Histochemical Differentiation of the Megakaryocytes in the Embryonic Liver

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The demonstration of chemical differentiation occurring prior to visible morphologic differentiation is one of the prime goals of the histochemical-hematologic investigator. If chemical differentiation precedes morphologic changes in cellular development, it might be possible to trace the origin of the various cells of the hemopoietic system more precisely and perhaps even to more primitive cells than have heretofore been recognized. Our preliminary observations strongly suggest that this is the case concerning the origin and differentiation of the megakaryocytes in the fetal liver.

Megakaryocytes have been considered to arise from a number of sources including mesenchymal cells,7,8 reticulum cells,5,13 hemocytoblasts or large lymphocytes,9,10,12,16 myeloblasts,2 megakaryocytoblasts,16 and occasionally from cells lining the sinusoids of the liver.1,2 Both intra- and extravascular formation of the megakaryocytes have been reported.4 It is customary for the hematologic investigator to believe that the megakaryocytes have only one cell of origin and that his "stem" cell is not necessarily related to those of other workers. Essentially all of the classic concepts of megakaryocytogenesis have been based on morphologic studies in which only general histologic staining methods were employed.

We have recently become interested in a reinvestigation of the origin and differentiation of the cells of the hemopoietic system using histochemical technics. In examining the livers obtained from fetal pigs following staining with the periodic acid-Schiff reaction, we noted the selective staining of the megakaryocytes which enabled us to establish clearly the mode of origin of the megakaryocytes in the fetal liver. It is the purpose of this report to present our finding concerning the origin of the megakaryocytes of the fetal liver of the pig which have been derived from both histochemical and morphologic studies (fig. 1).

Materials and Methods

Livers were obtained from fetal pigs between 35 and 40 mm. in length. The tissue was removed while the embryos were still warm and placed in the appropriate fixatives, including Bouin's, Zenker-formol, formol-sublimate-acetic, 10 per cent neutral formalin, formol-alcohol and 80 per cent ethanol. Our subsequent studies indicated that Bouin's solution was the most satisfactory fixative for the preservation of the cellular elements of the developing liver and was best adapted to our particular histochemical staining methods. Zenker-formol and formalin-sublimate-acetic were good but were less satisfactory than Bouin's solution. Paraffin sections cut at four μ were subjected to the periodic acid-Schiff reaction (10 minutes in 0.5 per cent aqueous periodic acid and 15 minutes in Schiff's
Fig. 1.—Schematic representation of the patterns of megakaryocytopoiesis in the fetal liver of the pig. Larger arrows indicate the principal lines of megakaryocyte differentiation.

Results

Megakaryocytes exhibited a moderately strong cytoplasmic reaction following the PA-S reaction or the bi-color PAS-Feulgen nucleic technic. This reactive material appeared as tiny granules evenly distributed throughout the cytoplasm of the megakaryocyte. Only a minimum amount of glycogen was present in the megakaryocytes following alcohol-formalin fixation as confirmed by salivary digestion. Hepatic cells, in contrast to the megakaryocytes, were extremely rich in saliva-labile glycogen and exhibited only a minimal PA-S-positive cytoplasmic reaction following saliva digestion on alcohol-formalin fixed preparations.

The use of aqueous fixatives, particularly Bouin's and formalin-sublimate-acetic solutions, not only provided excellent morphologic fixation but effectively removed the glycogen from the hepatic cells; only the water- and saliva-resistant mucopolysaccharides remained in the tissues. Therefore, following this procedure, hepatic cells exhibited only a very faint PA-S-positive reaction, in contrast with the strong PA-S-positive reaction present in the cytoplasm of the megakaryocytes. It became readily possible to distinguish the
megakaryocytes even under low magnification following this technic (figs. 2 and 3).

The only other cellular elements present in the fetal liver of the pig which exhibited a moderate PA-S reaction with the method described were a few of the phagocytic cells, macrophages, present in the hepatic sinusoids. Macrophages could be distinguished from megakaryocytes by the usual morphologic criteria including cell size, contour and the form and chromatin pattern of the nucleus. In addition, these phagocytic cells usually contained ingested foreign material (e.g., cellular debris or hemosiderin) and exhibited PA-S-positive granules of variable sizes while their hyaloplasm was only faintly PA-S-positive (fig. 8). The megakaryocytes, on the other hand, contained many tiny PA-S-positive granules evenly distributed throughout their cytoplasm (figs. 4, 5 and 8).

In tracing the histochemical development of the PA-S-reactive material of the cytoplasm of the megakaryocyte back through the immature stages, we observed that this material appeared extremely early in the differentiation process. In fact, we were able to identify cells undergoing the initial stages in megakaryocyte differentiation by the appearance of the PA-S-positive granules in the cytoplasm before these cells could be identified by morphologic criteria as revealed by conventional histologic staining methods.

Megakaryocytes were seen to arise in three separate locations in the fetal liver: (1) in peripheral mesenchyme, (2) between groups of hepatic cells, and (3) intravascularly in the liver sinusoids.

In the mesenchyme surrounding the developing liver, particularly in the area of the septum transversum, certain mesenchymal cells lying in relatively close approximation to the ingrowing hepatic cells begin to elaborate PA-S-positive granules in their cytoplasm. These cells (primitive megakaryocytes) retained their stellate contour and gradually increased their cytoplasmic volume and continued their elaboration of PA-S-positive granules (figs. 4 and 5). Without passing through morphologically typical “blast” cell stages, these cells continued to enlarge, undergo hypertrophy and lobulation of their nuclei and developed directly into typical young megakaryocytes and finally into mature megakaryocytes (figs. 4 and 5).

In most instances, however, the megakaryocytes of the fetal liver developed from reticulum cells (mesenchymal cells) which were surrounded and trapped by the hepatic cells during the proliferative growth of the liver. By position, these stellate cells are considered reticulum cells (figs. 9 and 11), but they did not appear to differ morphologically or histochemically (PA-S reaction) from typical mesenchymal cells (figs. 4 and 5). These reticulum cells may undergo three separate lines of differentiation: (a) to form erythroid elements, (b) to form granulocytes, or (c) to form megakaryocytes. In the formation of the megakaryocytes, these primitive stellate cells begin to form PA-S-positive granules in their cytoplasm and to undergo a histochemical and morphologic pattern of megakaryocyte differentiation, as described above (figs. 6, 10, 12 and 13), or less frequently, they may form a rounded cell with thickened nuclear membrane and prominent nucleoli (figs. 9 and 11). These
rounded cells may be classically considered as “blast” cells but must be termed megakaryocytoblasts since their chemical line of differentiation was established in a more undifferentiated stage of development, i.e., in the reticulum cell stage.

During the peripheral growth of the liver, the mesenchymal cells not surrounded and entrapped by the invading hepatic cells come to lie along side the hepatic cells. As the hepatic columns form and invade the mesenchymal territory, the mesenchymal cells extend along the liver strands. The regions between the liver columns or strands become relatively less cellular and soon

Plate 1. Liver of 20 mm. pig embryos fixed in Bouin’s solution, stained by the bi-color periodic acid-Schiff-Feulgen nucleal method and counterstained with orange G. (See individual legends on facing page.)
transform into hepatic sinusoids. Mesenchymal cells lying along side the hepatic columns thus form the endothelial cells (figs. 14 through 19) lining the sinusoids, while other mesenchymal cells in these areas transform into macrophages. The stellate cells lining the sinusoids of the liver are capable of differentiating either into macrophages or into megakaryocytes. Not infrequently, these stellate endothelial cells differentiate into megakaryocytes and undergo histochemical changes (PA-S reaction) followed by morphologic differentiation (figs. 14 through 16). Thus, megakaryocytes may form intra-vascularly, as well as extravascularly. Occasionally, the endothelial cells enlarge and grow into or between the hepatic cells (fig. 17). Megakaryocytes formed in this manner assume an extravascular position.

Megakaryocytes which developed in an extravascular position may gain access into the hepatic sinusoids by the processes of differential growth and pressure. In this manner, the giant cells are able to separate the hepatic cells and break through the delicate wall of the hepatic sinusoids.

Proof that megakaryocytes may be extravascular has been confirmed by our electron microscopic studies of the fetal liver. In these instances, the megakaryocytes were in direct contact with the hepatic cells, and no visible membrane was seen to separate them. Other megakaryocytes were identified in the hepatic sinusoids.

**DISCUSSION**

The PA-S reactivity of the megakaryocytes in the bone marrow has been established in both man and other animals. Our observations sub-

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Fig. 2.—Two definitive megakaryocytes are evident, one (a) in the hepatic sinusoid and the other (b) extravascularly lying between hepatic cells. The PA-S reactivity of the megakaryocytes illustrated is greater than the surrounding hepatic cells. The basement membrane (c) of the sinusoid is easily identified by its PA-S reactivity. Endothelial cells (d) lining the sinusoids are apparent and subendothelial reticulum cells (e) are frequently seen. (× 400)

Fig. 3.—Four megakaryocytes in the liver parenchyma illustrating various stages of maturation. One bi-lobed megakaryocyte is located in the hepatic sinusoid. Cytoplasmic borders are not as well delineated in this preparation as in figure 2, although the PA-S reactivity of the megakaryocyte is still apparent. Fixation formol-sublimate-acetic. (× 400)

Fig. 4.—A definitive megakaryocyte (a) developing in the mesenchyme of the septum transversum. A primitive megakaryocyte (b) in the initial phase of differentiation from mesenchymal cells is indicated. This cell has retained its stellate contour but possesses a strongly PA-S-positive reaction cytoplasm, as does the definitive megakaryocyte. Mesenchymal cells (e) and a hepatic cell (d) are indicated. (× 1200)

Fig. 5.—Similar to figure 3, illustrating a definitive megakaryocyte (a) and a very immature (primitive) megakaryocyte (b) surrounded by mesenchymal cells (c) in the septum transversum. Small PA-S-positive cytoplasmic granules are readily apparent in both megakaryocytes.

Fig. 6.—A definitive megakaryocyte (a) located between hepatic cells. The basement membrane of the sinusoid is well delineated, indicating that this megakaryocyte is extravascular in position. (× 1200)

Fig. 7.—A definitive megakaryocyte (a) with extended pseudo-pod and (b) a very immature megakaryocyte developing between the hepatic cells. (× 1200)
stantiate this finding in the livers of the fetal pig. Moreover, the periodic acid-Schiff reaction enables one to trace the origin and modes of differentiation of these giant cells much more critically and accurately than do the usual morphologic criteria. Thus, it was possible to demonstrate that chemical differentiation takes place prior to morphologic differentiation in the megakaryocytes in the embryonic liver.

Maximow\(^7\) and Knoll\(^9\) have observed megakaryocytes to arise directly from mesenchymal cells. Maximow,\(^5\) Kingsley,\(^6\) Rothermal,\(^12\) Potter and Ward,\(^10\) and Tocantins\(^16\) have shown that megakaryocytes arise from free rounded stem cells, termed hemocytoblasts or large lymphocytes. The myeloblast has
been described also as being capable of forming megakaryocytes.\(^2\) Other investigators consider that the megakaryocytes form from rounded stem cells which exhibit certain morphologic characteristics indicating that they will differentiate along the megakaryocytic lineage.\(^{13,16}\) These rounded cells have been considered as specific stem cells and have been termed by some investigators,\(^{13,16}\) megakaryocytoblasts. Bloom,\(^1\) Kölliker,\(^4\) and Downey et al.,\(^2\) have observed that under certain conditions megakaryocytes may arise from the cells lining the hepatic sinusoids. Zajicek\(^{19}\) has identified megakaryocytes and their precursors in the bone marrow of certain animals by their selective enzymatic staining following a modified acetyl cholinesterase technic. Zajicek\(^{19,20}\) has observed a few cells smaller than megakaryocytoblasts which he believes may differentiate along the megakaryocytic lineage.

Our studies have shown that megakaryocytes in the liver of the fetal pig arise from undifferentiated stellate cells: mesenchymal cells, primitive reticular cells (reticulum cells) and endothelial cells. These undifferentiated cells may develop into primitive megakaryocytes in three locations in the fetal liver: in the mesenchyme surrounding the developing liver, between groups of hepatic cells and from cells lining the hepatic sinusoids. During the differentiation process these undifferentiated cells may transform directly into megakaryocytes, primitive megakaryocytes, with the elaboration of PA-S-positive material, as well as cytoplasmic and nuclear mass. In other instances, the stellate cells, primitive megakaryocytes, which already exhibit histochemical signs of megakaryocyte differentiation may round up and pass through what may be considered a typical blast cell form. These rounded cells are not multipotential in the true sense as is suggested by such terms as hematoblasts, large lymphocytes and myeloblasts, but already have passed...
through the initial stage of megakaryocytic differentiation, and for this reason these rounded basophilic cells should be considered as megakaryocytoblasts. Figure 1 summarizes our finding concerning the pattern of origin and development of the megakaryocytes in the fetal liver of the pig.

In summary, our observations indicate that histochemical methods may provide an important approach for resolving some of the controversial problems concerning the origin, development, potentialities and interrelationships of the cells of the hemopoietic system.

Plate 3. Liver of 20 mm. pig embryos fixed in Bouin's solution, stained by the bicolor periodic acid-Schiff-Feulgen nucleal method and counterstained with orange G. (× 1200) (See individual legends on facing page.)
DIFFERENTIATION OF MEGAKARYOCYTES IN EMBRYONIC LIVER

Summario in Interlingua

Es reportate constatationes relative al origine del megacaryocyotos del hepate fetal de porcos super le base de studios histochimic e morphologic.

Le reactivitate a acido periodic de Schiff que ha essite reportate pro megacaryocytos del medulla ossee de humanos e altere animales esseva confirmate con respecto al megacaryocytos del hepate del porce fetal. Per medio de iste reaction il esseva possibile demonstrar que un differentiation chimic occurre in le megacaryocytos del hepate embryonic ante le initio de omne differentiation morphologic.

Esseva monstrate que le megacaryocytos in le hepate del porco fetal prende lor origine ab non-differentiate cellulas stellate, i.e. ab cellulas mesenchymal, primitive cellulas reticular, e cellulas endothelial. Iste non-differentiate cellulas pote disveloppar se in megacaryocytos primitive in tres sitos intra le hepate fetal, i.e. in le mesenchyma que circundla le hepate in le curso de su disveloppamento, inter gruppos de cellulas hepatic, e ab cellulas que revesti le sinusoides hepatic. In le curso del processo de differentiation, iste non-differentiate cellulas pote transforma se directemente in megacaryocytos, i.e. megacaryocytos primitive, con le elaboration de material de positivitate pro acido periodic de Schiff e etiam con massa cytoplasmic e nucleiari. In altere casos, le cellulas stellate—primitive megacaryocytos—que jam exhibi signos histochimic de differentiation megacaryocytic pote arrondar se e passar per un forma que pote esser considerate como tipicamente blastocytic. Iste cellulas arrondate non es multipotential in le ver senso del termino (como illo es continite in designationes como hemocytoblastos, large lymphocytos, e myeloblastos). Illos ha jam passate per le studies inicial del differentiation megacaryocytic, e pro iste ration tal arrondate cellulas basophilic deberea esser considerate e designate como megacaryocytobastos. Figura 1 summarisa nostre constatationes relative al origine e al disveloppamento del megacaryocyotos in le hepate fetal del porco.

Fig. 14.—A rounded cell (a) with PA-S reactive finely granular cytoplasm and a prominent nucleolus represents a very young megakaryocyte (megakaryocytoblast) present in the hepatic sinusoid. An endothelial cell (b) lines the sinusoid.

Fig. 15.—An immature megakaryocyte (a) in the hepatic sinusoid. A swollen endothelial cell lines the sinusoid, as indicated at (b). Nucleated erythroid elements also are present in the sinusoid.

Fig. 16.—A bi-lobed megakaryocyte (a) present in the sinusoid with an endothelial cell (b) lining the hepatic sinusoid.

Fig. 17.—Two megakaryocytes, (a) and (b), in the initial stage of differentiation with one surface against the sinusoid while the remainder of the cells are surrounded by hepatic cells (c). An endothelial cell (d) is shown between the two developing megakaryocytes and lines the hepatic sinusoid. Immediately above the endothelial cells is an intravascular normoblast in mitosis.

Fig. 18.—Two megakaryocytes (a) developing between hepatic cells and immediately under an endothelial cell (b) lining the hepatic sinusoid.

Fig. 19.—A definitive megakaryocyte (a) situated between the sinusoid and the hepatic cells. The thin cytoplasmic membrane of the endothelial cell (b) of the sinusoid overlies the megakaryocyte.
Nostre observationes indica que methodos histochemic es potentialmente capace a contribuer grandemente al resolution de certes del problemas controverse sublevate per le investigation del origine, del disveloppamento, del potentialitatites, e del interrelationes del cellulas in le systema hematopoietic.

REFERENCES


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