POSSIBLE DEVELOPMENTAL INTERACTIONS OF HEMATOPOIETIC CELLS AND HEPATOCYTES IN FETAL RAT LIVER

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ABSTRACT

In the fetal DA rat liver, hematopoiesis occurs around day 14 to day 20 of gestation, although the maximum number of hematopoietic cells are observed on day 18. After birth, the hematopoietic foci mostly disappear. Morphological analysis by light and electron microscopy reveals changes in hematopoiesis during gestation. Supporting techniques for histological study such as density fractionation and cell-scatter analysis are also employed for fractionation of fetal liver hematopoietic cells, but these methods are not sufficiently useful for clarifying the gestational changes in fetal liver hematopoiesis or for the enrichment of hematopoietic stem cells in fetal rat liver. Immunocytological and immunohistological methods using a monoclonal antibody against rat fetal liver hematopoietic cells (UB-12) established by us as well as other monoclonal antibodies (OX7 and W3/13) to rat hematopoietic cells are effective for analysis of rat hematopoietic tissues: The antigenic expressions detected by UB-12 are extensive when hematopoiesis in fetal liver is active, while decreases in parallel with the decrease of hematopoiesis. Using UB-12 monoclonal antibody and radioimmunoassay, it is possible to follow the shift of hematopoietic focus from fetal liver to adult bone marrow semiquantitatively. Some surface antigens of hematopoietic cells such as cell adhesion molecules are important for cell-cell interaction between hematopoietic cells and stromal cells and UB-12 antigen might be one of such molecules. The expression of antigens on hepatocytes detected with monoclonal antibodies directed against adult hepatocytes (HAM4 and others) is negative or quite low in the early gestational period and increases in later stages. However, even before birth the degree of expression is lower than the adult level. The morphological changes occurring with the transition from fetal-type to adult-type hepatocytes also become obvious towards the birth proving distinct difference between fetal hepatocytes and adult hepatocytes, and suggesting stromal function of fetal hepatocyte for the hematopoietic cells. These results suggest populational and cellular changes in both hematopoietic cells and hepatocytes during the gestation and it is possible to propose a theoretical cell-cell interaction through cell membrane molecules with adhesive function between hematopoietic cells and fetal hepatocytes. Also, paracrine type of cell-cell interaction may be important for fetal hematopoietic cells and hepatocytes to proliferate. Thus, the diminution of hematopoietic foci in the liver after day 19 of gestation can be explained partly by changes in the cell-cell interaction of hematopoietic cells with fetal hepatocytes, while the changes occurring in the whole body of the fetus in the perinatal period, such as the start of bone and bone marrow formation, and endocrinological or metabolic changes around birth may also be important. The mechanisms responsible for the shift of hematopoietic foci are discussed, focusing on the shift of hematopoietic foci from fetal liver to adult bone marrow.

INTRODUCTION

Among the many tissues, epithelial and mesenchymal interactions are considered to be important during fetal development (40). Hematopoietic tissues often develop in the microenvironment of endodermal origin of epithelial tissues (97). The thymus is also considered to be provided with microenvironmental structures bearing epithelial reticular tissues for thymocyte proliferation and

differentiation (79, 82).

In the fetal liver, initially, hepatomorphogenesis takes place through the effect of mesenchymal components on endodermal components (52). In mammals, hematopoietic tissues expand their descendants in epithelial hepatocytes during the fetal period, just after the events of hematopoiesis in the yolk sac. It is still not clear why hematopoietic tissue shows this sort of proliferation in fetal liver before proliferation in bone marrow and other

Fig. 1 Gestational changes in fetal liver weight and nucleated hematopoietic cell counts. Fetal liver on each gestational day was weighed and plotted (●—●). Hematopoietic cells were prepared from each liver, counted and plotted (●---●). Weight of fetal liver increased with gestation, particularly after about day 16. The number of nucleated hematopoietic cells also increased with the increase in liver weight, whereas after day 19, the number decreased rapidly.

sites, except that around the time of fetal liver hematopoiesis, bone formation may not yet be established and therefore hematopoietic cells are unable to proliferate in the bone marrow. As alternatives to fetal liver, many other tissues such as intestine and kidney could become candidates for sites of hematopoietic proliferation. However, only in fetal liver does intensive hematopoiesis occur. Thus, hematopoietic proliferation in the fetal liver might be essential even for hepatocyte proliferation or differentiation around the time of liver organogenesis in mammals.

Our intention in the present study is to answer these questions, initially by analyzing the events of fetal liver morphogenesis, that is, gestational changes in both hematopoietic cells and hepatocytes in the livers of fetal DA rats, using monoclonal antibodies reactive with either hematopoietic cells or hepatocytes.

ANALYSIS OF HEMATOPOIETIC CELLS IN FETAL RAT LIVER (COMPARISON WITH ADULT BONE MARROW CELLS)

Changes in Cell Number

As gestation proceeded the weight of the fetal liver increased (Fig. 1), and the number of hematopoietic cells within it changed. Around day 16–18, the number of hematopoietic cells increased in parallel with the weight increase of the liver. However, despite the further increase in fetal liver weight, the number of hematopoietic cells started to decrease rapidly on day 19 (Fig. 1), and around birth, the number of these cells was quite small. This result suggests that in the DA rat, hematopoiesis may start at about days 12–15, and gradually increase up to day 18, declining rapidly thereafter. Furthermore, the degree of hematopoiesis on days 12–16 may be quite intense, resulting in a maximal

Fig. 2 Light microscopical findings of fetal liver and fetal liver hematopoietic cells at day 14 of gestation. Figure A shows the HE-stained fetal liver. At day 14 fetal liver, among the hepatocytes, hematopoietic cells occur sporadically. Nucleated red blood cells are present within the sinusoids (S). Silver staining of 14-day fetal liver (B), showing some reticular fibers facing the sinusoids (S), although the meshwork of reticular fibers is quite poor compared with that in the adult. Hematopoietic cell suspensions were prepared from 14-day fetal liver, smeared and Wright-stained (C). Erythroid series and some other mononuclear cells are evident. A mitosis is observed. A, $\times 100$; B, $\times 200$; C, $\times 1,000$

number of hematopoietic cells on day 18. Therefore, at around day 12 in the fetal liver, some form of quite potent microenvironment may exist, catching the circulating pluripotent hematopoietic stem cells which might be released from the yolk sac into the circulation. Localization of this sort of microenvironmental tissues in fetal liver would be an important research aim when analyzing hematopoiesis in fetal liver.

Morphological Changes

To analyse the populational changes in hematopoietic cells occurring in the fetal liver during the gestational period, several methods could be employed. Among these, study of morphological changes in the liver might be a convenient approach.

For several decades, many researchers have observed fetal hematopoiesis in humans and rodents using light microscopy (24, 63, 71, 78, 103). Erythropoietic cells were observed mostly among fetal hepatocytes in DA rat (Figs. 2-4). Granulocyte and monocyte hematopoiesis was evident to a lesser degree. Lymphocyte hematopoiesis may not be easy to observe, but fetal liver contains multipotential hematopoietic cells which can differentiate into T and B lymphocytes (11, 12, 16, 42, 76). In semithin sections of fetal liver at days 14, 15, 18 and 20 of gestation, hematopoietic foci appeared to differ from each other (Fig. 3, A-D). On day 14, sporadic hematopoietic foci exist among hepatocytes (Fig. 2A and Fig. 3A). On day 15 the number of hematopoietic cells in liver had increased (Fig. 3B). On day 18 hematopoiesis in the liver was quite active, but by day 20 had become considerably reduced (Fig. 3, C and D). At day 18 of gestation many hepatocytes still appeared round and were present solitarily.

As an alternative form of light microscopical analysis of fetal hematopoietic cells, cell smears from free cell suspensions of fetal hematopoietic cells were prepared and Wright-stained. A variety of hematopoietic cells including small round cells and blastoid cells as well as mature or immature erythroid cells were observed on day 14 (Fig. 2C). This analysis suggested that erythroid hematopoiesis was the main form of hematopoiesis in fetal liver, and that the degree of hematopoiesis varied during gestation.

To analyse in further detail the populations of hematopoietic cells in fetal liver, electron microscopy has often been employed in mice (2, 63, 78)

Fig. 3 Semithin sections of fetal liver at different gestational ages (A, B, C and D represent days 14, 15, 18 and 20 of gestation, respectively). Sections were stained with toluidine blue. The number and population of hematopoietic cells change with gestation. On day 20, hepatocytes form hepatic cords as in adult liver, and the hepatocytes are no longer round as in the early gestational period. A, B, C, D; $\times 372$

and humans (103). Medlock and Haar described in detail the early liver formation (63). Some close contact of hematopoietic cells and hepatocytes was reported, suggesting some cell-cell interaction of hematopoietic cells and hepatocytes in mice (2, 63). Ohnishi and Emura described electron microscopic features of fetal liver hematopoietic stem cells in human as well as close contact of erythrocytic cells and hepatocytes (71). In the DA rat, a typical relationship between hematopoietic cells and hepatocytes was observed (Fig. 4, A and B).

Density Gradient Fractionation

To analyse hematopoietic stem cell populations, density gradient fractionation has often been employed (100). We investigated gestational changes in fetal liver hematopoietic cells using density gradient fractionation, and comparison was made with

the fractionation pattern of adult bone marrow in the gravity range of 1.02–1.10 (Fig. 5). However, this method appeared not to be useful for purifying particular cell types, since the changes in the percentages of each fraction revealed no obvious gestational changes (Fig. 5), and the morphological features of each cell fraction varied on cell smears. Furthermore, the colony-forming activity (CFU-C) of these cells varied among fractions, although fractions 3 and 4 appeared to have relatively higher CFU-C than other fractions (data not shown).

Most fetal liver hematopoietic cells are considered to be of erythroid lineage, and binding and the effect of erythropoietin to fetal liver cells or bone marrow cells are considered to be important (8, 81). It has been demonstrated that erythropoietin receptors exist on fetal liver erythroid cells in mice and rats (59, 90). Therefore, the ability of Percoll-fractionated components of fetal rat liver to bind to

Fig. 4 Ultrastructural features of hematopoietic cells and hepatocytes in fetal liver. Fourteen-day fetal liver was fixed and prepared for transmission electron microscopy. Hepatocyte (H) forms a rosette with hematopoietic cells. The hepatocyte has a nucleus with distinct nucleoli and rough-surfaced endoplasmic reticulum. The shape of the hepatocyte is not like that in adult liver. Compared with the hepatocyte, erythroblasts have scanty cytoplasm (A). B shows hepatocytes (H) and a macrophage (M) where an erythroblast appears to have started enumeration. Bar, 1 μ m

erythropoietin was measured preliminarily using ^{125}I -labeled erythropoietin (Amersham) and radioimmunoassay. Fractions 1, 2, 3 and 4 showed binding activity of 2,223, 2,249, 2,228 and 2,299 cpm per 5×10^6 cells, respectively. Compared with fetal liver hematopoietic cells, the ability of eryth-

rocytes and thymocytes to bind to erythropoietin was 350 and 399 cpm, respectively. These results also showed that although fetal liver hematopoietic cells have the ability to bind erythropoietin, the binding ability was not concentrated in any particular cell fraction prepared using Percoll gradient.

Fig. 5 Fractionation of fetal liver hematopoietic cells and adult bone marrow cells by density fractionation. Fetal liver hematopoietic cells (days 14, 16, 18 and 20) and adult bone marrow cells were layered on a Percoll gradient and centrifuged. The specific gravity of each fraction was: Fr 1: 1.02–1.04, Fr 2: 1.04–1.06, Fr 3: 1.06–1.08 and Fr 4: 1.08–1.10. Figure shows the percentage of each fraction on each gestational day and that in adult bone marrow. The patterns of days 14–18 appear similar to that of adult bone marrow but that of day 20 appears different.

Cell Scatter Analysis and Lectin Binding

As a method similar to, but far more useful than density gradient separation, flow cytofluorometry has been established for blood cell analysis. Using this method, changes in hematopoietic cells in fetal liver were analysed (Fig. 6). Cell scatter of fetal liver (days 15 and 20) and bone marrow suggested the presence of different populations of hematopoietic cells between day 15 and day 20 of fetal liver. The density gradient fractions (1–4) of 15-day fetal liver cells were also studied by cell scatter analysis (Fig. 7). The profiles of each fraction varied, but no particular distribution pattern was obtained.

The surface lectin binding characteristics of blood cells are sometimes useful for analysis. For example, peanut agglutinin-binding cell populations (74) and wheat germ agglutinin (WGA)-binding cell populations (85) were considered to repre-

sent particular differentiation stages of the cell in bone marrow. One study demonstrated enrichment of hematopoietic stem cells using density fractionation and sorting by the cell scatter or FITC-conjugated WGA in mice (54). Nicola *et al.* reported the isolation of hemopoietic progenitor cells using FITC-conjugated-pokeweed mitogen (69). However, with regard to fetal rat liver cells, not many studies of this type have been performed. We therefore analysed the WGA binding characteristics of rat fetal liver hematopoietic cells, since in mice hematopoietic stem cells were reported to be stained positively with WGA (94). In rats, WGA labeled a broad range of cells, although the peak of Percoll fraction 2 appeared to show brighter fluorescence than other fractions (Fig. 7). Thus, at least for identifying the population of brightly stained cells including stem cells, this method might be useful to some extent, but its use appears quite limited because of the broad staining profiles.

Production of a Monoclonal Antibody against Fetal Liver Hematopoietic Cells (UB-12)

Monoclonal antibody has proved extremely valuable for analysis of cell surface antigens since the establishment of the technique by Köhler and Milstein in 1975 (47). Williams, Galfrè and Milstein later succeeded in producing monoclonal antibodies to rat thymocyte membrane antigens (96), and subsequently many monoclonal antibodies reactive with leukocytes have facilitated recognition of numerous antigens which are now called clusters of differentiation (CD) in humans (50). The antigen recognized and clarified are serially classified by CD number, such as CD4 for helper T cell antigen and CD 8 for suppressor T cell antigen. There are also several monoclonal antibodies reported for hematopoietic stem cells in humans and rodents. CD34 may be one of such antigens (80). Several monoclonal antibodies against rat lymphocytes are available (95), but not many against pluripotent hematopoietic cells. Therefore, we tried to raise monoclonal antibodies against fetal liver hematopoietic cells. Fifteen-day fetal rat liver hematopoietic cells were used to immunize mice, and monoclonal antibodies against them (UB-11 and UB-12) were established by Fujikura, Kuniki and Fukumoto (20). The characteristics of these monoclonal antibodies are shown in Table 1 (20–22). UB-12 appeared specific for fetal liver hematopoietic cells. Radioactive

Fig. 6 Light-scatter analysis of fetal hematopoietic cells and adult bone marrow cells. Cell suspensions prepared from fetal liver (day 15 and 20) or bone marrow were analysed by flow cytometry. The vertical axis represents forward light scatter (FLS) and the horizontal axis shows 90° LS. Profile of fetal liver at day 15 (A) appears different from that at day 20 (B). The profiles of fetal liver hematopoietic cells from both days 15 and 20 of gestation are also different from that of bone marrow (C), but the profile for day 15 appears closer to that for bone marrow than that for day 20. Bit map 1 includes red blood cells, bit map 2 includes a lymphocyte-like population, and bit map 3 may represent the population of granulocyte-like cells.

Fig. 7 Cell scatter and WGA-positivity of density-fractionated cells. Fractionated cell populations of 15-day fetal liver cells on a Percoll gradient were analysed by a flow cytometer. Fr 3 and 4 contained distinct lymphocyte-like populations. All four fractions contained both strongly and weakly WGA-positive populations.

Table 1 *Monoclonal Antibodies Reactive with Rat Fetal Liver Hematopoietic Cells*

Monoclonal antibody	Recognition	Some particular specificities (references)
UB-12	Antigen on fetal liver hematopoietic cells or some bone marrow cells	Not react to brain, thymus and red blood cells (20, 22)
UB-11	Antigen on fetal liver hematopoietic cells or red blood cells	Not react to brain, react to thymus and red blood cells (20)
OX7	Thy-1 antigen	React to brain, thymus, and some bone marrow cells (57)
W3/13	Leukosialoglycoprotein	React to thymus cells, T cells and granulocytes (95, 96)
OX26	Transferrin receptor	(44)

Fig. 8 Binding inhibition assay of UB-12 with adult rat tissues or fetal liver. Homogenates from each organ were prepared in saline (0.5 g wet weight of tissue/ml saline). Then, culture supernatant of UB-12 diluted 5 times was absorbed with each serially diluted tissue homogenate overnight at 4°C. The absorbed antibody was reacted with glutaraldehyde-fixed fetal liver hematopoietic cells for 1 h. After washing the cells with PBS, cell-bound UB-12 was assayed with ¹²⁵I goat F(ab')₂ anti-mouse IgG, and radioactivity associated with the cells was counted in a gamma-counter. Fetal liver inhibited UB-12 strongly whereas UB-12 was not inhibited by rat red blood cells or thymus (quoted from reference 20).

Fig. 9 Immunohistochemical staining of fetal liver with UB-12. Frozen sections of fetal liver at various gestational stages (A, B, C and D for days 14, 16, 18 and 20, respectively) were stained with UB-12, and then HRP-conjugated goat F(ab')₂ anti-mouse IgG and diaminobenzidine. Sections were further counter-stained with hematoxylin. On day 14, UB-12-positive cells are present sporadically, and on day 16, an appreciable number of UB-12-positive cells are evident. On day 18, the number and expression of UB-12 antigen-positive cells has decreased, and on day 20, only focal UB-12-positive hematopoietic foci are observed among the hepatocytes. $\times 372$

binding inhibition assay of UB-12 using tissue homogenates from several organs as absorbing tissues showed that UB-12 was quite specific for fetal liver hematopoietic cells, whereas UB-12 also reacted with some cell populations in the bone marrow (Fig. 8) (22). The molecular or antigenic determinant recognized by UB-12 was then examined. From the positive distribution in fetal liver and bone marrow, and not in other tissues, the molecule which UB-12 recognizes appeared to be different from leukosialoglycoprotein (LSGP) recognized by W3/13 (96) or Thy-1 antigen recognized by OX7 (57), as Thy-1 antigen exists in thymus and brain and LSGP is expressed on polymorphonuclear leukocytes, whereas UB-12 antigen is not (Table 1, Fig. 8).

Immunohistochemical Analysis Using Monoclonal Antibodies against Blood Cells

As shown in Fig. 9, at day 14 of gestation, UB-12-positive cells were distributed among hepatocytes (Fig. 9A). On day 16, UB-12-positive cells increased in number, and among them UB-12-negative hepatocytes were distributed (Fig. 9B). On day 18, the number of UB-12-positive cells was still quite high, but the staining intensity with UB-12 was lower than that on day 16 (Fig. 9C). On day 20, focal UB-12-positive cell clusters were observed among the hepatocytes, which were forming hepatic cords (Fig. 9D). As shown in Fig. 10, in the yolk sac of fetal rat, hematopoietic cells were also stained with UB-12 antibody on day 10 of gestation,

Fig. 10 Expression of UB-12 antigen on hematopoietic cells in blood islands of the yolk sac. From a 10-day fetus, yolk sac regions were obtained, sectioned and immunohistochemically stained with UB-12. Epithelial cells (endoderm) and hematopoietic cells (mesoderm) are evident in (A). In (B), UB-12-positive cells have accumulated in the mesodermal region of the yolk sac. Cells in the endoderm (E) are negative. A, (HE) $\times 400$; B, $\times 1,000$

suggesting a phenotypic similarity of hematopoietic cells to fetal hematopoietic cells in the yolk sac, and the usefulness of UB-12 antibody for detecting hematopoietic cells in the yolk sac. UB-12 also stained satisfactorily some hematopoietic foci in adult bone marrow (22).

In fetal liver hematopoiesis, Thy-1- or W3/13 (96)-positive cells are stained sporadically during the gestational period. The number of cells stained and intensity of staining was high on days 14–18 (22). Thy-1- or W3/13-positive cells were considered to include relatively immature hematopoietic cells, especially stem cells (42, 43, 60, 61).

Degree of Expression of UB-12, Thy-1 and LSGP (W3/13) Antigens on Hematopoietic Cells Analysed by Flow Cytofluorography

The expression of UB-12, Thy-1 and LSGP (W3/

13) antigens on fetal liver hematopoietic cells from rats at days 12–20 of gestation was analysed by a flow cytofluorometry (Fig. 11, A–C). Intense expression of UB-12 antigen was observed around day 16 of gestation, and thereafter the intensity of the antigen expression decreased (Fig. 11A). Thy-1 antigen expression was high during early gestation (days 13–14) and then decreased rapidly (Fig. 11C). Compared with these antigens, W3/13 antigen expression was high on day 12 and then decreased gradually (Fig. 11B). Dual-parameter analysis of hematopoietic cells (UB-12 and Thy-1) was performed in fetal liver and adult bone marrow (Fig. 12). In the UB-12-positive population, Thy-1 low positive, Thy-1 strongly positive and Thy-1-negative populations appeared to exist in adult bone marrow (Fig. 12D), although on day 14 the largest number of Thy-1 low positive (Thy-1^{lo}) populations was observed among the fetal liver profiles (Fig. 12, A–C). In adult bone marrow, more cells were strongly Thy-1-positive and weakly UB-12-positive cells were also observed (Fig. 12D). Thy-1 low positive cells are considered to include multipotential hematopoietic cells in mice (93). These results suggest that hematopoiesis may be quite active around days 12–14 and that thereafter the number of proliferated fetal liver cells may increase up to day 18 and thus the number of recovered fetal liver hematopoietic cells was maximal on day 18 of gestation (Fig. 1).

The cells reactive with UB-12 were mostly Thy-1-negative (60%) in adult bone marrow but Thy-1-positive and UB-12-positive cells also existed in adult bone marrow (about 5%) (Fig. 12D) and in the 14-day fetal liver (1.5%) (Fig. 12A). These cells might be of interest because of their ability to proliferate in lethally irradiated recipients. We are currently examining this possibility. Hunt showed that Thy-1 positive cells in fetal rat liver at day 19 of gestation had the CFU-S activity, and were progenitors of the B lymphoid cell line (43). Changes in major histocompatibility complex (MHC) class I antigen on murine hematopoietic progenitor cells have also been reported by Okamoto *et al.* (72). Purified hematopoietic progenitor cells from mouse bone marrow have been prepared using a combination of several monoclonal antibodies against blood cells and cell sorting with a fluorescence-activated cell sorter (66, 85, 93).

Shift of Hematopoietic Cells from Fetal Liver to Bone Marrow

Fetal liver hematopoietic cells as well as about 30%

Fig. 11 Gestational changes in UB-12, Thy-1 and W3/13 antigen expressions in fetal liver hematopoietic cells. Fetal liver hematopoietic cells were labelled with UB-12 (A), Thy-1 (B) and W3/13 (C), followed by FITC-labelled anti-mouse IgG and analysed by flow cytofluorometry. The percentage labelling and degree of expression of UB-12 antigen have decreased after 17 days of gestation (A). Thy-1 antigen expression and percentage of Thy-1-positive populations have decreased rapidly after 14 days of gestation (B). The percentage labelling and degree of expression of W3/13 antigen decreased gradually after day 13 (C).

of bone marrow nucleated cells were stained with UB-12. As described in the previous section, this antibody also labelled hematopoietic cells in the yolk sac (Fig. 10). Therefore, we used this antibody to follow the movement of hematopoietic foci using radioimmunoassay. Fig. 13 shows the profile of radioactive binding, which was obtained from the data on bound radioactivity (cpm) for suspensions of 10^7 cells prepared from each organ at different periods of gestation and ages after birth. The radioactivity in liver decreased after day 16 of gestation and became quite low after birth, whereas the binding activity of bone marrow cells and spleen cells increased around the time of birth. Before day 70 after birth, the binding activity in the spleen decreased rapidly, while that in the bone marrow remained to be high. These semiquantitative results clearly showed a shift in hematopoietic foci from fetal liver to spleen and bone marrow, and subsequently the hematopoietic foci in bone marrow remained as the main site of hematopoiesis throughout life.

Fig. 14 shows the initiation of hematopoiesis in fetal bone marrow (A, gestational day 19 and B, gestational day 20) followed by definitive and

extensive hematopoiesis after birth (C, day 1 and D, day 4). These results appear to be consistent with those shown in Fig. 13.

In a study of hematopoietic cell shift in mice, Moore and Metcalf (65) demonstrated changes in the numbers of *in vivo* colony-forming cells (CFU-S) in the yolk sac, circulation and liver: the peak of CFU-S in the yolk sac was reached on day 11 (about $10^2/10^5$ cells), that in the circulation on day 12 (about $10/10^5$ cells) and that in the fetal liver on day 13 ($10^4/10^5$ cells). This result suggests that pluripotent hematopoietic cells tend to shift from the yolk sac into the circulation and then into the fetal liver. It is reported that in mouse spleen, hematopoiesis starts around day 15 of gestation (15). In our study hematopoiesis in the spleen appears to start on days 18–19 of gestation in DA rats (Fig. 13). There are also reports describing the distribution of hematopoiesis in the human embryo and fetus (5). In the human fetus in the 105 mm crown-rump length stage, hematopoietic cells are recognizable in the bone marrow (6). Functional studies of fetal liver hematopoiesis have been made by several researchers (56, 64, 89). Micklem *et al.* showed that fetal liver hematopoietic cells had higher self-renewal

Fig. 12 Gestational changes in fetal liver hematopoietic cells (A–C) and adult bone marrow cells (D) revealed by OX7 and UB-12 double labelling. Fetal liver hematopoietic cells were labelled with OX7 and UB-12. The double-positive cell population in 14-day fetal liver (1.5%) (A) were higher than those in 16-day fetal liver (0.24%) (B) and 18-day fetal liver (0.09%) (C).

capacity than adult bone marrow cells (64). Lucarelli *et al.* (56) and Kelemen *et al.* (45) showed that fetal liver hematopoietic cells could be substituted for adult bone marrow cells in lethally irradiated mice. Thomas, Smith and Sumpster (89) showed the equation of $(Dm/Dl) = (Sl/Sm) \times (Vl/Vm)$ for explaining the results of CFU-S analysis (donor derived colony forming cell numbers established in irradiated host spleen) in mice; numbers of cells required to effect a 50 mg increase in spleen weight for each, adult bone marrow cells and 16 day fetal liver cell are expressed as Dm and Dl, respectively; number of colony forming units (CFU-S) per 10^5 cells for bone marrow and fetal liver are expressed as Sm and Sl, respectively; and size of colonies formed in the spleen by cells derived from bone marrow and fetal liver are expressed as Vm and Vl, respectively. The experimental values for each were; $Dl = 3.0 \times 10^6$ and $Dm = 2.2 \times 10^6$, $Sm = 9.6$, $Sl = 3.7$, $Vm = 1.2 \text{ mm}^3$ and $Vl = 2.6 \text{ mm}^3$. From these

values they concluded that adult bone marrow cells contained two and a half times as many stem cells as the same number of fetal liver cells ($Sl/Sm = 3.7/9.6$), but that the number of progeny derived from a single fetal liver stem cell was rather more than double the number of progeny derived from a single bone marrow stem cell ($Vl/Vm = 2.6/1.2$) during 10 days following transplantation to the spleen of a lethally irradiated recipient (89). In our preliminary study, two different sources of fetal rat liver hematopoietic cells were prepared (days 15–16 and days 18–19 of gestation). Similar numbers of cells in each group and adult bone marrow cells (5×10^7) were used to reconstitute lethally irradiated rats. The cells obtained on days 18–19 failed to save the animals, whereas the cells obtained on days 15–16 of fetal liver and adult bone marrow cells succeeded in saving them (Tokuda *et al.* in preparation).

Fig. 13 Changes in UB-12 binding activity in fetal liver, spleen and bone marrow at various ages determined by radioimmunoassay. Cell suspensions from fetal liver, spleen and bone marrow were prepared. The cells (1×10^7) were incubated with UB-12 and then with ^{125}I -labelled goat F(ab')_2 anti-mouse IgG. Cell-bound radioactivity (cpm) was plotted against age (days). The results are plotted for fetal liver as ----, spleen —, and bone marrow —, respectively. Results are expressed schematically. The data show that, UB-12 activity was high in fetal liver during the fetal period, and then the reactivity shifted to the spleen and bone marrow. Subsequently, after day 50, only the bone marrow showed constant extensive UB-12 binding. These results appear to reflect well the movement of hematopoietic foci in the rat. An arrow shows day 14 of gestation.

ANALYSIS OF HEPATOCYTES IN FETAL RAT LIVER (COMPARISON WITH ADULT HEPATOCYTES)

Hematopoietic foci appear after hepatocytes start to proliferate in the liver and then disappear around birth when the number and functional activity of hepatocytes increase markedly. Therefore, one way to study the reasons for the occurrence of hematopoiesis in fetal liver, is to analyse the changes in morphology and function of hepatocytes. Thus, in the following section, I will consider morphological and phenotypic changes in fetal hepatocytes at different gestational ages in comparison with those of adult hepatocytes with no hematopoietic activity.

Histological Features of Fetal Hepatocytes

In the fetal liver at day 14 of gestation a few hematopoietic cells were observed among the hepatocytes (Fig. 3A). The hepatocytes appeared round,

and showed weak cytoplasmic staining. At this stage, hepatic cords were not well developed and when the tissues were stained with silver, not many reticular fibers were observed along the sinusoidal space compared with the adult (Fig. 2B). At day 15 of gestation, many hematopoietic cells had infiltrated into the liver (Fig. 3B), and cells undergoing mitosis were observed. The hepatocytes appeared round and were closely opposed to either hepatocytes or hematopoietic cells. Electron microscopy at day 14 of gestation showed that the cytoplasm of hepatocyte had expanded and each was in close proximity to several erythroid cells (Fig. 4A). At day 16, some hepatocytes were located closely adjacent to hematopoietic cells, but in other areas, hematopoietic cells were closely associated with mesenchymal cells. At day 18, some hepatocytes appeared rectangular and were juxtaposed with similar hepatocytes, whereas other hepatocytes were round and located close to hematopoietic cells (Fig. 3C). On day 20, distinct morphological changes in hepatocytes were obvious and hepatic

Fig. 14 Histological features of bone marrow around the perinatal period. Bone marrow was obtained from the femur, and paraffin-embedded, sectioned and stained with HE. On day 19 of gestation (A), bone marrow appears scarce and fibrocyte-like cells, possibly functioning as stromal cells, are dominant. On day 20 (B) and one day after birth (C), hematopoietic foci have become obvious. On day 4 after birth (D) the structure of the bone marrow has come to resemble that in the adult. HE, $\times 332$

cords become established. The polarity of hepatocytes became dominant, and their cytoplasmic staining appeared quite different from that at the previous gestational ages (Fig. 3D).

Monoclonal Antibodies against Rat Hepatocytes

In comparison with monoclonal antibodies against leukocytes, not many monoclonal antibodies against hepatocytes have been available for humans and rodents. The most obvious reason for this is that for purposes of analysis the hepatocytes are not easy to obtain, whereas leukocytes can be taken easily from blood.

To characterize the features of rat hepatocytes and to analyse their differentiation, we have raised monoclonal antibodies against adult rat hepato-

cytes. Monoclonal antibodies directed against cell membrane glycoproteins, prepared using a lentil-lectin affinity column, were called HAM1, HAM2 and HAM3 (31, 33). A monoclonal antibody against rat liver cell membrane glycoproteins, which were prepared from wheat germ agglutinin, was called HAM4 (87). Using fetal liver as an antigen, we obtained monoclonal antibody HAM5, which was found to bind to both fetal and adult hepatocytes (20). Recently, we also established a monoclonal antibody (HAM8) against rat gap junction (23). The characteristic features of these monoclonal antibodies against rat hepatocytes are shown in Table 2. Hepatocytes have three faces: sinusoidal, contiguous, and bile-canalicular (Fig. 15A) (98). The sinusoidal face borders the sinusoid of the liver and is an important site of absorption

Table 2 *Characteristic Features of Monoclonal Antibodies against Rat Hepatocytes or Hyperplastic Liver Nodules*

Monoclonal antibody	Antigen	Antigens used to immunize (references)
HAM1	*	Liver membrane glycoprotein binding to lentil lectin (31)
HAM2	MHC class I	Liver membrane glycoprotein binding to lentil lectin (31, 33)
HAM3	*	Liver membrane glycoprotein binding to lentil lectin (31)
OX61	Dipeptidyl peptidase IV	(62)
HA4	11 KDa molecule	(41)
HAM4	*possibly dipeptidyl peptidase IV	WGA binding liver glycoprotein (58, 83, 87, 101)
HAM5	*	Fetal liver cells (20)
HAM6	*	Hyperplastic liver nodules (73)
HAM7	*	Hyperplastic liver nodules (73)
HAM8	Connexin 32	Gap junction molecule (23)

* The molecular nature of antigen is not clarified.

and secretion between the hepatocyte and the sinusoid (Fig. 15B, arrowheads). Several receptors for hormones, such as insulin receptor (10), have been reported to exist on hepatocytes. The contiguous face is a cell-cell adhesion area where gap junctions exist. Other molecules, possibly components of junctional complexes, may be located on this face. The bile-canalicular face is where bile is secreted into bile canaliculi (Fig. 15B). Thus, hepatocytes are quite different from cells such as lymphocytes which have no polarity. Ontogenically, hepatocytes are derived from the endodermal epithelium of the intestine, and their features are quite similar to those of the intestinal epithelium. The bile-canalicular face of the hepatocyte is equivalent to the luminal surface of the intestine, the contiguous face is equivalent to that of intestine, and the basal membrane of the intestine appears to be similar to the sinusoidal face of the hepatocyte with regard to their common functional situation of molecular exchange between cells and blood vessels.

HAM4 recognizes predominantly the bile canalicular face of hepatocytes (Fig. 16, A, B, C and Fig. 18A). The molecule recognized by this antibody is considered to be dipeptidyl peptidase IV (Table 2). A few monoclonal antibodies equivalent to HAM4 have been reported, including HA4 and OX61 (41, 62). We have investigated the ultrastructural distribution of HAM4 antigen using immunoelectron microscopy (101). The antigen recognized by this

antibody is distributed not only on the bile-canalicular face but also the sinusoidal face, although the distribution tends to be far more predominant on the former (101). When isolated hepatocytes were prepared from adult rat liver and cultured, the HAM4 antigen was distributed around the surface of hepatocytes (101). The formation of couplets of hepatocytes induces the formation of bile canaliculi as well as HAM4 localization on their surfaces (83). When isolated hepatocytes were prepared from adult rat liver and injected directly into the spleen of partially hepatectomized rats, hepatic cords were reestablished and HAM4 expression was observed on the bile-canalicular face (Fig. 16C) (Fujikura *et al.* in preparation). HAM2 antigen is distributed on the sinusoidal, bile-canalicular and contiguous faces, although predominantly on the sinusoidal face (101). We succeeded in purifying the HAM2 antigen molecule using HAM2 affinity chromatography (33), and the purified antigen cross-reacted with OX18, which is a monoclonal antibody against rat MHC class I antigen (32).

HAM1 recognized predominantly the sinusoidal face, but also the bile canalicular face to some extent, as confirmed using the immunogold technique (Fig. 15C) (Fukumoto *et al.* in preparation). In light-microscopical immunohistochemistry of normal rat liver, HAM1 and HAM3 staining was not useful, as staining was quite weak. HAM1 antigen expression analysed by flow cytofluorography and

Fig. 15 Architecture of the liver. (A) shows a schematic representation of the three faces of hepatocytes; the sinusoidal face, bile-canalicular face, and contiguous face. Facing the sinusoidal face is Disse's space. Scanning electron microscopy of rat liver shows hepatic cords where bile canaliculi are present (an arrow) and facing the sinusoid (S), and sinusoidal face of hepatocyte, Disse's space can be seen (arrowheads) (B). Immunoelectron microscopy of adult liver using HAM1-gold (C). The antigen is localized predominantly on the sinusoidal and bile canalicular faces of hepatocytes. Bar, 1 μ m

Fig. 16 Immunofluorescence and immunohistochemical staining of adult rat liver. Adult DA-strain rat liver was frozen-sectioned. Staining was done with HAM4 and subsequently with FITC-labelled goat $F(ab')_2$ anti-mouse IgG. The stained sections were examined by fluorescence microscopy. Strong fluorescence is evident along the bile canaliculi (A). In immunohistochemical staining using HAM4 and HRP-conjugated goat $F(ab')_2$ anti-mouse IgG, a similar staining pattern to that in (A) is evident. This section is counterstained with hematoxylin (B). In transplanted and reconstituted hepatocytes in the spleen, strong immunohistochemical staining of HAM4 antigen is seen along the bile-canalicular face (arrows) (C). A, $\times 800$; B, $\times 800$; C, $\times 1,000$

Fig. 17 Immunohistochemical staining of fetal liver with HAM4. Frozen section of fetal liver (A, B, C and D for days 14, 16, 18 and 20, respectively) was stained with HAM4 ascites. Then the sections were stained with HRP-conjugated affinity-purified goat F(ab')₂ anti-mouse IgG, and treated with diaminobenzidine. HAM4 antigen expression is not obvious on day 14. On day 16, antigen expression has increased, showing scattered bile-canalicular structures. On day 18, the staining of HAM4 has become stronger than that on day 16, and on day 20 a typical HAM4 staining network similar to that of adult liver is evident (Fig. 16, A and B). $\times 336$

radioimmunoassay was extensive on rat hepatoma cell lines, although most hepatocyte membrane antigens recognized by monoclonal antibodies against normal hepatocytes were expressed less on rat hepatoma cell lines than on the cell surface of normal hepatocytes (31, 87). HAM8 recognizes rat connexin 32, and the distribution of this antigen is sporadic along the contiguous face of the hepatocyte (23). The expression of HAM8 antigen is also less on rat hepatoma cell lines (23). To examine the degree of expression of hepatocyte antigens detected by these monoclonal antibodies on fetal liver, immunohistochemical staining was conducted.

Immunohistochemical Staining of Adult and Fetal Hepatocytes

Fetal hepatocytes were examined using several monoclonal antibodies against hepatocytes, macrophages or antigens of other cells (Tables 1, 2 and 3). On day 14, HAM4 antigen was not obvious but observed on some hepatocytes located near hematopoietic cells (Fig. 17A). On day 16 it was clearly observed among some hepatocytes (Fig. 17B). The reactive areas were sparsely scattered. As gestation proceeded, the degree of expression increased (Fig. 17, C and D; Table 3). HAM2 antigen expression was first observed about day 16 of gestation, and thereafter became intense (Table 3). Immunoelec-

Table 3 *Antigen Expression of Fetal Liver Hematopoietic Cells and Hepatocytes in the Rat Analysed by Monoclonal Antibodies and Immunohistochemistry*

Monoclonal antibody	Antigen expressions (gestational day)			
	14	16	18	20
ED1	—	—	±	+
ED2	—	—	±	+
ED3	—	—	±	±
Mar3	—	±	+	+
HAM4	—~±	±	+	+
HAM2	—	±	±	+
HAM8	—	—	±	+
HAM5	+	+	+	+
OX7	+	±	±	±
UB-12	++	++	+	±
UB-13*	++	+	—	—

References: for ED1, ED2 and ED3 (14), Mar3 (102), HAM4 (87), HAM2 (31), HAM8 (23), HAM5 (20), OX7 (57), UB-12 (22) and UB-13 (91). The degree of antigen expressions were expressed as — negative, ± weakly or sporadically expressed, + expressed significantly, ++ expressed strongly. * This antigen was not on either hematopoietic cells or hepatocytes, but on some fibrous tissues.

tron microscopic examination of HAM4 antigen was conducted on fetal liver (Fig. 18B). The antigen was found to be expressed at the later stages of gestation, but the degree of expression was much reduced compared to the adult levels (Fig. 18).

Thus, we found that most surface antigen molecules on the adult rat hepatocytes detected with monoclonal antibodies were not yet expressed on fetal hepatocytes or if present, were expressed only weakly (Figs. 17 and 18; Table 3). These included MHC antigen class I, hepato-renal antigen or gap junction molecules (23, 58, 87). Thus, it was evident that the hepatocyte membrane antigens of adult rats were largely different from those of fetal rats, suggesting some functional alterations of hepatocytes between the fetal and adult periods. To clarify this point further, we attempted to raise monoclonal antibodies against rat fetal liver cells, as we considered that fetal hepatocytes might have some unique membrane molecules. It was not possible to raise a specific monoclonal antibody which could differentiate between fetal and adult hepatocytes, but HAM5 antibody, recognizing both cell types, was obtained (20).

During the early gestational period up to day 14, no particular staining was observed with the antibodies used, although UB-13-reactive tissues were most evident in day-14 fetal liver, (Fig. 19C) and

the reactive area appeared to become more faint and scattered as gestation proceeded (Fig. 19D; Table 3). Specific staining of fibrous tissues in fetal liver with UB-13 may represent an important feature of hematopoiesis since reticular fiber-like structures in normal rat thymus showed staining with UB-13 (91), and the thymus in the sublethally irradiated and recovering rats showed much intense staining with UB-13 than in normal, suggesting that these structures may help to create a microenvironment suitable for thymocyte re proliferation (Kuniki *et al.*, in preparation). Therefore, the reduction in intensity of UB-13 staining in fetal liver from day 14 of gestation onwards might be related to some changes in some types of hematopoiesis in fetal liver. As the thymus starts to grow with thymocyte production around day 14 in DA rat (51), the strong staining with UB-13 in early fetal liver before day 14 may indicate the existence of some T-cell equivalent cell growth in fetal liver.

ED1-, ED2-, ED3- (14) or Mar-3- (102) reactive cells thought to be macrophages, were examined extensively as it is generally considered, that reticulum cells or macrophages are important cells for the stromal cells of hematopoiesis; for example, in Bessis's hemocytoblastic islets (4), usually macrophage like cells are considered as stromal cells. However as shown in Table 3 and Fig. 19, cells reac-

Fig. 18 Immunoelectron microscopic localization of HAM4 antigen in adult and fetal liver. A hepatocyte suspension was prepared from day 18 fetal liver or adult hepatocytes following the method described by Yamaguchi *et al.* (101). The hepatocytes were treated with HAM4-gold, embedded in Epon, ultrathin-sectioned and examined by electron microscopy. HAM4 antigen detected with immunogold (25 nm and 45 nm, see reference 101) is evident along the bile-canaliculi of adult rat hepatocyte (arrows) (A). As in the adult, in 18-day fetal liver hepatocytes immunogold (15 nm) was seen on some areas of hepatocytes (arrows) (B). Bar, 1 μ m

tive to monoclonal antibodies to macrophages were not possible to detect before day 18 although hematopoiesis was quite active even before that day. The numbers of these reactive cells appeared to increase as gestation proceeded (Table 3). Fig. 19 shows the expression of ED1 antigen in fetal liver at day 18 of gestation and no particular staining was observed on day 14 of gestation (Fig. 19A).

POSSIBLE DEVELOPMENTAL INTER-ACTIONS BETWEEN HEMATOPOIETIC CELLS AND HEPATOCYTES

In DA rats, hematopoiesis was observed on days 12–20 of gestation in fetal liver (Figs. 1–5, Figs. 11–13). The morphological changes in fetal liver suggested the following gestational changes in hemato-

poiesis (Fig. 20): just before the initiation of hematopoiesis before day 12, mainly hepatocytes exist (stage 1), intensive hematopoiesis on days 12–16 (stages 2, 3), maximum number of hematopoietic cells on day 18 but the morphological and functional changes of hepatocytes started (stage 3), and the diminution of hematopoietic foci on days 19 and 20 (Fig. 1, Fig. 9 and Fig. 20, stage 4).

To clarify the reasons for these marked changes in hematopoiesis in fetal liver within such a short period, we analysed the gestational changes in cell surface membrane antigens of both hematopoietic cells and hepatocytes using monoclonal antibodies reactive with either cell type, since it is considered that the changes in their interaction were instrumental in the shift of hematopoiesis in fetal liver, and that cell surface molecules were involved in

Fig. 19 Immunohistochemical staining of fetal liver with an anti-rat macrophage monoclonal antibody (ED1) (A, B) and UB-13 (C, D). The section was stained with monoclonal antibody and then with HRP-conjugated affinity-purified goat $F(ab')_2$ anti-mouse IgG. No or weak staining with ED1 is present on day 14 (A). On day 18 (B), some strong staining was evident sporadically (arrows). Within the UB-13-positive network (arrows), hepatocytes and hematopoietic cells exist on day 14 liver (C). No particular staining was observed on day 18 liver (D). $\times 400$

Fig. 20 Schematic drawing of gestational changes in hematopoietic cells and hepatocytes in the fetal rat. Before day 12 of gestation, immature-type hepatocytes form primitive hepatic cords along the sinusoids, and no clear hematopoietic foci are observed (stage 1). On days 12–15, hematopoiesis, especially erythropoiesis, occurs among the hepatocytes. UB-12 antigen expression on hematopoietic cells is intense. The liver consists of non-systematical cell mass of both hepatocytes and hematopoietic cells. Both cells may interact or combine each other through the weak and loose forces. Morphologically the hepatocytes are still primitive, and around them, rosette formation by erythroid cells is evident. The expression of HAM4 antigen is not obvious (stage 2). On days 16–18, some hepatocytes appear well polarized, and the staining of UB-12 on hematopoietic cells appears decreased in comparison with that on days 14–15. HAM4 antigen expression on some hepatocytes is obvious (stage 3). On days 19–20, the morphological features of hepatocytes have changed markedly. The hepatocytes show obvious polarity and their cytoplasm is stained intensely. The staining of HAM4 begins to resemble that in adult, but is still not as strong. The UB-12-positive cell population has decreased markedly (stage 4).

these interactions.

Some Membrane Antigens on Hematopoietic Cells Mediate Important Roles for Cell-Cell Interactions

Recently Lewinsohn *et al.* reported that the hematopoietic progenitor cells expressed H-CAM (homing-associated adhesion molecule) (CD44) in humans (53). Arkin *et al.* reported the expression of intercellular adhesion molecule-1 (CD54) on hematopoietic progenitors (1), suggesting the important cell-cell interactions between hematopoietic progenitor cells and stromal cells. In our experiment the results clearly showed that the antigenic changes on the hematopoietic cells detected with monoclonal antibodies including UB-12 paralleled the morphological changes in hematopoietic foci in fetal liver; when hematopoiesis was intensive (days 12–15), the degree of expression of UB-12 antigen was quite intense (Fig. 11) and when hematopoiesis became reduced (days 18–20), the expression of the antigen also became weak (Fig. 11). Although the nature of the antigenic determinant recognized by UB-12 was not clarified, it appeared to be different from any rat antigens reported previously. Judging from the changes in expression of UB-12 antigen on hematopoietic cells, and hematopoietic cells are usually closely contacted to either hematopoietic cells or hepatocytes, we considered that the antigen (or antigenic determinant) might be important for interaction of hematopoietic cells with stromal cells in fetal liver. In fact there is a report showing the interactional changes in fibronectin and fibronectin receptor during the transition between erythroblast and erythrocytes (75). It appears that UB-12 antigen is different from functional molecules such as the transferrin receptor (recognized by monoclonal antibody OX26) (44) or the erythropoietin receptor. At present the possibility of the adhesion between UB-12-positive hemopoietic cells and fetal hepatocytes is intensively explored to examine the effects of UB-12 antibody on these cell interactions *in vitro* and *in vivo*.

Fetal Hepatocytes Are Different from Adult Hepatocytes

We have analysed the expression of membrane antigens on fetal hepatocytes, since in fetal liver, close contact between hematopoietic cells and hepatocytes was observed (Fig. 4A), and several

other researchers have reported that such interactions might be important for hematopoiesis (2). Monoclonal antibodies against hepatocyte membrane antigen, have also been reported by several groups (31, 41, 62). HA4 was the one of the earliest monoclonal antibodies produced against rat hepatocytes, recognizing an 11-KDa molecule (Table 2) (41). We have also reported several monoclonal antibodies against rat hepatocyte membrane glycoproteins since 1984 (Table 2; 20, 23, 31, 73, 87), and these have been designated HAM1~8. Immunohistochemical and immunoelectron-microscopical analysis showed that, on rat hepatocytes, there are three types of membrane antigen; first, there are membrane antigens localized predominantly on the bile-canalicular face. Second, there are membrane antigens associated with the sinusoidal face of hepatocytes, which are important for molecular transport or as receptors for circulating bioactive materials such as hormones and interleukins (ILs). Third, there are molecules contributing to junctional complexes, which are present on the contiguous face of hepatocytes. To maintain hepatocyte function normally, it appears to be important that cell polarity is preserved (sinusoidal, contiguous and bile-canalicular polarity) and that the proper membrane molecules are expressed on each face of normal adult rat hepatocytes. However, transformed-type hepatocytes, often show a loss of polarity, and the membrane antigens specific to each cell face show a decrease in expression (58, 73, 87). Some cells which have lost their polarity such as oval cells (17) may be important as hepatocyte stem cells (77, 84). Some transformed cell or tissue of liver may express the unique antigens (73). In fetal liver, similar phenomena were found to occur. Most hepatocytes had no definite polarity, and the degree of expression of membrane antigens detected with our monoclonal antibodies against adult hepatocytes was very low, if present. This finding suggests that fetal hepatocytes are functionally different from adult hepatocytes. We have reported previously that immunoglobulin catabolism takes place in the liver (25, 27-29). Furthermore catabolism of immunoglobulin G occurs in the liver of adult but not fetal sheep, suggesting a functional difference between fetal and adult liver (26, 30). Greengard (35) reported gestational changes in hepatic enzymes which were divided into three developmental periods; late fetal (e.g. UDPG-glycogen glucosyltransferase), neonatal (e.g. phosphoenolpyruvate carboxylase) and late suckling (e.g. tryptophan oxygenase). In parallel with these

periods, the functional changes in hepatic enzymes were observed, suggesting that fetal hepatocytes did not function in the same way as adult hepatocytes. Furthermore, Nakamura, Nagao and Ichihara have reported autocrine-type growth ability of fetal hepatocytes but not adult hepatocytes (67). However, if fetal hepatocytes are in contact with adult hepatocytes, the former appears to lose the ability to carry out autocrine cell growth (67). These results may show that fetal hepatocytes do not function like those in adult liver, and instead have a unique ability to proliferate. The main signal determining the difference between adult and fetal hepatocyte might be expressed on the cell membrane. Among the specific cell membrane molecules important for cell-cell communication in fetal hepatocytes, some types of cell adhesion molecule such as cadherin may be important (86). Our results clearly showed a distinct difference of fetal hepatocytes with regard to the expression of cell membrane antigens detected with monoclonal antibodies reactive to adult hepatocytes.

Possible Role of Fetal Hepatocytes as Stromal Cells for Hematopoietic Cells

Recently, long-term bone marrow hematopoietic culture has become possible using stromal cell lines as well as several growth factors such as colony-stimulating factors (CSF) and interleukins. From mice, Kodama *et al.* (46) and Ogawa *et al.* (70) have established adipose cell lines or fibroblast-like cell lines which can support the growth of bone marrow cells in culture. Ogawa *et al.* have shown that ST2 cells can support stem cell-like cell growth *in vitro* (70). Similar cell lines from fetal rat liver may also be useful for hematopoiesis. Thus, we tried to use ST2 cells for culturing rat bone marrow cells or fetal liver cells. However the ST2 cell line was found not to be useful for rat bone marrow or fetal rat liver cells (data not shown).

Bessis described the role of macrophage-like cells in supporting red blood cell hematopoiesis in erythroblast islets (4). The erythroid cells are apparently surrounding the macrophages. These morphological features were also evident in bone marrow, fetal liver, and young spleen. In fetal liver there are many close contacts of fetal hepatocytes with hematopoietic cells, suggesting some cell-cell interactions (1, 63). As previously described the low or lack of expression of adult-types of membrane antigens on fetal hepatocytes as well as poor polarity of fetal hepatocytes could be considered favor-

able for the close contact of hematopoietic cells with fetal hepatocytes compared with the presence of adult-type hepatocyte membrane antigens such as MHC class I or hepato-renal antigens. Thus, in fetal liver, instead of macrophages, fetal hepatocytes may act as stromal cells for fetal hematopoiesis. In fact, recently Hata *et al.* (37) has established a new cell line of fetal hepatocytes which can support hematogenesis in mice. For the first time they showed that cloned cell lines of fetal hepatocytes can support hematopoiesis *in vitro*, and that they secrete factors to stimulate the growth of granulocyte-macrophage cell lines. On the other hand, it has been reported that adult or fetal hepatocytes have the potential to produce erythropoietin (18, 19, 49). Thus, it is quite possible that some other types of fetal hepatocytes could act as stromal cells for erythroid hematogenesis. The morphological changes of hepatocytes following gestation such as hepatic cord formation may obviously not favor the presence of hematopoietic cells in the liver, as the hepatocytes are in tight contact each other by the junctional complexes and the areas where hematopoietic cells grow may become quite narrow (Fig. 20, stage 4). Subsequently, the hematopoietic focus may disappear from the liver around the birth.

It is reasonable to consider that the main stromal cells in fetal liver would be fetal hepatocytes. However, mesenchymal components such as fibroblasts, adipose cells and macrophages or endothelial cells might also be carefully considered as stromal cells for fetal liver hematopoiesis (3). In fact Crocker, Morris and Gordon showed that some adhesion receptors play important roles in the interactions between macrophages and hematopoietic cells in bone marrow and fetal liver (9).

It has also been reported that stem cell factor has been isolated from rat liver-cell-conditioned medium (55).

Besides the use of fetal hepatocytes and mesenchymal cells as stromal cells, there is some evidence that fibrous components such as reticulin fibers or collagen fibers in fetal liver may play a role in hematopoiesis in the same way that interactions of hematopoietic cells and fibronectin are considered important (34, 36, 39, 75, 104). Our findings shown in Fig. 19 (C, D) might be also important from such viewpoint.

Why Does Shift of Hematopoiesis Occur in Fetal Liver?

Several reports have described a shift of hemato-

poiesis in mammals (6, 88). This may happen due to changes in the hematopoietic microenvironment (13, 92, 99). However, details of the mechanism responsible for this shift remain unclear. Therefore, we tried to predict some of these mechanisms by analyzing the changes in fetal liver hematopoiesis. When fetal liver hematopoiesis is maximal at day 18 of gestation, bone formation progresses in DA rats (Fig. 14). After bone formation, bone marrow hematopoiesis starts. After birth, the histological features of hematopoiesis are dominant in bone marrow. These results suggest that circulating pluripotent hematopoietic cells at around day 18 may be trapped in newly established bone tissue, and that thereafter they may proliferate as bone marrow. Thus, a significant number of blood cells could be supplied by bone marrow gradually at a later stage. This may reduce the demand of hematopoiesis in fetal liver, and the speed of hematopoiesis may thus slow down; subsequently the stromal potential of fetal liver may be reduced because of less stimulation by the hematopoietic cells. As described previously around this time, membrane antigen expressions, and morphological and functional changes of hepatocytes coincide and these changes may be unfavorable for the hematopoietic cells. These sorts of environmental changes result in reduction of hematopoiesis in fetal liver and subsequent disappearance of hematopoiesis in liver after birth. From this analysis, the shift of hematopoiesis can be explained by extensive hematopoiesis in new hematopoietic foci (in this case, newly established bone marrow) and subsequent gradual reduction of hematopoiesis in the 'old' hematopoietic foci (in this case, fetal liver).

Now, relating to the shift of hematopoiesis, I would like to consider one question here, that is; would it be possible for hematopoietic foci to become reestablished in adult liver? In some diseases such as leukemia and aplastic anemia, extramedullary hematopoiesis in the adult liver has been reported (7, 48). Therefore, one method for reestablishing hematopoiesis in liver may be to inactivate the bone marrow, or to stimulate blood cell hyperproduction by the body. Estrogen can induce hematopoiesis in adult mouse liver which was shown by Hayama *et al.* (38). This may be due to an effect on both hepatocytes and hematopoietic cells. They also mentioned the important role of estrogen-activated Kupffer cells for hematopoiesis. Judging from the fetal liver hematopoiesis in liver, it might be possible to create hematopoiesis in adult liver if the hepatocytes are very immature and

bone marrow hematopoiesis is inactivated. Partial hepatectomy induces hepatocyte proliferation and at that time the hepatocytes appear morphologically and functionally immature, although, usually no clear extramedullary hematopoiesis was reported in the regenerating liver. Thus, at the moment to reestablish intensive hematopoiesis in adult liver might be quite difficult.

CONCLUSION

Hematopoiesis takes place in the fetal liver of mammals. It is still not clear why hematopoiesis should occur in the fetal liver and not in the adult liver, and why this is a feature peculiar to mammals fetal liver.

To help clarify these aspects, we analyzed changes in hematopoiesis in terms of morphology, flow cytofluorometry and immunohistochemistry using monoclonal antibodies reactive with either hematopoietic cells or hepatocytes. We considered it important to analyze both hematopoietic cells and hepatocytes, since the occurrence of hematopoiesis in the fetal liver might be due to some beneficial effect on both hematopoietic cells and hepatocytes. Furthermore, the mechanisms governing the changes occurring in the fetal liver may depend largely on cell-cell interactions among hematopoietic cells and hepatocytes, which may be mediated through the molecules expressed on the membranes of both cell types.

We used several monoclonal antibodies against rat blood cells, since the use of monoclonal antibodies against leukocytes in humans, mice and rats has become available. In rats, however, not many monoclonal antibodies are available for hematopoietic cells especially for the stem cells except OX7 (anti-Thy-1 antigen) or W3/13 (anti-leukosialoglycoprotein) antibodies.

Therefore, we raised a monoclonal antibody, UB-12 against fetal liver hematopoietic cells. The nature of the antigenic determinant appeared to differ from the molecules reported previously, being distributed in a range of cells from yolk sac hematopoietic cells, to about 30% of adult bone marrow cells, and it was quite useful for analyzing the shift in hematopoiesis semiquantitatively (Fig. 13). We analyzed the expression of UB-12 antigen immunohistochemically and immunocytologically in both fetal liver hematopoietic cells and adult bone marrow cells, and found that the expression of surface antigens on the two cell types were fundamentally similar but some functional differ-

ences were shown, especially following gestations. Monoclonal antibodies including OX7 and W3/13 were also used for comparison between fetal hematopoietic cells and adult bone marrow cells. The multipotent hematopoietic cells were thought to be Thy-1^{lo}-positive, and this population comprised about 10% of the cells in adult bone marrow. In the fetal liver, a significant amount of Thy-1 expression was observed around days 14–16 (Fig. 12), and thereafter the Thy-1-positive population was reduced suggesting a decline of hematopoiesis. The pattern of expression of UB-12 antigen was quite similar to that of hematopoietic changes. Thus, the interactional function of UB-12 antigen with stromal cells could be considered. In a functional study, fetal liver also had CFU-C and CFU-S activity compatible with adult bone marrow, but in the late stage of gestation, at days 18–20, the activity of CFU-S was reduced.

In addition, we attempted to analyse changes in hepatocyte membrane antigen expression during gestation to clarify alterations in fetal hematopoiesis, since we considered it important to analyse the interaction between these cell types. Electron microscopy had revealed a close association between hepatocytes and hematopoietic cells. In comparison with hematopoietic cells, a number of monoclonal antibodies against rat hepatocytes are not available, although the number of cell membrane antigens on hepatocytes might be equivalent to that on leukocytes. Unfortunately, however, there is no CD-like information on hepatocytes in humans and rodents. I believe CD like antigen classification of hepatocyte cell surface molecules will be more useful for such polarized cell as hepatocytes than for non-polarized cell as lymphocytes. Since 1984, we have been trying to raise monoclonal antibodies against rat hepatocyte membrane antigens (HAM1~8). Using these monoclonal antibodies, we tried to analyse the gestational changes in hepatocyte membrane antigens in comparison with those of adult hepatocytes. The results clearly showed that fetal hepatocytes are completely different from adult hepatocytes, especially at days 14–18 of gestation. After day 18, the adult-type antigenic expression appears gradually. On the basis of these results and other morphological features (fetal hepatocytes have no or less polarity and this suits the low level or absence of hepatocyte membrane antigens, as particular antigens distribute to the particular cell faces of hepatocytes), we consider that fetal hepatocytes might provide a favorable environment for hematopoietic cells.

Hepatocytes with adult-type membrane antigen expression and polarity may not be conducive for growth of hematopoietic cells. Thus we consider that fetal hepatocytes may act as stromal cells for hematopoietic cells, while conversely hematopoietic cells may also exert a beneficial effect for proliferation of fetal hepatocytes. Some studies have provided support to this hypothesis: for example, Itoh's group established a hepatocyte cell line that allowed the growth of hematopoietic cells (37), and Nakamura proved that fetal, but not adult hepatocytes have the potential to proliferate by themselves (67). Furthermore, some factors from platelets which originate from mesenchymal tissues stimulate the proliferation of adult hepatocytes (68). It thus seems likely that both fetal hepatocytes and hematopoietic cells secrete growth factors stimulating their mutual proliferation by paracrine type of secretion in fetal liver. Thus, in mammals, hematopoiesis in fetal liver may be essential for hematopoietic cells and hepatocytes to regulate or to be regulated on their proliferation and differentiation by each other.

A change of systemic hematopoietic site (such as the appearance of bone marrow) may affect the potential of fetal liver hematopoiesis (i.e. the need for fetal hematopoiesis may be reduced), and subsequently, fetal liver hematopoiesis may decrease. At this time, functional changes in fetal hepatocytes may also occur (around day 18 of gestation), accompanied by development of cell polarity, and this would reduce the space available for hematopoietic cells. Through these types of events hematopoietic foci in the liver may disappear. The clear aim of future work will be to prove this sort of change in cell-to-cell interaction directly using *in vitro* models as well as demonstrating genetic changes in each cell type during gestation. Besides, I would like to stress the importance of comparative cytological studies such as comparison with membrane antigen expressions between lymphocyte and hepatocyte, although the antigens of the former was systematically examined as CD.

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