Immunomagnetic exclusion of PECAM-1-positive endothelial cells in fetal mouse liver cell cultures causes impaired growth and gene expression of hepatoblasts and stellate cells

Yoshinori Sugiyama, Yurie Takabe, Shinomi Yagi, Toru Koike, and Nobuyoshi Shiojiri
Department of Biology, Faculty of Science, Shizuoka University, Oya 836, Suruga-ku, Shizuoka City, Shizuoka 422-8529, Japan

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ABSTRACT

Previous studies using mice having defective VEGF signaling have demonstrated that vascular development is indispensable for early hepatic organogenesis. However, not only whether its action lasts during later hepatic development, but also what molecules are involved in that action remains to be determined. The present study was undertaken to examine the effects of primitive sinusoidal endothelial cells on hepatic growth and maturation in primary culture of fetal mouse liver cells, and to determine their molecular mechanisms. When endothelial cells were excluded from E12.5 liver cell cultures by using PECAM-1-antibody-coated magnetic beads, the growth of hepatoblasts and stellate cells was conspicuously reduced and hepatic maturation was also suppressed. Conditioned medium prepared from fetal liver cell cultures containing almost all hepatic cell types stimulated the growth and gene expression of hepatoblasts and stellate cells similarly to the cultures in the presence of endothelial cells. HGF mRNA expression was downregulated in endothelial cell-free cultures of fetal liver cells, and the addition of HGF to the culture medium rescued the cells from the effects of endothelial cell depletion. These data suggest that humoral factors, including HGF, which are produced by endothelial cells or stellate cells, are involved in fetal hepatocyte growth and maturation.
BMPs, oncostatin M, TNFα and hepatoma-derived growth factor (HDGF), and extracellular matrices may be involved in such cellular interactions (1, 8–10, 12, 19).

PECAM-1 (CD31) is a member of the immunoglobulin superfamily that has distinctive features of an immnoreceptor based upon its genomic structures and the presence of intrinsic immunoreceptor tyrosine inhibitory motifs in its ligand binding (6, 16). It is expressed at high density at the lateral borders of endothelial cells and at a lower density on the surfaces of hemopoietic and immune cells, including platelets (6, 16). In fetal mouse livers, endothelial cells of all blood vessels, including those of portal and hepatic veins and sinusoids, express this molecule (13, 14, 22). Thus, a proper antibody reacting with cell-surface PECAM-1 could exclude the endothelial cell population in fetal mouse livers by using immunomagnetic methods, to elucidate cellular interactions that involve endothelial cells (17).

In the present study using an immunomagnetic method, PECAM-1-positive endothelial cells were excluded from E12.5 liver cell cultures to examine their role in liver development. The exclusion of endothelial cells resulted in poor growth and gene expression of hepatic constituent cells such as hepatocytes and stellate cells. Their effect could be restored by the addition of a conditioned medium prepared from fetal liver cell cultures or HGF.

MATERIALS AND METHODS

Animals. C3H/HeSlc mice (SLC, Hamamatsu, Japan) were used. Animals were kept in a controlled light-dark cycle and fed a standard chow diet and water ad libitum. Animals were mated during the night, and noon of the day when a vaginal plug was found was considered 0.5 days of gestation (E0.5). Mouse fetuses at E12.5 were used for cell culture studies. E12.5, E14.5, E17.5, neonatal (P0) and adult livers were used for immunohistochemistry for PECAM-1 and histology. All animal experiments were carried out in compliance with the “Guide for Care and Use of Laboratory Animals” of Shizuoka University.

Exclusion of PECAM-1-positive endothelial cells. Exclusion of endothelial cells from fetal liver cell suspensions was performed using immunomagnetic methods according to the manufacturer’s instructions. Immunomagnetic beads (Dynal, A. S., Oslo, Norway) coupled with sheep anti-rat IgG antibodies, were incubated with 0.1% gelatin/20 mM Tris/150 mM NaCl/10 mM CaCl2 (TBS) to suppress their nonspecific binding to cells (15). After washing in 1% bovine serum albumin (BSA)/TBS, the beads were incubated with a rat anti-mouse PECAM-1 antibody (1/250 dilution) at room temperature for 30 min, washed twice with 10% fetal bovine serum (FBS) (Sigma Chemical Co., St. Louis, MO, USA)/DM-160 (Kyokuto Suryaku Co. Ltd., Tokyo, Japan), and then used for cell exclusion experiments.

Livers were dissected out from E12.5 mouse fetuses and then diced (24). Diced livers were treated with 10 mM O,O’-bis(2-aminoethyl)ethylenglycol-N,N,N’,N’-tetraacetic acid (EGTA) dissolved in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Grand Island, NY, USA) containing 10% FBS on ice for 30 min. After removing the EGTA solution, tissues were then treated with 1000 U/mL dispase (Godo Shusei Co. Ltd., Tokyo, Japan) dissolved in DMEM/10% FBS for 30 min at 37°C. After gentle pipetting with a Pasteur pipette, undigested tissues were removed from the cell suspension by filtration using a nylon mesh filter (132 μm pore size) (Nihon Rikagaku Kikai Co. Ltd., Tokyo, Japan). Cells were collected by centrifugation at 50 × g for 10 min and the resultant cellular pellet was washed twice with DM-160 containing 10% FBS and 0.01% deoxyribonuclease I (Worthington Biochem. Corp., Freehold, NJ, USA). Cells were resuspended in 10% FBS/DM-160 (10^6 cells/mL), to which 10 mM EGTA was added. The viability was more than 95% by the trypan blue exclusion test. The cell suspension was incubated with anti-PECAM-1 antibody-coupled beads (10-fold the cell number) for 30 min at 4°C. As a control, cell suspensions were incubated with immunomagnetic beads that were pretreated with 1% BSA/TBS in place of the anti-PECAM-1 antibody. By using a magnetic particle concentrator (MPC), PECAM-1-positive cells were excluded from the cell suspension. The PECAM-1-positive cell-depleted fraction was washed twice with 10% FBS/DM-160 by centrifugation, and resuspended in 10% FBS/DM-160 containing 10^{-7} M dexamethasone and antibiotics. This suspension was adjusted to the original volume before mixing with immunomagnetic beads.

Cell culture. Cell suspensions of 70 μL, from which PECAM-1-positive cells were excluded, and control cell suspensions were cultured for 5 days on Teflon-coated slides (AR Brown Co. Ltd., Tokyo, Japan) at 37°C in a water-saturated atmosphere containing 5% CO2. The medium was changed at days 1 and 3. In some experiments, HGF (20 ng/mL; Genzyme-Techne Corp., Boston, MA, USA) or a conditioned me-
also stained with H-E. Control incubations were carried out in PBS containing 1% BSA in place of the primary antibodies.

**Immunohistochemistry.** Liver tissues for immunohistochemistry of PECAM-1 were frozen in n-hexane cooled with dry ice-ethanol. Frozen sections were cut at 8 µm thickness and fixed in cold acetone (−20°C) for 10 min.

Hydrated sections and cultured cells were incubated for 1 h at room temperature with the primary antibodies listed in Table 1. α-Fetoprotein (AFP), albumin and carbamoyl phosphate synthase I (CPSI) are specifically expressed by fetal mouse hepatoblasts and hepatocytes, although AFP is an immature marker and CPSI is a mature hepatocyte marker (22). Fetal hepatoblasts and hepatocytes also express E-cadherin and cytokeratins, whereas other hepatic cells do not express these proteins except for biliary epithelial cells. Adult hepatocytes do not express cytokeratins recognized by anti-calf keratin antiserum used in the present study (22). Desmin is specifically expressed in stellate cells in fetal livers. F4/80 expression was used as a marker for Kupffer cells. After thorough washing with PBS, sections were incubated with a Cy3- or fluorescein-labeled donkey anti-rabbit or rat IgG antibody (Jackson ImmunoResearch Lab., West Grove, PA, USA) (1/500 dilution for Cy3-labeled antibodies and 1/50 dilution for fluorescein-labeled antibodies) for 1 h at room temperature, washed again, and mounted in buffered glycerol containing p-phenylenediamine (7). The specific immunofluorescence in the section was observed with a fluorescent microscope (model BX50 equipped with BX-FLA; Olympus, Tokyo, Japan). Sections were also stained with H-E. Control incubations were carried out in PBS containing 1% BSA in place of the primary antibodies.

**Immunoblotting.** First, cultured cells on day 5 were fixed in 10% trichloroacetic acid at 4°C for 30 min (17). Extracts were obtained according to the method described (17). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on 10% acrylamide slab gels under reducing conditions following the method of Laemmli (11). Following electrophoresis, proteins were transferred to PVDF membranes. After blocking with 5% skim milk for 2 h (17), the membranes were incubated with rabbit anti-mouse AFP (1/10000 dilution), mouse albumin (1/10000 dilution), mouse CPSI (1/5000 dilution), or chicken desmin (1/2000 dilution) antisera, or a rat anti-E-cadherin antibody (1/2000 dilution). Following washing with TBST, a horseradish peroxidase-conjugated goat antibody against rabbit IgG (1/2000 dilution; Organon Teknika) or rat IgG (1/10000 dilution; Jackson ImmunoResearch) was used as the secondary antibody. Incubation of the membranes with these antibodies was for 2 h at room temperature. ECL detection was conducted with Amersham Pharmacia Biotech (Buckinghamshire, England) reagents according to the manufacturer’s recommendations. The data were quantified by using NIH image.

**RT-PCR.** Total RNAs were extracted from E17.5 livers and cell culture samples on day 3 using Isogen (Nippon Gene, Tokyo, Japan). Complementary DNA was synthesized from total RNA (0.5 µg) in 10 µL of reaction mixture containing 2.5 µM oligo

### Table 1  Primary antibodies used in immunohistochemistry and immunoblotting

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Dilution*</th>
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<tr>
<td>Rabbit anti-mouse AFP antiserum</td>
<td>ICN Biochemicals, Aurora, OH</td>
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<tr>
<td>Rabbit anti-mouse albumin antiserum</td>
<td>Cappel Laboratories, West Chester, PA</td>
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<tr>
<td>Rabbit anti-mouse carbamoyl phosphate synthase I (CPSI) antiserum</td>
<td>Nitou et al. (2002)</td>
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<tr>
<td>Rabbit anti-calf keratin antiserum</td>
<td>Dako, Carpinteria, CA</td>
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</tr>
<tr>
<td>Rat monoclonal anti-mouse E-cadherin antibody (ECCD2)</td>
<td>Takara Biomedical, Otsu, Japan</td>
<td>1 : 100</td>
</tr>
<tr>
<td>Rat monoclonal anti-mouse PECAM-1 antibody</td>
<td>eBioscience, San Diego, CA</td>
<td>1 : 100</td>
</tr>
<tr>
<td>Rabbit anti-chicken desmin antiserum</td>
<td>Progen Biotechnik Gmbh, Heidelberg, Germany</td>
<td>1 : 100</td>
</tr>
<tr>
<td>Rat anti-mouse F4/80 antibody</td>
<td>BMA Biochemicals AG, Augst, Switzerland</td>
<td>1 : 100</td>
</tr>
<tr>
<td>Rabbit anti-mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody</td>
<td>AbFrontier, Seoul, Korea</td>
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* Dilution for immunohistochemistry. *The antibody was not used in immunohistochemistry.
by the immunomagnetic method (Fig. 3A). Phase-contrast microscopy showed no significantly different cell numbers between both PECAM-1-positive cell-excluded fractions and control fractions (Fig. 3C, D). Because the major cell type of E12.5 livers are hemopoietic cells, their number may not differ between the fractions. Magakaryocytes were excluded from PECAM-1-positive cell-depleted fractions whereas some megakaryocytes were observed in control fractions ( insets in Fig. 3C, D). Immunoblotting analyses of E-cadherin, desmin and GAPDH contents demonstrated that other cell types such as hepatoblasts and stellate cells were similarly contained in both cell fractions (Fig. 3B).

On days 1 and 5 after beginning culture, PECAM-1-positive cells were totally excluded when PECAM-1-positive cell-depleted fractions were cultured (Fig. 4B, D). By contrast, in control cultures, PECAM-1-positive cells were detectable as cell aggregates on day 1, and formed fine branching capillary networks on day 5 (Fig. 4A, C). Because megakaryocytes differentiated from hemopoietic cells during culture, they were observed even in PECAM-1-positive cell-depleted fractions (insets in Fig. 5A, B). Their number in PECAM-1-positive cell-depleted fraction was approximately half times smaller than that in control fraction during culture.

Exclusion of PECAM-1-positive cells results in poor growth and gene expression of other hepatic cells

Morphologically, cell growth and spreading in cultures of PECAM-1-positive cell-excluded fractions and control fractions was remarkably different. In culture of cell fractions from which PECAM-1-positive endothelial cells were excluded, adhesion of spherical aggregates on the glass slide, which were formed by hepatoblasts, was late at early stages of culture, compared with control cultures. Their spreading on the glass slide was also delayed at 1 day (Fig. 5A, B), and much poorer growth was seen at 5 days of culture, compared with control experiments (Fig. 5C, D). Immunohistochemical analyses also demonstrated that the numbers of E-cadherin-, CPSI-, desmin- and F4/80-positive (Kupffer cells) cells decreased and their staining intensities also weakened in cultures of endothelial cell-depleted fractions (Fig. 6A–H).

Immunoblot analyses indicated that GAPDH, which may be present at a constant level in cells and was used as an internal control, was expressed to a much lesser amount in a culture well of endothelial cell-depleted fractions on day 5, compared with that in a control well (Fig. 7A), suggesting that the cell

**RESULTS**

**Expression of PECAM-1 during liver development**

Throughout liver development, vascular structures, including portal veins, central veins and sinusoid structures, were well developed even in immature livers in fetal stages (Fig. 1A, C, E, G). In E12.5 livers, PECAM-1 expression was detectable in all vascular endothelial cells, megakaryocytes and some hemopoietic cells (Fig. 1B). The PECAM-1-positive endothelial cells were located very close to desmin-positive stellate cells and cytokeratin-positive hepatoblasts (Fig. 2A–D). The PECAM-1 signals were strong in sinusoidal endothelial cells of E14.5 livers, but those became weaker in E17.5 livers (Fig. 1D). In neonatal livers, PECAM-1 expression was strong in endothelial cells of both portal and hepatic veins, but was significantly low in sinusoidal endothelial cells (Fig. 1F). In adult livers, sinusoidal endothelial cells very weakly expressed PECAM-1, and endothelial cells of portal veins, central veins and hepatic arteries strongly expressed PECAM-1 (Fig. 1H).

**Exclusion of PECAM-1-positive endothelial cells from E12.5 liver cell suspensions**

PECAM-1-positive cells were excluded as cell aggregates from the E12.5 liver cell suspensions without drastically affecting other cell type compositions by the immunomagnetic method (Fig. 3A). Phase-contrast microscopy showed no significantly different cell numbers between both PECAM-1-positive cell-excluded fractions and control fractions (Fig. 3C, D). Because the major cell type of E12.5 livers are hemopoietic cells, their number may not differ between the fractions. Magakaryocytes were excluded from PECAM-1-positive cell-depleted fractions whereas some megakaryocytes were observed in control fractions (insets in Fig. 3C, D). Immunoblotting analyses of E-cadherin, desmin and GAPDH contents demonstrated that other cell types such as hepatoblasts and stellate cells were similarly contained in both cell fractions (Fig. 3B).

On days 1 and 5 after beginning culture, PECAM-1-positive cells were totally excluded when PECAM-1-positive cell-depleted fractions were cultured (Fig. 4B, D). By contrast, in control cultures, PECAM-1-positive cells were detectable as cell aggregates on day 1, and formed fine branching capillary networks on day 5 (Fig. 4A, C). Because megakaryocytes differentiated from hemopoietic cells during culture, they were observed even in PECAM-1-positive cell-depleted fractions (insets in Fig. 5A, B). Their number in PECAM-1-positive cell-depleted fraction was approximately half times smaller than that in control fraction during culture.
Fig. 1 Development of vascular system and expression of PECAM-1 during mouse hepatic organogenesis. (A, C, E, G) H-E staining. (B, D, F) Immunohistochemistry for PECAM-1. (H) Immunohistochemistry for PECAM-1 (red) and cytokeratin (green) (blue, DAPI staining). Primitive sinusoids (arrows) are well developed at E12.5, but are short in sections (A, B). Megakaryocytes (asterisk) are also PECAM-1-positive. Sinusoid structures (arrows) become elongated at E17.5 in section (C, D). In neonatal liver, PECAM-1 signals are low in sinusoidal endothelial cells, but are strong in endothelial cells of portal and central veins (E, F). Although the expression of PECAM-1 is downregulated in sinusoids (arrows) in the adult stage, endothelial cells of the hepatic artery and biliary vessels are strongly PECAM-1-positive in addition to those of portal and central veins (G, H). Arrowheads indicate hemopoietic cells (C, E). bd, bile duct; cv, central vein; ha, hepatic artery; pv, portal vein; v, portal vein or central vein. Bars indicate 100 μm.
Fig. 2  Double immunofluorescent analyses of PECAM-1 (red) and cytokeratin (green) (A, B), or PECAM-1 (red) and desmin (green) (C, D) in E12.5 livers. (A, B) PECAM-1-positive endothelial cells are located close to cytokeratin-positive hepatoblasts. (C, D) PECAM-1-positive endothelial cells are supported by desmin-positive stellate cells, and both cells build primitive sinusoids. Bars indicate 50 μm.

Fig. 3  Exclusion of PECAM-1-positive cells in E12.5 liver cell suspensions. (A) Aggregates of PECAM-1-positive endothelial cells (arrows) separated by the immunomagnetic bead method from E12.5 liver cell suspensions. (B) Immunoblotting analyses of E-cadherin, desmin and GAPDH contents in control fractions and fractions excluding PECAM-1-positive cells from E12.5 liver cell suspensions. The E-cadherin, desmin and GAPDH contents are not significantly different between the fractions. (C, D) Phase-contrast micrographs of control fraction and the fraction from which PECAM-1-positive cells were excluded, respectively. The cell numbers of the fractions are not significantly different. Some megakaryocytes are observed in the control fraction (arrow), but are absent from the PECAM-1-positive cell-excluded fraction (insets in C, D). Bars indicate 100 μm.
Fig. 4 Cultures of E12.5 liver cells from which PECAM-1-positive cells are excluded (B, D) and control cultures (A, C) on days 1 and 5. Endothelial cells are not detectable in cultures from which endothelial cells were excluded at cultivation (B, D), whereas endothelial cells are observed at 1 day (A) and form fine capillary networks at 5 days (C). Red, PECAM-1 immunostaining; blue, DAPI staining. Bars indicate 100 μm.

Fig. 5 Decreased cell growth and spreading in PECAM-1-positive cell-depleted E12.5 liver cell cultures. (A, B) Phase-contrast micrographs. (C, D) H-E staining. Spherical cell aggregates (arrows) are still observed and their spreading on the glass slide is not extensive in the PECAM-1-positive cell-depleted culture on day 1 (B), whereas liver cells already extensively spread on the glass slide in the control culture (A). Some megakaryocytes (arrowheads) are observed in cultures of the control fraction and PECAM-1-positive cell-excluded fraction on day 1 (insets in A, B). The cell density in the control culture (C) is higher than that in the PECAM-1-positive cell-depleted culture on day 5 (D). Bars indicate 100 μm.
Fig. 6 Immunohistochemical analyses of expression of markers for each hepatic cell type in PECAM-1-positive cell-depleted E12.5 liver cell cultures on day 5. E-cadherin expression in hepatocytes is lower in the PECAM-1-positive cell-depleted culture than in the control culture (A, B). Expression of CPSI in hepatocytes is much weaker in the PECAM-1-positive cell-depleted culture than in the control culture (C, D). The numbers of desmin-positive stellate cells and F4/80-positive Kupffer cells are decreased in the PECAM-1-positive cell-depleted culture (F, H), compared with control cultures (E, G). Bars indicate 100 μm.
growth was impaired without endothelial cells. Expression of E-cadherin and desmin was weak in cultures without endothelial cells, compared with control cultures (Fig. 7A), indicating poor growth of hepatoblasts/hepatocytes and stellate cells, respectively. Concerning hepatocyte maturation, expression of albumin and CPSI was low while AFP expression was high in cultures without PECAM-1-positive cells, compared with control cultures (Fig. 7A, C, D) when expression of each protein was standardized by the GAPDH content. RT-PCR analyses gave a similar result, showing that expression of AFP, albumin and CPSI mRNAs was suppressed in cultures without PECAM-1-positive cells (Fig. 7B).

**Effects of the conditioned medium and HGF on hepatocyte growth and maturation**

When the conditioned medium prepared from E12.5 liver cell cultures, which had all hepatic cell type components, was added to the culture medium for PECAM-1-positive cell-depleted fractions at a concentration of 50%, the growth of the fetal liver colonies and protein expression of AFP, E-cadherin, albumin, CPSI, desmin and GAPDH recovered to
Fig. 8 The conditioned medium (CM) and HGF restore the effect of PECAM-1-positive endothelial cells on the growth and gene expression of hepatoblasts and stellate cells in E12.5 liver cell cultures on days 3 (D) and 5 (A, B). CM (50%) or HGF (20 ng/mL) was added to the culture media of E12.5 liver cells, which lacked PECAM-1-positive cells. (A) Although control cultures express strongly or weakly markers of hepatocytes and stellate cells, those without PECAM-1-positive cells show poorer expression of hepatocyte and stellate cell markers. The addition of CM to cultures without PECAM-1-positive cells remarkably promotes expression of hepatocyte and stellate cell markers. (B) The addition of HGF has a similar effect on the protein expression of hepatocytes in the experiments, in which CM is added (A). (C) Quantification of protein expression of hepatocyte markers shown in B. The addition of HGF inhibits AFP expression similarly to control cultures. (D) Expression of HGF mRNA in cultures of E17.5 liver cells and those excluding PECAM-1-positive cells, which have much lower expression of HGF mRNA.

control levels (Fig. 8A). Because adhesion and migration of aggregates of hepatoblasts or hepatocytes on the glass slide was delayed in cultures of endothelial cell-depleted fractions (Fig. 4A, B), mRNA expression for HGF, which has been shown to be involved in cell migration (3, 26), was examined. RT-PCR analysis showed that HGF mRNA was at a low level in cultures of endothelial cell-depleted fractions, compared with control cultures (Fig. 8D). HGF mRNA was more strongly expressed in E17.5 livers than control cultures. When HGF was added to endothelial cell-depleted cultures, it strongly stimulated the growth and protein expression of hepatocytes (Fig. 8B, C). The addition of the conditioned medium or HGF reduced AFP expression and promoted hepatocyte maturation (Fig. 8C).

DISCUSSION

In the present study, we succeeded in excluding...
PECAM-1-positive endothelial cells from fetal liver cell cultures. Megakaryocytes were also excluded with endothelial cells, but could be differentiated from hemopoietic cells during culture. Their cell number in cultures of PECAM-1-positive cell-depleted fractions was somewhat smaller than that in control cultures. Even in control cultures containing whole hepatic cell compositions, PECAM-1-positive megakaryocytes were not abundant throughout culture. Thus, their exclusion at the start of cultivation may not have a significant role in hepatic organoid development in our culture system. Although the effect of megakaryocytes on hepatic growth and differentiation in vitro should not be ignored, as discussed later, we could perfectly exclude endothelial cells, at least, from fetal liver cell suspensions, which may lead to elucidation of the role of endothelial cells in hepatic organogenesis. Other cell populations such as hepatoblasts, stellate cells and hemopoietic cells were not different between the fractions.

The present study demonstrated that the exclusion of PECAM-1-positive endothelial cells from fetal liver cell suspensions resulted in impaired growth and gene expression of other hepatic cells such as hepatoblasts/hepatocytes, stellate cells and Kupffer cells, suggesting that the maturation of hepatocytes might be controlled by their cellular interactions with endothelial cells. These data are well consistent with the work using Flk-1 knockout mice by Matsumoto et al. (13), in which the presence of Flk-1-expressing endothelial cells was shown to play a decisive role in hepatic primordium morphogenesis. During liver organogenesis, hepatic endothelial cells develop along the primordium, and are located close to hepatic cords and stellate cells throughout development (18, 23, 24). The hepatic vascular system, including the portal veins, central veins and primitive sinusoids, develops with different phenotypes from early stages of the organogenesis, and primitive sinusoid structures, in which endothelial cells, stellate cells and hepatoblasts have a configuration similar to that in hepatic lobules of the adult, are already formed in fetal livers (14, 22, 28). Consequently, endothelial cells may also interact with hepatoblasts and stellate cells in normal liver development. Our preliminary experiments using a Flk-1 (VEGF receptor) kinase inhibitor in cultures of E12.5 liver cells to block the growth of endothelial cells also revealed an effect on the growth and gene expression of hepatoblasts/hepatocytes and stellate cells similar to that shown in the present study.

It has not been reported what molecules are involved in endothelial cell-other hepatic cell interactions. The present study demonstrated that the effects of exclusion of PECAM-1-positive cells on the growth and gene expression of other hepatic cells could be reversed by the addition of a conditioned medium prepared from conventional fetal liver cell cultures, suggesting that humoral factors produced by PECAM-1-positive cells may act on the growth and gene expression of other hepatic cells, including hepatoblasts and stellate cells. Although HGF mRNA expression was weak in control cultures, it was remarkably reduced in PECAM-1-endothelial cell-depleted cultures, and HGF could replace the effect of the conditioned medium. Therefore, HGF may be one of such humoral factors that mediate endothelial cell-other hepatic cell interactions. As it has been demonstrated that HGF could be produced by stellate cells, and works on hepatocyte growth and maturation (10), endothelial cells may produce other humoral factors, which may lead to upregulation of HGF in stellate cells. However, endothelial cells themselves may produce this growth factor (4, 27). In any event, it is intriguing why stellate cells could not produce sufficient HGF for hepatoblast/hepatocyte growth and maturation without endothelial cells. A cellular interaction network such as endothelial-stellate cell-hepatoblast interactions may operate to increase the HGF concentration among hepatic constituent cells via other humoral factors or HGF itself. In fetal livers, HGF mRNA was expressed as shown in the present study and previous studies, and c-Met protein is also expressed (5, 10, 20), suggesting that HGF-cMet signaling actually works during fetal liver development. Our data agree well with those in mice lacking the SF/HGF gene, which fail to complete development and die in utero (20, 25). The mutation affects not only the placenta, but also the embryonic liver, which is reduced in size and shows extensive loss of parenchymal cells.

On the other hand, when E9.5 liver primordia were cultured in vitro with an HGF-neutralizing antibody, using the same concentrations and preparations that inhibited HGF in Transwell cultures of lung explants, no inhibition of vascular development or of tissue growth was observed (13), suggesting that factors other than HGF may be involved in vascular development and liver growth in the early stages. This result may not be consistent with our data using E12.5 liver cells. Different molecules might work in endothelial-hepatoblast or endothelial-stellate cell interactions at various developmental stages such as the primordium formation stage and midgestational growth stage of the liver.
Although we successfully excluded PECAM-1-positive endothelial cells from E12.5 liver cell suspensions by using immunomagnetic beads, megakaryocytes, which were not abundant in the fetal livers, were also excluded from the cell suspension and might be important for cell-cell interactions during liver development. To demonstrate their involvement in hepatic development, the development of cell separation protocols excluding only endothelial cells or megakaryocytes is required. This is one of questions to be resolved in the future.

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