substantial number of progenitors and possibly stem cells are found in peripheral blood, probably migrating from bone marrow under conditions of "stress," after chemotherapy or infusion of cytokines; also, abnormal hematopoiesis may develop in long bones, spleen, and liver, in particular in myeloproliferative disorders and in chronic anemias of diverse origins.

Studies on the differentiation pathways taken by the progeny of colony-forming units in spleen (CFU-s) after infusion into lethally irradiated mice led Wolf and Trentin to hypothesize the existence of hematopoietic-inductive microenvironments. Specifically, macrophage-like cells were associated with the generation of erythroblasts, while stellate cells were related to that of granulocytes. The generation of two-dimensional in vitro systems, the long-term marrow cultures, considerably reinforced the hypothesis of microenvironments inductive of hematopoiesis, since in these systems the maintenance of stem cells and progenitors is related to the presence of microenvironmental cells. Microenvironmental cells may be defined by phenotype as macrophages and as nonhematopoietic stromal cells. In humans the identity of the latter remains debated. Because of their property to synthesize numerous glycoproteins of the extracellular matrix, they may be defined as connective tissue-forming cells. Such cells may remain in a relatively undifferentiated state as poorly characterized "mesenchymal cells," i.e., vimentin-positive cells with more or less specific markers for "fibroblasts" such as intracytoplasmic proly-5-hydroxylase and membrane expression of glycoproteins recognized by the antibody 1B10. On the contrary, more differentiated stromal cells may be easily recognized as cells with endothelial or osteoblastic features, as adipocytes or as myofi-broblasts similar to immature vascular smooth muscle cells. An issue more unanimously resolved by the studies of long-term marrow cultures concerns the regulation of hematopoiesis by microenvironmental cells, indicating a tight paracrine control, where stromal cells and hematopoietic cells are in close contact.

Although these in vitro studies are informative, there is a need for their extension in vivo. Study of trephine marrow biopsies provides a static picture since adults are in steady phase...
state conditions when normal, or in slowly evolving conditions when diseased. Studies performed in cases where hematopoiesis is rapidly expanding would be more informative. For example, a study of the marrow architecture of mice with stress hematopoiesis enabled Weiss and Geduldik to define "barrier cells" as a significant microenvironmetal cell population. Barrier cells, restricted in number and in location in normal mice, were increased and strongly represented at the vicinity of sinuses and along bone trabeculae in animals treated with interleukin-1.

We reasoned that the early developing fetal marrow in long bones might prove to be an appropriate model for the study of microenviroment to hematopoietic cell relationships, since (1) in this situation hematopoiesis is a highly dynamic process, in which stem cell recruitment, expansion, and differentiation may be much more active than at the adult stage; and (2) the early settlement of blood cells in the bone cavities proceeds in an environment that is much easier to describe than that of the blood cell-packed adult marrow.

Bone marrow is the last blood-forming tissue that develops in ontogenesis, when hematopoiesis is already extinct in the yolk sac, transiently proceeding in the liver and actively developing in the thymus. The early ontogeny of the bone marrow cavity has been described in a few histologic studies in the animal and human embryo and fetus. In the process of endochondral ossification, surrounding mesenchymal cells and blood vessels invade the cartilaginous bone rudiments leading to the formation of ossifying trabeculae separated by vascular spaces and loose mesenchymal areas. The analysis of blood cell development in avian embryo rudiments leading to the formation of ossifying trabeculae proceeded in an environment that is much easier to describe than that of the blood cell-packed adult marrow.

Bone marrow is the last blood-forming tissue that develops in ontogenesis, when hematopoiesis is already extinct in the yolk sac, transiently proceeding in the liver and actively developing in the thymus. The early ontogeny of the bone marrow cavity has been described in a few histologic studies in the animal and human embryo and fetus. In the process of endochondral ossification, surrounding mesenchymal cells and blood vessels invade the cartilaginous bone rudiments leading to the formation of ossifying trabeculae separated by vascular spaces and loose mesenchymal areas. The analysis of blood cell development in avian embryo rudiments leading to the formation of ossifying trabeculae proceeded in an environment that is much easier to describe than that of the blood cell-packed adult marrow.

Bone marrow is the last blood-forming tissue that develops in ontogenesis, when hematopoiesis is already extinct in the yolk sac, transiently proceeding in the liver and actively developing in the thymus. The early ontogeny of the bone marrow cavity has been described in a few histologic studies in the animal and human embryo and fetus. In the process of endochondral ossification, surrounding mesenchymal cells and blood vessels invade the cartilaginous bone rudiments leading to the formation of ossifying trabeculae separated by vascular spaces and loose mesenchymal areas. The analysis of blood cell development in avian embryo rudiments leading to the formation of ossifying trabeculae proceeded in an environment that is much easier to describe than that of the blood cell-packed adult marrow.

RESULTS

The development of the human long bone marrow has been divided into five distinct stages. Stage I (6 to 8.5 gestational week [gw]) is that of the entirely cartilaginous rudiment; at stage II (8.5 to 9.0 gw), chondrolysis is actively proceeding and perichondral vascular, osteoblast, and osteoclast precursors invade the forming marrow cavity; stage III (9.0 to 10.5 gw) is characterized by the development of the vascular bed between the ossifying trabeculae, in the absence of discernible hematopoiesis; stage IV (10.5 to 15 gw) is characterized by the development of intrinsic medullary hematopoiesis; and stage V (16 gw onwards) is the transition to the final organization of the long bones. The number of specimens studied is indicated in Table 1. Specimens included proximal (femur and humerus) and distal (tibia, fibula, radius, and ulna) bones.

Table 1. Long Bone Rudiments Analyzed

<table>
<thead>
<tr>
<th>Stage</th>
<th>Developmental (postfertilization)</th>
<th>Total Specimens</th>
<th>Proximal Bones*</th>
<th>Distal Bones</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>6-8.5</td>
<td>8</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>II</td>
<td>8.5-9</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>III</td>
<td>9-10.5</td>
<td>5</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>IV</td>
<td>10.5-15</td>
<td>21</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>V</td>
<td>16-onward</td>
<td>6†</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>46</td>
<td>28</td>
<td>17</td>
</tr>
</tbody>
</table>

* Femur or humerus.
† Tibia, fibula, radius, or ulna.

Antibodies. Monoclonal antibodies to CD31 (IC/70A), CD68 (KP-1), smooth muscle-specific α actin (oSM actin) (oSM-1, clone 1A4), glycoporphin A (JC159) and von Willebrand factor (F8/86), were purchased from Dako (Glostrup, Denmark); those to CD34 (HPCA-1) and CD45 (Hle-1) from Becton-Dickinson (San Jose, CA); the one to CD15 (80H5) from Immunotech (Marcelle, France); and the one to fibroblast-associated antigens (1B10) from Sigma (St Louis, MO). An antibody, Me20-4, to the low-affinity nerve growth factor receptor (NGFR) was purchased from Amersham (Little Chalfont, UK). The Stro-1 hybridoma was obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA).

Tissue processing and staining. Tissues were immersed overnight in Bouin's fixative, then included in paraffin. Five micrometer-thick sections were deparaffinized, dehydrated, and endogenous peroxidases were inhibited for 20 minutes in methanol containing 0.2% (vol/vol) hydrogen peroxide. Sections were then washed with phosphate-buffered saline (PBS) with 0.25% (vol/vol) Triton X-100 and nonspecific staining was blocked with nonimmune goat serum. The primary antibody was added overnight at 4°C. After washing with PBS-Triton X-100, incubation was carried out for 1 hour at room temperature with, first, biotinylated goat antimouse Ig antibody (Dako) and subsequently with peroxidase-labeled streptavidin (Dako). Peroxidase activity was revealed with 0.025% (vol/vol) 3,3'-diaminobenzidine (Sigma) in PBS containing 0.015% (vol/vol) hydrogen peroxide. Slides were counterstained with Harris' hematoxylin and mounted in aqueous medium (BioGenex Laboratories, Dublin, CA) for examination and photography on an Optiphot-2 microscope (Nikon, New York, NY).
Marrow Ontogenesis

Uncolonized, entirely cartilaginous long bone rudiments were analyzed on eight different samples ranging from 6 to 8.5 weeks of gestation. The epi-, meta-, and diaphyseal regions were already patterned, with chondrocytes being dilated in the two latter areas. Numerous blood vessels and capillaries were seen dispersed in the perichondral limb mesenchyme. They could be easily identified since all endothelial cells were brightly stained for CD34 (Fig 1A), whereas pericytes consistently expressed αSM actin (not shown). In the immediate vicinity of the cartilaginous diaphyseal shaft, capillaries were more numerous and arranged parallel to the bone rudiment (Fig 1B). In the same location, CD68⁺ cells were also concentrated, whereas they were sparsely scattered in the rest of the limb mesenchyme (Fig 1C). Since, according to the Fourth Workshop on Leucocyte Differentiation Antigens, CD68 is represented mainly on cells from the monocyte-macrophage lineage, we assume that the CD68⁺ cells found at this stage belonged to this lineage.

Stage II: Appearance of bone marrow cavities (week 8.5-9). As observed previously by Streeter,²⁵ the chondrolysis/cell invasion process that marks the appearance of bone marrow spaces proceeds very rapidly. In three samples studied at 8.5 and 8.8 weeks of gestation, cartilage was degraded in most of the dia- and metaphyseal regions, with only minor territories still constituted of solid cartilage (Fig 2A). The loose tissue resulting from the chondrolysis process con-
tained newly immigrated cells. Osteoblasts closely surrounded the cartilage islets, to which numerous CD68+ cells, some of them multinucleated, were also adherent, suggesting their osteoclastic nature (Fig 2B). No other hematopoietic cells could be identified; in particular, although rudimentary vascular structures were already detected, no round CD34+ cells were present to suggest colonization by hematopoietic precursor cells (not shown).

Stage III: Development of the vascular bed (week 9-10.5).

In the space left vacant by the receding cartilage a loose connective tissue developed within which angiogenesis proceeded, as observed in five samples.

From week 9 to week 16 there were noticeable differences in development between the diaphysis and metaphysis. In the former, cartilage regression, angiogenesis, and hematopoiesis proceeded quickly in well-defined sequential steps. On the contrary, cartilage regression was slow in the metaphysis and the timing for angiogenesis and hematopoiesis was less clearcut. We shall therefore focus on the development of angiogenesis and hematopoiesis in the diaphyseal region.

The most striking feature of vascular development in this region was the formation of primary logettes. These structures, as observed by week 9, are shown in Fig 3. At the center of the logette is an arteiole comprising an intima made of CD34+ endothelial cells (Fig 3A and B) and a media made of αSM actin+ smooth muscle cells (Fig 3C). The logette is limited from the sinus by a lining of CD34+ endothelial cells (Fig 3B), some of which display, on their abluminal side, αSM actin+ myoid cells (Fig 3C). The arteiole is surrounded by a loose connective tissue made of cells that do not express CD34 (Fig 3B), αSM actin (Fig 3C), CD45 (Fig 3D), 1B10, Stro-1, and NGFR (not shown) and are located within a fine fibrillar network. It must be emphasized that we did not observe any round CD34+ cells at that stage either; the only CD34+ cells detected were endothelial cells from arteries and sinus linings.

From week 9 to 10 arterioles increased in size, becoming true arteries; logettes and sinuses also enlarged. A qualitative change occurred by week 10.5 when extravascular hematopoietic CD15+ cells were first observed.

Stage IV: Development of intrinsic hematopoiesis (week 10.5-15).

This stage has been studied in 21 specimens. Typical week 11 to 12 logettes are shown in Fig 4. These logettes are elongated parallel to the bone long axis. They are limited by large vascular sinuses except at one point where they attach by a short pedicle to the tissue adjacent to a bone/cartilage formation (Fig 4A). The center of the logette is occupied by an artery with its intima of CD34+ endothelial cells (Fig 4D) and its media of αSM actin+ smooth muscle cells (Fig 4A). The outer border of the logette is made of CD34+ endothelial cells (Fig 4D) with more or less numerous abluminal αSM actin+ myoid cells (Fig 4A, B, and C). A few αSM actin+ myoid cells are also found within the logettes, with a bulky body and a tapered, long winding tail (Fig 4B).

Cell density in the logettes is much higher at this stage (Fig 4A) than at the previous one. The morphology and consistent CD45 expression (Fig 5A) of the numerous round cells present clearly indicate their hematopoietic origin. The abundance of hematopoietic cells in the logette is in striking contrast with the low frequency of these in the tissue adjacent to the bone/cartilage formation (Fig 4A). Moreover, there are few hematopoietic cells in the vascular sinuses (Figs 4A and 5A). Careful examination of the logette also reveals a
Fig 5. Development of intrinsic hemopoiesis; CD45\(^+\), and CD34\(^-\) cells within the primary logettes. (A) Diaphyseal region of a 11.5 gw humerus. Staining with Hle-1. The logette (indicated by a large arrowhead) is visible on the upper part of the photograph. It is filled with many CD45\(^+\) round cells located within the pedicle (left) or close to the central arteriole (right). Within the large surrounding sinus only one CD45\(^+\) cell is observed on the luminal side of the endothelial lining that limits the loose connective-tissue adjacent to the bone. Three CD45\(^+\) cells surround the bone/cartilage formation, two of these are multinucleated. These cells, which are similar to CDW cells (vide Fig 68) in terms of morphology and location, probably correspond to osteoclasts. (Original magnification × 90.) (B) Diaphyseal region of a 11 gw femur. Staining with HPCA-1. Within the logette (centered by a capillary comprising CD34\(^+\) endothelial cells), there are numerous CD34\(^-\) hematopoietic cells (most of these clearly belong to the granulocytic lineage). Only one CD34\(^-\) round cell (arrow) is seen. This cell with high nuclear to cytoplasmic ratio is smaller than most of the other CD34\(^-\) round cells. Morphology and expression of CD34 allow to define this cell as an hematopoietic precursor. (Original magnification × 250.)

Fig 6. Few flattened cells among the round ones (Fig 4B, C, and D). These do not express CD45 (not shown), CD34 (Fig 4D), or αSM actin (Fig 4B and C). These flattened cells are often aligned (Fig 4B, C, and D), delineate more or less clearly capillary lumina that frequently contain hematopoietic cells (Fig 4C) and are sometimes in contact with αSM actin\(^+\) myoid cells (Fig 4B). Sometimes aspects of capillaries streaming into the sinus are observed (not shown). These observations strongly suggest that these flattened cells, although CD34\(^-\), are endothelial.
Primary logettes were found at week 11 to 12 in the diaphysis only. Their number was usually less than 10 per long bone and they were the only sites where hematopoietic cells were observed. These cells expressed CD45 (Fig 5A) and included morphologically identifiable erythroblasts and polymorphonuclear granulocytes. Blood cells within logettes were further characterized by their expression of lineage markers (Fig 6). Most of them were CD15+ cells (Fig 6A); the cell morphology and the intensity of staining led us to consider these as belonging to the granulocytic lineage.31 There were also a few glycophorin A+ erythroblasts (Fig 6C and D); within the logette erythroblasts were at the earliest El and E2 differentiation stages (Fig 6D); more mature erythroblasts, corresponding to the E3 and E4 stages, were seen only within the sinuses where they often lined endothelial cells (Fig 6D). Occasionally CD68+ monocytic cells were present in the logettes (Fig 6B). Megakaryocytes as recognized by morphology and intensity of CD31 staining31 were even rarer (not shown). Stage IV was also the first stage at which nonendothelial, presumably hematopoietic CD34+ cells were observed (Fig 5B). However, these cells were present at such a low frequency that they could not be observed in most samples tested. In three specimens entirely devoted to CD34 staining, a few small and round CD34+ cells evoking hematopoietic precursors were seen inside logettes, among other hematopoietic cells in the space between the central arteriole and the sinusuline lining (Fig 5B).

Hematopoiesis developed within progressively enlarged logettes until week 15. A 15-week fully developed logette is shown in Fig 7. The fibula is penetrated on its equatorial plane by an artery (Fig 7A). This artery streams into a central artery that follows the long axis of the bone (Fig 7A). The logette is organized around the arterial shaft, and is surrounded by a large and convoluted sinus (Fig 7A). Hematopoietic cells are found almost exclusively within that logette; they include diffuse CD15+ cells (Fig 7B) and clustered glycophorin A+ erythroblasts (Fig 7C). Almost no hematopoietic cells are found within the large sinus surrounding the logette (Fig 7A, B, and C).

At this stage, as in the preceding one, we did not notice differences in the development of logettes according to proximal or distal bones (Table 1).

Stage V: The final organization of the long bones (week 16 onward). This stage has been studied in nine specimens. From week 16 onward, bone increasingly replaced the hematopoietic logettes in the outer region of the diaphysis. Bone trabeculae left space at regular intervals for more or less extended cavities of loose connective tissue containing CD34+ capillaries but without remaining hematopoietic cells, except some CD68+ mononucleated and multinucleated cells (probably macrophages and osteoclasts).

Hematopoietic cells at this stage were found in the inner region of the diaphysis, at the diaphysis to metaphysis junction, and in the metaphysis itself. Hematopoietic cell expansion was such that logettes, as described previously, were no longer detected. Areas packed with hematopoietic cells contained numerous arteries and arterioles, which no longer appeared as organizing centers. Large sinuses were still visible. Of note was the presence within the hematopoietic cord of numerous aSM actin+ myoid cells, more or less clearly connected to vascular structures.

By week 16 cartilage trabeculae, bordered by numerous CD68+ osteoclasts, were still visible in the metaphysis. These trabeculae delineated large parallel sinuses.

DISCUSSION

This study performed on 46 human specimens from 6 to 28 weeks of gestation indicated that hematopoiesis in fetal long bones is strictly time dependent. It begins by week 10.5 and remains confined to the diaphyseal region until week 15. The onset of intrinsic hematopoiesis is preceded by two stages: that of chondrolysis from week 8.5 to week 9 and that of vascular bed development from week 9 to week 10.5. In the outer region of the diaphyseal area, from week 16 onward, hematopoiesis progressively disappears being replaced by calcified trabecular modeling adult-type bone.

In long bones, and in particular in the diaphyseal area where development was easier to follow because of its high pace within a limited time frame, hematopoiesis presented some specific features: (1) CD34+ hematopoietic precursors were extremely rare, if present at all, in all samples tested; (2) granulopoiesis was the major lineage represented, largely exceeding erythropoiesis; and (3) granulopoiesis developed in distinct solid structures that we called primary logettes, the large vascular sinuses surrounding these logettes being almost devoid of hematopoietic cells. The little release of hematopoietic cells into the sinuses is in agreement with what has been recently described when human hematopoietic stem cells were injected in utero into the sheep fetus.32 Although human cells homed into the marrow cavities, circulating donor cells were few before birth, suggesting inefficient hematopoiesis.

The primary logettes were few, usually less than 10 per long bone. They were clearly distinct from the bone/cartilage islands, and attached by these to a short pedicle. They ap-
peared therefore immersed into large, interconnected sinuses. They were outlined by cells from the vasculature, being centered by an arteriole/artery and limited from the surrounding sinus by an endothelial lining flanked by abluminal myoid cells. Logettes appeared as early as week 9, but were devoid of hematopoietic cells up to week 10.5. With the development of hematopoiesis, logettes increased in size and merged, giving rise by week 15 to few, if not a single, elongated logette(s) that extended throughout the diaphyseal region.

The prevalent cell population expressing CD34 that we observed was, by far, that of elongated endothelial cells from arterioles and arteries and from sinus linings. Endothelial cells from capillaries were not always CD34+. In particular, in logettes, most flattened cells linearly ordered and delineating more or less clearly a capillary lumen were CD34-. CD34 is a well-known marker of endothelial cells in humans35-36 and a recent report has shown that murine embryonic endothelial cells also express this antigen.37 The heterogeneous expression of CD34 from one tissue to another,34,38 or even within a tissue,35 has already been described but is not explained yet. We found variations in expression for other endothelial cell markers; von Willebrand factor was detected almost exclusively in endothelial cells lining sinuses and CD31 was rarely expressed and, when so, almost solely by endothelial cells from arteries.

The scarcity of detectable CD34+ round hematopoietic precursors at the key stages of marrow development was an unexpected result, which raises several questions. First of all, there were no technical reasons for this. CD34+ round cells have been easily detected by us in the yolk sac, fetal liver, and close to the ventral wall of the aorta from 5-week embryos.39 In the latter case we could distinguish between CD34/45 double-positive round hematopoietic precursors bulging into the vessel lumen and CD34+Europaeus mans33-36.

Fig 7. Development of intrinsic hematopoiesis; a fully developed, late-stage logette (fibula from a 15 gw sample). (A) Staining with aSM-1. On this section, cut at mid-diaphysis along the central longitudinal plane of the bone, smooth muscle aSM actin+ cells underscore the arterial network. A perforating artery enters the bone at mid-diaphysis. This artery streams into a central artery parallel to the bone axis. The central artery is very long, extending from the diaphysis toward the metaphysis. The primary logette organizes along the arterial network. The pedicle is large, corresponding to the perforating artery. The primary logette forms a more or less regular sheath surrounding the central artery. Notice the presence of numerous myoid aSM actin+ abluminal cells underlining the logette/sinus border. (Original magnification ×125.) (B) Staining with 80H5. This section is cut along a longitudinal plane more peripheral as compared with that used in A. There is therefore a bony spicule separating the central cavity into two regions. There are many round CD15+ cells within the logette. On the contrary, the large sinus surrounding the logette is devoid of CD15+ cells. (Original magnification ×135.) (C) Staining with JC 159. The plane of section is slightly more peripheral as compared with that used in A. One can observe therefore part of the perforating artery (bottom), but the central artery is not visible. One can notice numerous glycophorin A+ red cells within the sinus. On the contrary, most of the round cells within the hematopoietic cord are glycophorin A-. However, there are three foci of glycophorin A+ erythroblasts within the cord. (Original magnification ×125.)
they have seeded the primary logettes, and/or by marrow colonization by already committed, CD34+ and lineage+ (mostly CD15+) cells. On the contrary, in the hypothesis of intrinsic stem cell development, one may assume that CD34+ endothelial cells differentiate into hematopoietic precursors. This event would be reminiscent of what probably occurs in the yolk sac where hemangioblasts would give rise to hematopoietic cells within the hematopoietic island and to endothelial cells limiting the island.65 In any case, the observations made did not support our original assumption that the early developing human bone marrow might be a privileged site for primitive stem cell expansion.

This study provides some clues on the nature of the microenvironmental cells that may be instrumental for the early development of hematopoiesis.

Observations on 6- to 9-week long bones strongly suggest that CD45/CD68 double-positive cells from the macrophage lineage (from monocytes to osteoclasts) are involved in chondrolysis, a prerequisite for the development of the vascular bed. We confirmed the observation of Streeter65 concerning the rapidity of the chondrolysis process since the appearance of marrow cavities took place within a few days only (from week 8.5 to 9) and therefore could be observed in no more than three samples. The role of macrophages in this process was strongly suggested by the numerous CD68+ cells found within the forming cavities in apposition to cartilage rudiments, a location where large multinucleated osteoclasts were observed at later stages throughout the study. On the contrary, immunostaining of 11- to 15-week long bones did not provide obvious arguments that monocytes may facilitate the development of in situ hematopoiesis, since CD68+ ovoid cells were observed very rarely in primary logettes, in contrast to CD15+ granulocytes and, to a lesser extent, glycophorin A+ erythroblasts.

Marrow vascular cells are probably essential for the development of local hematopoiesis since (1) they delineated the vascular bed of primary logettes 2 weeks before inception of hematopoiesis; (2) they increased in number when hematopoiesis occurred, and both the central artery and sinus/logette border considerably enlarged; (3) they presented contacts with hematopoietic cells developing within the logette; and (4) virtually no nucleated hematopoietic cells were observed within the large sinuses surrounding the logettes. Vascular cells were of three kinds, including CD34+ endothelial cells from arteries and sinus/logette borders, αSM actin+ smooth muscle cells from artery media, and myoid cells located on the abluminal side of the endothelial cell lining and within the logette cord, and finally flattened cells delineating more or less clearly capillary lumina and sometimes streaming into the surrounding logettes. Although CD34+, these latter cells were probably endothelial. Myoid αSM actin+ cells were more frequent than in marrow from adults57; this observation made already by Schmitt-Graff et al on fetal marrows of at least 15gw2 was confirmed in this study of earlier stages.

Besides vascular cells what microenvironmental cell type might be involved in long bone fetal hematopoiesis? Two populations may be readily discarded: adipocytes and osteoblasts.

Adipocytes were never observed in our study, not even after week 15 when hematopoiesis expanded to the extent that it wiped out the logette architecture. At that stage and in that location, the appearance of the bone marrow was close to what is observed in trephine biopsies from adults with myeloproliferative disorders.45 As early as 1882, Ernst Neumann noticed that adipocytes did not develop before birth and several reports since have confirmed this observation.46 However, Emery and Follett46 showed that adipocytes could be found in the toes from most full-term infants, suggesting that these cells appear before birth, although lately in gestation. The lack of fat cells throughout most of the fetal life is not well understood. An in vitro study on long-term liver cultures has shown that it was not due to the lack of steroid receptors on stromal cells.46

Our study also suggests that osteoblasts are likely not involved in the development of hematopoiesis since very few hematopoietic cells were found in tissues adjacent to bone/cartilage structures in 10.5- to 15-week long bones. This was in striking contrast with the density of hematopoietic cells within the primary logettes.

The last microenvironmental cell subset that deserves more attention is that of the poorly defined "fibroblasts." We noticed in early nonhematopoietic primary logettes (from 9 to 10.5 gw) a few cells located in the vacant space between the central arteriole and the sinus border. These cells were CD45- and CD34-, unlike some human and murine embryonic and fetal extramedullary fibroblasts.34,36,47 They were also different from myoid cells, lacking the winding tail suggestive of a contractile function and being αSM actin-. They were enmeshed within a fine fibrillar network, whose composition has not been studied in this work. We tried to characterize these interstitial cells with antibodies specific for membrane antigens of (myo)fibroblasts (1B10)46 or stromal cells (Stro-1)58 and for the low-affinity nerve growth factor-receptor, NGFR.59 1B10 and Stro-1 did not give specific staining. The anti-NGFR antibody labeled, by week 11 to 12, mostly fibrillar structures and a few cells in the arterial adventitia; these were sometimes observed within the primary logettes; more frequently they were found in the vicinity of the remaining bone/cartilage areas where few (if any) hematopoietic cells could be visualized. It appears to us therefore unlikely that NGFR+ cells would constitute a major microenvironmental cell population as suggested by Cattonetti et al.50

In conclusion, we must emphasize the peculiarities of hematopoiesis observed in fetal long bones, in particular the predominance of granulopoiesis from week 10 to 15 in the diaphyseal area. Microenvironmental cells that may seem operative at that stage, such as vascular cells, may be less critical in the different context of multilineal and permanent hematopoiesis from flat bones or vertebrae. Nevertheless, the previous work of Chen and Weiss20 on fetal hematopoiesis in vertebrae allows us to underscore some similarities in blood cell development from both locations. These authors indicated that (1) mononuclear cells (probably macrophages) were present mainly in the early stage when chondrolysis occurred and hematopoiesis was not yet established; (2) a dilated central vascular sinus was present.
before the onset of hematopoiesis, which was later divided into branched and interconnected channels; (3) hematopoietic cells, as they began to appear, concentrated in the vicinity of an arteriole; on the contrary, most of the tissue immediately adjacent to the cartilage and bone was devoid of hematopoietic cells; and (4) at no time from 13.2 gw to 17.3 gw were adipocytes detected. The role played by vascular cells in the development of hematopoiesis in vertebræ appeared also therefore to be essential, although no clear indications were given as to the existence of primary logettes.

ACKNOWLEDGMENT

We are indebted to Drs Philippe Blot, Jean-François Oury, François Narcy, Nicole Mulliez, and Jean-Yves Couturier for providing the fetal samples.

REFERENCES

3. Ward HP, Block MH: The natural history of agnogenic myeloid metaplasia (AMM) and a critical evaluation of its relationship with the myeloproliferative syndrome. Medicine 30:357, 1971
22. Henry Holt, 1952
36. Greaves MF, Brown J, Molgaard HV, Spurr NK, Robertson
D, Delia D, Sutherland R: Molecular features of CD34: A hematopoietic progenitor cell-associated molecule. Leukemia 6:31, 1992 (suppl 1)


47. Brown J, Greaves MF, Molgaard HV: The gene encoding the stem cell antigen, CD34, is conserved in mouse and expressed in hematopoietic progenitor cell lines, brain, and embryonic fibroblasts. Int Immunol 3:175, 1991


Early ontogeny of the human marrow from long bones: an immunohistochemical study of hematopoiesis and its microenvironment [see comments]

P Charbord, M Tavian, L Humeau and B Peault