The Development of Vertebral Bone Marrow of Human Fetuses

By Li-Tsun Chen and Leon Weiss

The development of the bone marrow of the thoracic vertebrae in seven human fetuses ranging from 95 to 150 mm in crown-rump length (CRL) was studied using light and electron microscopy. In the 95-mm CRL, hypertrophy of the chondrocytes occurred in the central region of the vertebrae, and blood vessels penetrated there from dorsal and ventral sides of the vertebral body. The primary marrow was represented by liberated cartilage lacunae, occupied by the thin-walled blood vessels and a few mesenchymal cells and mononuclear cells containing granules or vacuoles (GMC). In the 99-mm CRL, chondroclasts were active in removing the cartilage near the central region of the vertebrae. Consequently, a large cavity was formed and occupied by a dilated sinus. GMC were numerous. Osteoblasts and osteocytes were increased in number. Reticular cells with long processes containing large amounts of glycogen began to appear in the extravascular space and formed the loosely arranged cellular meshwork of the hematopoietic compartment. Bundles of collagen fibrils were scattered in the meshwork. Hematopoietic cells were recognizable only in the 105-mm-CRL fetus and increased in number in the 120-mm-CRL fetus. The sinus endothelium was very thin and continuous without apertures except where blood cells crossed the wall. The developing blood cells lying against the outside of the sinus endothelium indented it. At points, collagen fibrils attached to the outside of endothelial cells and appeared to function as the anchoring filaments of lymphatics. The physiologic implications of the association of stromal cells, vascular sinuses, and hematopoietic cells are discussed in relationship to the microhematopoietic environment of the bone marrow.

THE BONE MARROW provides a preferred microenvironment for hematopoietic stem cells. They reside there and differentiate into various cell lines. However, very little information about the marrow microenvironment is available.

Fetal bone marrow is an ideal tissue to begin a study of the marrow vasculature and stroma, major structural elements in the hematopoietic microenvironment. One is able to follow the formation of the stroma prior to the presence of stem cells and then its interactions with differentiating blood cells.

The development of marrow in long bones has been studied by light microscopy in human fetuses of 70-mm CRL and other stages, and in other mammals. Only Maximow and Hammar attempted to study marrow histogenesis. Gilmour found the preservation of his material too poor for a histologic study of histogenesis, including the stroma elements, and confined himself to counts of hematopoietic cells. Kalpaktsoglou and Emery also studied the differential counts of cell types in human fetal rib marrow during the last 3 mo.
of intrauterine life. We have found no electron-microscopic studies of human fetal marrow.

In long bones, the encasement of marrow by bone and the presence of bony spicules make it difficult to obtain the delicate marrow without damage. In vertebrae, however, the marrow is encased in cartilage instead of bone through much of fetal development. The cartilage can be trimmed away easily, rendering the tissue readily accessible to fixatives, without disruption of the marrow. Decalcification can be carried out after fixation.

Histogenesis of marrow is initiated earlier in long bones than in vertebrae. Thus, it has been possible to study the earliest prehematopoietic bone marrow in the thoracic vertebrae of the 95-mm-CRL fetus, the youngest in our series, while the long bones at 70-mm CRL, the stage studied by Maximow, have advanced to moderate levels of hematopoiesis.

In this report, we describe the fine structure of vascular sinuses, hematopoietic compartments, and of stromal and hematopoietic cells during the development of the bone marrow in the vertebrae of human fetuses from 95- to 150-mm CRL. We carry out descriptions from the primary bone marrow, consisting of vascular sinuses and nonhematopoietic stroma, through stages of stem cell penetrance and diverse large-scale hematopoiesis and blood cell delivery. We believe that our observations provide clues as to the nature of the hematopoietic microenvironment of the developing marrow and to the interactions of reticular cells, macrophages, and stem cells in hematopoiesis.

MATERIALS AND METHODS

Fetuses

Human fetuses were obtained by therapeutic abortions done by hysterectomy at the Johns Hopkins Hospital. Seven fetuses were studied. Their size, measured as crown-rump length (CRL), and the fertilization age, calculated from data in Patten's text\textsuperscript{7} are: 95-mm CRL (13.2 wk), 99-mm CRL (13.5 wk), 105-mm CRL (13.9 wk), 120-mm CRL (15 wk), 138-mm CRL (16.3 wk), and 150-mm CRL (17.3 wk). The bone marrow used in this work was obtained from fetuses that provided spleen and lymph nodes\textsuperscript{8} for studies of fetal hematopoiesis which originated in this laboratory.

Electron Microscopy

Thoracic vertebrae were used throughout this study. Single vertebrae were separated from the vertebral column and the intervertebral cartilage was carefully trimmed away under a dissecting microscope without disrupting the marrow. The tissue was then fixed in Karnovsky's glutaraldehyde-parafomaldehyde mixture\textsuperscript{9} in cacodylate buffer. The vertebrae then were postfixed in 1% osmium tetroxide in cacodylate buffer for 2-4 hr and decalcified in 10% EDTA\textsuperscript{10} in the buffer for 3 days at 4°C. The EDTA solution was changed daily. The vertebrae were then dehydrated through graded ethanol, cleared in propylene oxide, and embedded in Araldite (Durcupan).\textsuperscript{11} Thick sections (1 μ) were stained with 0.5% toluidine blue\textsuperscript{12} in borax solution (15%, sodium borate in distilled water). Thin sections were stained with uranyl acetate\textsuperscript{13} and lead citrate\textsuperscript{14} and examined in a Siemens Elmskop I.

RESULTS

The thoracic vertebral bodies of the human fetuses ranging from 95- to 150-mm CRL appeared as a column of blocks joined together with short dimension in the cephalo-caudal direction. The development of the primary bone
marrow occurred near the center of the vertebral body and depended upon calcification of the cartilage matrix, hypertrophy of the chondrocytes, and invasion of the lacunae by the blood vessels. The shape and the dimension of the marrow space were revealed after removal of the peripheral cartilage of the vertebral body under a dissecting microscope. It was that of a jug with a narrow dorsal neck (cartilage canal). There were smaller cartilage canals from the ventral, lateral, and dorsal sides of the vertebra. A cartilage canal generally contained one arterial vessel and one dilated venous vessel (Figs. 1-3). The general shape of the marrow space remained the same as the fetuses grew and the vertebrae increased in size.

The vestige of notochord (VN) was recognizable in the center of the vertebral body of fetuses up to 120-mm CRL (Figs. 2 and 3). The primary bone marrow developed dorsal and ventral to the VN (Fig. 1) and then expanded through the central region of the vertebral body (Fig. 2). No ossification of the perichondrium of the vertebral bodies occurred.

In the 95-mm-CRL fetus, the primary bone marrow was represented by blood vessels occupying cartilage lacunae and accompanied by a few mesenchymal cells and mononuclear cells containing granules or vacuoles (GMC) (Fig. 4). Many GMC were present in the cartilage canal as well as in the marrow (Fig. 4). A few osteoblasts laid down bone matrix. Very few osteocytes occurred. Chondroclasts and hematopoietic cells were not evident.

In the 99-mm-CRL fetus, hematopoietic cells were not yet observed in the marrow. Many GMC were present in the marrow (Fig. 6). Osteoblasts and osteocytes increased in number. Chondroclasts were most numerous in the central region of cartilage matrix. A large cavity was formed near the center. It was occupied by a dilated sinus (Fig. 2). The central cavity continued to expand, and a larger one was present in the 120-mm-CRL fetuses (Fig. 3).

In the 105-mm-CRL fetus, a few hematopoietic cells were present and initiated the hematopoietic compartment of the marrow. Most of them appeared to be in the vicinity of the arterial vessel (Fig. 10). More hematopoietic cells were present in the 120-mm-CRL fetus (Fig. 13). By the time the fetuses reached 150-mm CRL, there were great numbers of hematopoietic cells. Consequently, the hematopoietic compartment expanded, pressed upon the dilated sinuses, and rendered them into a system of branching, interconnected channels (Fig. 14). As hematopoietic cells increased in number, GMC decreased. Even at the periphery of marrow, it was difficult to locate the GMC.

Mononuclear Cells Containing Granules or Vacuoles (GMC)

GMC had a rather smooth contour and were round or elongated. Their granules measured about 1 μ in diameter, contained homogenous granular substance, and were bounded by membranes (Fig. 6). The granules often became vacuolated and greatly increased their size to 4–5 μ. The fusion of granules or vacuoles commonly occurred (Figs. 6 and 7).

The nuclei of GMC were more heterochromatic than those of other cell types (Figs. 4 and 6). A rim of heterochromatin was apparent along the nuclear membrane (Fig. 6). The nucleus, located centrally or eccentrically, was often indented by large vacuoles (Figs. 4 and 7). The GMC contained rough endo-
Fig. 1. Fetus of 95-mm CRL. The thoracic vertebral body shows the hypertrophy of the cartilage in the central region and the invasion of the blood vessels through a large dorsal and a small ventral cartilage canal. An arterial vessel (A) is shown in the middle of the dorsal cartilage canal. The venous vessels (V) have a dilated lumen. This section is cut sagittally. Toluidine blue. ×84.
plasmic reticulum, a Golgi apparatus, moderate numbers of mitochondria, and no glycogen. They divided. Occasionally the GMC contained phagosomes (Fig. 7).

The relative percentage of GMC appearing in the marrow of 95–150-mm-CRL fetuses is shown in Table 1. In comparison to the older fetuses, young individuals contained fewer hematopoietic cells and higher percentages of GMC. The GMC were almost entirely located extravascularly in marrow and cartilage canals. They were one of the major cell types found in the primary bone marrow.

Vascular Sinus

The blood vessels entered and left the marrow through a large dorsal cartilage canal and three to five small ones. After entering the marrow, an arterial vessel branched. Its branches traveled toward the periphery of the marrow where venous sinuses lay. In comparison to sinuses, the arterial vessels had a smaller lumen, higher endothelium, basement lamina underlying the endothelium, and a layer of pericytes (Figs. 5, 10, 15). The lumen of the sinus was
especially large near the center of the marrow where it could be 400 μm in width in the 120-mm-CRL fetus (Fig. 3). The endothelial cells of the sinuses were extremely thin, even 200 Å in thickness (Figs. 11 and 12). A complete basement lamina and the adventitial cells outside the endothelium were not observed in the fetuses studied here. The endothelial cells were attached by junctional complexes (zonula adherens). The junctions were in the form of a mortise joint (mortise and tenon) (Fig. 8), a lap joint (overlapping) (Figs. 7 and 8), or end to end (Figs. 12 and 13). There were no gaps or apertures in the endothelium except when cells were in passage through it (Fig. 17).

Endothelial cells extended processes into the hematopoietic compartment and frequently surrounded the developing blood cells (Fig. 16). The sinus endothelial cells appeared pliant since they were indented by the developing blood cells (Fig. 13).

At points, bundles of collagen fibrils were attached to the abluminal side of the endothelium and appeared to hold the endothelial cells to the reticular cells (Fig. 12).

Cytoplasmic filaments were a common organelle in the sinus endothelial cells. In addition, a distinctive tubular structure containing lamellar substructure was observed (Fig. 9). The endothelial cells divided.
Fig. 4. Fetus of 95-mm CRL. Invasion of liberated lacuna by the capillary (Ca). GMC, and mesenchymal cell (M). Liberated degenerate chondrocytes (DC) are not yet removed. C, cartilage. x4400.

Hematopoietic Compartments

The hematopoietic compartment of the marrow was an extravascular space lying between the sinus and the cartilage and bone, where hematopoietic cells proliferated and differentiated in a loose cellular meshwork. In the 95-mm-CRL fetus, a loose cellular meshwork was not yet established. GMC and mesen-
Fig. 5. Fetus of 99-mm CRL. The primary bone marrow. The reticular cells (R) with long processes begin to appear in the hematopoietic compartment and form the cellular meshwork. They contain a large amount of glycogen (G). An arteriole (A) is partially covered by a pericyte (P). S, sinus. x6600.

Table 1. Occurrence of GMC (Mononuclear Cells Containing Granules or Vacuoles) in the Marrow of the Thoracic Vertebrae of Five Fetuses

<table>
<thead>
<tr>
<th>Size of Fetus (Crown-rump Length in mm)</th>
<th>Per Cent of GMC</th>
<th>Per Cent of Others*</th>
<th>Number of Cells Counted†</th>
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<tr>
<td>95</td>
<td>41.8</td>
<td>58.2</td>
<td>1100</td>
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<tr>
<td>99</td>
<td>16.2</td>
<td>83.8</td>
<td>1842</td>
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<td>105</td>
<td>3.5</td>
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<td>6006</td>
</tr>
<tr>
<td>150</td>
<td>&lt;0.05</td>
<td>&gt;99.95</td>
<td>6557</td>
</tr>
</tbody>
</table>

*Including undifferentiated cells, chondroclasts, osteoblasts, reticular cells, and the developing blood cells.
†Cells counted were in the hematopoietic compartment.
Fig. 6. (Top) Fetus of 99-mm CRL. A group of GMC outside the sinus (S) in the primary bone marrow. Two GMC in the center contain spherical granules of different sizes. The granules of the GMC at the bottom of the figure have lost their contents and have become swollen into large vacuoles. The granules of the GMC at the upper center have also lost all their content and are larger than intact granules in size. Loss of granule content may represent an artifact of histologic preparation. The fusion of empty granules is commonly seen (arrow). ×6000.

Fig. 7. (Bottom) Fetus of 99-mm CRL. The primary bone marrow. Two GMC lying outside the sinus (S) contain phagosomes (P). It commonly occurs that one granule indents another (arrow) or coalesces with another (*). Reticular cells with long processes form a loosely arranged cellular meshwork outside the sinus (S). A few bundles of collagen fibrils (CF) are scattered in the meshwork. The junction of the sinus endothelial cells in the form of a lap joint is indicated by a thick arrow. R, reticular cell. ×6000.
chymal cells occupied the compartment. Reticular cells with long processes were not present (Fig. 4). At 99-mm CRL, reticular cells with long broad processes were observed, and a loosely arranged cellular meshwork began to take shape. Collagen fibrils were scanty (Figs. 5 and 7). Recognizable hematopoietic cells were not present. In the 120-mm-CRL fetus, more bundles of collagen fibrils appeared in the hematopoietic compartment. They were not embedded in or surrounded by reticular cells, but rather scattered in the meshwork. Hematopoietic cells were easily identified. The reticular cells in the hematopoietic compartment were generally oriented with their long cellular processes parallel to the sinus endothelium. Reticular cells contained a fair amount of cytoplasmic filaments. They also contained a large amount of glycogen, both in the perinuclear region and in its processes (Figs. 5, 7, 8, 11, 12). The glycogen content in the reticular cells decreased as hemopoiesis increased.

**Hematopoietic Cells**

Recognizable hematopoietic cells were observed in the 105-mm-CRL or larger fetuses (Figs. 10–12). The earliest identifiable hematopoietic cells were similar to the candidate stem cells described by Van Bekkum and others.15,16 and
Fig. 10. Fetus of 105-mm CRL. The hematopoietic cells begin to appear in the hematopoietic compartment and concentrate in the vicinity of an arteriole (A). Most of the hematopoietic compartment immediately adjacent to the cartilage and bone is devoid of hematopoietic cells. A few GMC (arrows) are present in the meshwork of the hematopoietic compartment. B, bone; C, cartilage; Os, osteoblast; CH, chondroclast; and S, sinus. ×375.

lay in the meshwork of the hematopoietic compartment (Fig. 11). They were only loosely associated with the reticular cells. The more differentiated hematopoietic cells, on the other hand, were rather closely confined by the cellular reticulum and were in contact with reticular cells (Fig. 12).

DISCUSSION

In order to obtain the most accurate information of the delicate structure of the fetal bone marrow, mechanical disruption of the marrow or the delay of penetration of fixative must be minimized. We find that the vertebral marrow is superior to that in long bones for this study because, as indicated in the Introduction, it can be prepared with little or no distortion.

The bone marrow in the younger fetuses in this study consisted of a large
Fig. 11. Fetus of 120-mm CRL. The hematopoietic compartment, prior to the massive invasion and proliferation of the hematopoietic cells, consists of a loosely arranged cellular meshwork lying between the sinus and the cartilage and bone. The meshwork is composed of reticular cells with long processes (R) and bundles of collagen fibrils (CF). The cellular processes contain a great amount of glycogen (G) which may be preserved or washed-out. Two hematopoietic cells similar to the candidate stem cells described by Van Bekkum et al. appear in the meshwork. ×4000.
Fig. 12. Fetus of 120-mm CRL. Four normoblasts lie between the cellular processes of the reticular cells in the hematopoietic compartment. The lower three appear to push against the sinus. Bundles of collagen fibrils (arrows) bind the sinus endothelial cells to the reticular cells. The processes of the reticular cells are thin and broad. In cross section, they look like a thin line; in oblique section they look like a broad sheet (X). The end-to-end junction of the endothelial cells is indicated by a thick arrow. x6000.
Fig. 13. Fetus of 120-mm CRL. The increase in developing blood cells in the hematopoietic compartment results in expansion of the hematopoietic compartment into the sinus. Two of the developing blood cells push against and indent the endothelium. The indentation of the endothelium coincides with the shape of the cells indenting it. Along the endothelium, the indented region is thinner than that of the rest. This reflects the flexibility of the endothelium. The endothelium appears to be held to its place by a group of collagen fibrils cut in cross section (CF), while it is pushed toward the lumen of the sinus by the developing blood cells. EJ, end-to-end joint of the endothelial cells; Mit, cells in mitosis; and R, reticular cells. (A) ×4400, (B) ×13,200.
Fig. 14. Fetus of 150-mm CRL. The developing blood cells greatly increase in number in the marrow. Consequently, the hematopoietic compartment expands and divides the sinus into branching interconnected channels. A few megakaryocytes are readily recognizable. Embedded in methacrylate and stained with hematoxylin and eosin (Ruddell: Hydroxylethyl methacrylate combined with polyethylene glycol 400 and water; an embedding medium for routine 1-2 micron sectioning. Stain Technol 42:119, 252, 1967). B, bone; C, cartilage; and S, sinus. ×464.
Fig. 15. Fetus of 150-mm CRL. An arteriole (thick arrows) is present in an hematopoietic compartment which contains various types of the developing blood cells. Cell junctions of the sinus endothelial cells are indicated by thin arrows. ×4400.
Fig. 16. (Top) Fetus of 150-mm CRL. Sinus endothelial cells extend their processes (arrows) into the hematopoietic compartment. The processes often surround or cover the developing blood cells. L, lymphocyte; R, reticular cells. x4000.

Fig. 17. (Bottom) Fetus of 150-mm CRL. Two granulocytes are in passage through the endothelium. The one on the left (G) is partially revealed. Apertures in the endothelium are only observed when the blood cells migrate through the endothelium. M, developing megakaryocyte; R, reticular cell. x4000.
central vascular sinus surrounded by a loose reticular connective tissue which, in the older fetuses, became hematopoietic. In early stages, the central sinus was large and dilated, virtually filling the marrow cavity. In later stages, the marrow cavity expanded, and the periphery of the sinus became crenelated. In fact, it received tributaries originating at the periphery of the cavity. In early stages, the reticular stroma surrounding the central sinus was a thin connective-tissue envelope about the sinus system. It contained no hematopoietic cells but was rich in ground substance and granulated mononuclear cells (GMC). The encasing cartilage was beset with chondroclasts, almost regularly spaced, on the marrow surface. Later the cartilage recedes and ossifies, the central sinus system draws to the center of the marrow and receives tributaries, and hematopoietic stem cells enter the perivascular reticular stroma and proliferate and differentiate there.

The endothelium of the vascular sinus was so thin over so large a surface that it would appear that its microfilaments must play a major role in maintaining its delicate structure. Its patency must also depend upon bundles of collagenous fibrils attached to the basal endothelial surface, extending out into the surrounding reticular connective tissue, and to a lesser extent upon long cytoplasmic processes extending from the basal endothelial surface into the perisinus tissue. In many respects these sinuses are structurally similar to lymphatics. Where vascular sinuses occur, as with the lobule of the liver, red pulp of the spleen, and bone marrow, lymphatic vessels are absent. This finding suggests that vascular sinuses may assume the role of lymphatic vessels as well as that of blood vessels. The endothelial cells about the periphery of the marrow are rather frequently observed in mitotic division, suggesting that the blood vessels grow and fill the newly liberated lacunae by multiplication of endothelial cells.

Reticular cells in our material lay in a clear ground substance which is likely gelated. They possess multiple, far-reaching, thin, broad processes which form a meshwork. Those reticular cells close to vascular sinuses appear held to the outside endothelial surface by bundles of collagen fibrils.

The extravascular space is compartmentalized by the reticular cell processes, and differentiating hematopoietic cells lie within the compartments. Over broad areas, cell membranes of the hematopoietic cells and reticular cells are in close apposition. Metabolites or signals critical to hematopoiesis may be exchanged across these contiguous membranes. The hematopoietic cells appear immobilized in the reticular meshwork, a condition which may be necessary for their differentiation. Thus, in studies in vitro of marrow, hematopoietic colonies fail to develop in a liquid culture medium, but do appear in soft agar. The close relationship of differentiating blood cells and reticular cells may constitute an important element in the hematopoietic microenvironment, inducing the differentiation of stem cells into a particular cell line. Indeed, the distinctive organization of stroma and vasculature represent the hematopoietic inductive microenvironment (HIM) characterized by Trentin's valuable work.

The reticular meshwork in the fetal marrow is different from that of the fetal spleen. The meshwork of the spleen is a vascular space and a blood filter. That of the marrow, on the other hand, is a scaffolding for hematopoiesis and
may play a role in spacing and supporting sinuses. In the spleen, unlike the marrow, many cell junctions of the adherent type are present between reticular cells, and the cells contain plaques of microfilamentous bands. In the adult, the reticular meshwork in the spleen is tighter and associated with a better developed fibrillar reticulum than in marrow.

The existence of hematopoietic stem cells was established without knowledge of their morphology by establishing, in lethally irradiated recipient mice, the clonal nature of spleen cell colonies derived from stem cells in the marrow suspension provided by lightly irradiated donors. Van Bekkum and his colleagues increased the number and concentration of stem cells in the donor bone marrow in spleen-colony assay by pretreating the donor with vinblastine and then subjecting this stem cell-enriched suspension to further enrichment by centrifugation. They identified, by electron microscopy, a cell type in the marrow suspension whose numbers appeared proportionate to the increased numbers of spleen colonies. They termed this cell type a candidate stem cell. Those cells we have recognized as the earliest hematopoietic cells in the reticular meshwork of the primary marrow are morphologically similar to those described by Van Bekkum and his colleagues. We surmise that these early cells we observed are likely stem cells.

Granulated mononuclear cells are present in large numbers early in our series and, as hematopoiesis increases, their percentage decreases. In the 95-mm-CRL fetus, they are almost 42% of the nucleated cells of the marrow, while in the 150-mm-CRL fetus, they are less than 0.05%. The GMC appear to decrease in absolute number as well, as indicated by the difficulty in finding them in intermediary fetal stages when relatively few hematopoietic cells are present. The GMC were described by Maximow as a vacuolated wandering cell which he thought to be phagocytic. In our material they appear by electron microscopy to be granulated cells containing a uniformly sized, moderately dense, membrane-bound, secretory-like granule. In some respects they resemble mucous-producing cells. These granules coalesced and lost their content, probably as a result of fixation-induced artifact. The presence of some heterolysosomes suggests, as Maximow believed, that they may be phagocytes. While they contain fewer heterolysosomes than other clearly identifiable phagocytes in our material, we believe these cells are macrophages.

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REFERENCES


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