Structural and Functional Differentiation of Sinusoidal Endothelial Cells During Liver Organogenesis in Humans

By Anne Couvelard, Jean-Yves Scoazec, Marie-Christine Dauge, Annie-France Bringuier, François Potet, and Gérard Feldmann

During fetal life, human liver sinusoids, which differentiate between 4 and 12 weeks of gestation from capillaries of the septum transversum, must support an important hematopoietic function and acquire the structural and functional characteristics of adult sinusoids. To gain insight into their differentiation process, we studied the expression of (1) markers of continuous endothelium, absent from adult sinusoidal endothelial cells (PECAM-1, CD34, and 1F10); (2) functional markers of adult sinusoidal endothelial cells (CD4, ICAM-1, CD32, and CD14); and (3) extracellular matrix components (laminin, tenascin, fibronectin, and thrombospondin) in 37 fetuses of different gestational ages. We identified two successive differentiation events. (1) An early structural differentiation, occurring from 5 to 12 weeks of gestation, was characterized by the loss of continuous endothelial cell markers and a reduction in the perisinusoidal amount of laminin and in the deposition of tenascin, fibronectin, and thrombospondin; at the end of this process, fetal liver sinusoids present structural characteristics comparable to those of the sinuses in adult hematopoietic bone marrow. (2) A later functional differentiation was characterized by the acquisition of the markers of adult sinusoidal endothelial cells, initiating at 10 weeks of gestation and completed by 20 weeks of gestation; this process likely contributes to adapt liver sinusoids to the specific functions of the adult hepatic tissue.

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Table 1. Antibodies Used in the Study

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Clone</th>
<th>Source</th>
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<tr>
<td>Markers of continuous microvascular endothelial cells</td>
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<tr>
<td>PECAM-1</td>
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<td>CD34</td>
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<td>1F10</td>
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<td>NCL-CD62</td>
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<td>Decay-accelerating factor</td>
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<td>Serotec</td>
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<tr>
<td>Markers specific for adult sinusoidal endothelial cells</td>
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<tr>
<td>CD4</td>
<td>SK3-SK4</td>
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<tr>
<td>ICAM-1</td>
<td>84H10</td>
<td>Immunotech</td>
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<td>FcR IgG II</td>
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<td>Becton Dickinson</td>
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<td>Extracellular matrix components</td>
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<td>Laminin</td>
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<td>Tenascin</td>
<td>6044</td>
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<td>Fibronectin</td>
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<td>Thrombospondin</td>
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adult liver sinusoidal endothelial cells, ie, CD4, ICAM-1, CD32, and CD14; and (3) extracellular matrix components, including laminin and tenascin, which were selected for their characteristic distribution in the adult perisinusoidal matrix, and fibronectin and thrombospondin, which were selected for their specific involvement in the composition of hematopoietic microenvironments. For these purposes, we studied samples from 37 human embryos and fetuses of various gestational ages (from 5 to 40 weeks of gestation) by using immunohistochemical and Western blotting techniques.

MATERIALS AND METHODS

Tissue Samples

Liver samples were obtained from 37 human embryos and fetuses of various gestational ages from 5 to 40 weeks of gestation. Fetuses were obtained from legal voluntary, therapeutic, or spontaneous abortions. The ages of the fetuses were determined according to the time from ovulation to the day of abortion. Ages were corrected according to crown-rump, hand, and foot lengths. The absence of significant liver histologic lesion was verified before the inclusion in the study group. We selected 2 cases at 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, and 22 weeks of gestation and 1 case at 19, 21, 23, 25, 30, 35, and 40 weeks of gestation.

Liver samples were either frozen in isopentane prechilled in liquid nitrogen and stored at -80°C until use or fixed in 10% formalin and embedded in paraffin. At early stages of growth (5, 6, and 7 weeks of gestation), only formalin-fixed material was available. From 8 to

Fig 1. Morphologic characteristics of the fetal human liver at 5 and 9 weeks of gestation. At 5 weeks of gestation (a), the hepatic diverticulum (HD) is located between the heart (H) and the digestive tract (DT). It is in close contact with the mesenchyme of the septum transversum (ST). The hepatic diverticulum is formed by loose cords of hepatoblasts (arrows) separated by sinusoid-like vessels (S) containing numerous circulating blood cells. At 9 weeks of gestation (b), well-formed hepatocyte cords are separated by sinusoid vessels. Portal vein branches (PV), which are surrounded by an abundant mesenchymal tissue, are visible in portal spaces limited by the ductal plate (arrowheads). Many hematopoietic cells are present, in close contact with hepatoblasts (arrows). Hematoxylin-eosin staining was used. Original magnification (a) × 70 and (b) × 150.
They were purchased from Affinity Research (Nottingham, UK), Becton Dickinson (Mountain View, CA), and Sigma (St Louis, MO). The following antibodies were used: (1) markers of the liver samples available for study were frozen. In addition, samples from 11 livers taken, respectively, at 9, 11, 15, 16, 18, 19, 20, 23, 30, 35, and 40 weeks of gestation were fixed by immersion in 4% paraformaldehyde (Merck, Darmstadt, Germany) in 0.1 mol/L phosphate buffer, pH 7.4; frozen in liquid nitrogen prechilled in isopentane; and then stored at -80°C until use.

Immunohistochemistry

Antibodies

The primary antibodies used in the study are listed in Table 1.

Table 1. Expression of Endothelial Cell Markers and of Extracellular Matrix Components in Fetal Liver Sinusoids According to the Gestational Age in Weeks

<table>
<thead>
<tr>
<th>Markers of continuous endothelial cells</th>
<th>Gestational Age (wk)</th>
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<tr>
<td></td>
<td>5-7</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>+</td>
</tr>
<tr>
<td>CD34</td>
<td>+/-</td>
</tr>
<tr>
<td>IF10</td>
<td>+/-</td>
</tr>
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<td>P-selectin</td>
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<td>DAF</td>
<td>ND</td>
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<tr>
<td>Markers of adult sinusoidal endothelial cells</td>
<td></td>
</tr>
<tr>
<td>CD4</td>
<td>ND</td>
</tr>
<tr>
<td>FcRlgG II</td>
<td>ND</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>ND</td>
</tr>
<tr>
<td>CD14</td>
<td>ND</td>
</tr>
<tr>
<td>Extracellular matrix components</td>
<td></td>
</tr>
<tr>
<td>Laminin</td>
<td>+</td>
</tr>
<tr>
<td>Tenascin</td>
<td>-</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>ND</td>
</tr>
<tr>
<td>Thrombospondin</td>
<td>ND</td>
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</table>

Abbreviations: ND, not determined; +, positive; +/-, intermediate staining; -/-, negative; + to +++, semiquantitative evaluation of staining intensity.

15 weeks of gestation, frozen and formalin-fixed material was available in, respectively, 7 and 9 cases. After 16 weeks of gestation, all the liver samples available for study were frozen. In addition, samples from 11 livers taken, respectively, at 9, 11, 15, 16, 18, 19, 20, 23, 30, 35, and 40 weeks of gestation were fixed by immersion in 4% paraformaldehyde (Merck, Darmstadt, Germany) in 0.1 mol/L phosphate buffer, pH 7.4; frozen in liquid nitrogen prechilled in isopentane; and then stored at -80°C until use.

**Light Microscopic Immunohistochemistry**

An indirect immunoperoxidase technique was applied for all antibodies on sections of either frozen or paraffin-embedded tissues. Sections of frozen tissues 4 μm thick were cut on a cryostat, dried overnight at room temperature, and then fixed for 10 minutes in cold acetone. Sections were rehydrated in 0.1 mol/L phosphate-buffered saline (PBS), pH 7.4 and then incubated for 90 minutes at room temperature with the primary antibody in an appropriate dilution in PBS. After washing in PBS, sections were then incubated for 90 minutes at room temperature with goat polyclonal species-specific peroxidase-labeled antimouse, antirat, or antirabbit Ig F(ab')2 antibodies (Immunotech). After washing in PBS, peroxidase activity was shown with diaminobenzidine (FcR IgG; CD32), and lipopolysaccharide-binding protein receptor (CD14); and (3) extracellular matrix components: laminin, tenascin, fibronectin, and thrombospondin.

**Fig 2. Expression of PECAM-1, CD34, and IF10 antigen in the human fetal liver according to the gestational age.** (a) and (b) show, respectively, the expression of PECAM-1 (a) and CD34 (b) at 6 weeks of gestation. PECAM-1 (a) is detected on the endothelial lining of the capillary vessels in the septum transversum (ST; arrows) and along the sinusoids (S; arrowheads). Note the presence of hepateoblasts (open arrows) invading the mesenchyme of the septum transversum and surrounding the capillary vessels. Like PECAM-1, CD34 (b) is expressed along the capillary vessels of the septum transversum (ST; arrows) as well as along the sinusoids (S; arrowheads) lying between the cords of hepatoblasts. The apparent level of expression of CD34 is slightly higher on the endothelial lining of the capillary vessels of the septum transversum (arrows) than on sinusoidal endothelial cells (arrowheads). (c) and (d) show, respectively, the expression of PECAM-1 (c) and IF10 antigen (d) at 7 weeks of gestation. Note the heterogeneous distribution of endothelial cell markers on the large vessels of septum transversum (ST). The expression of both PECAM-1 (c) and IF10 (d) is faint or undetectable along the part of the vessel coming in close contact (arrowheads) with the hepatic diverticulum (H). (e) shows the expression of CD34 at 9 weeks of gestation. CD34 is detected along the capillary vessels of the portal tract (PT). Its expression along sinusoids (S) is restricted to the sinusoidal endothelial cells in contact with the portal tract (arrows) and is undetectable at a distance (arrowheads). (f) shows the expression of PECAM-1 at 11 weeks of gestation. PECAM-1 is detected only along portal vessels (arrowheads). No staining is visible along the hepatic sinusoids. Note the presence of positive megakaryocytes (arrow). Indirect immunoperoxidase followed by nuclear counterstaining with Mayer’s hematoxylin was used. Original magnification: (a) × 140, (b) × 120, (c) × 150, (d) × 150, (e) × 180, and (f) × 70.
Fig 2.
Fig 3.
in paraffin-embedded tissue were those directed against PECAM-1, CD34 protein, IF10 antigen, laminin, and tenascin. An indirect immunoperoxidase technique was applied to 3-μm-thick deparaffinized sections. Before immunostaining with monoclonal antibodies (MoAbs) against PECAM-1 and CD34 protein, sections were rehydrated and incubated in a microwave oven three times for 5 minutes each in 10 mM/L citrate buffer, pH 6.2. Before immunostaining with the MoAb to laminin, sections were rehydrated and treated with 0.1% protease type XIV (Sigma) in PBS for 10 minutes at +37°C. Before immunostaining with the MoAb to tenascin, sections were rehydrated and treated with 0.02% protease type XXIV (Sigma) in 0.05 M/L Tris, pH 7.6, 0.025% CaCl2, for 10 minutes at room temperature. After washing in PBS, the same technique described above was performed.

Control sections. Negative controls consisted of the omission of the primary antibody replaced by PBS and of incubation with isotopic lgs. They were constantly negative. According to the gestational age, positive internal controls consisted of hematopoietic cells, of capillary vessels located in the septum transversum, or of the vessels of the developing portal tracts observed in the same sections.

Ultrastructural Immunohistochemistry

Ultrastructural immunohistochemistry was performed for the following antigens: PECAM-1, CD4, and tenascin. As previously described, an indirect pre-embedding immunoperoxidase technique was performed. Briefly, 12-μm-thick sections of paraformaldehyde-fixed tissue were incubated overnight at +4°C with the primary antibody in appropriate dilution, rinsed, and then incubated for 3 hours at room temperature with peroxidase-labeled species-specific antimouse Ig antibody (Amersham, Les Ulis, France). Peroxidase activity was shown according to the method of Graham and Karnovsky. Sections were then postfixed in 1% osmium tetroxide, dehydrated in graded alcohols and in propylene oxide, and then embedded in epoxy resin (Agar 100; Agar Aids, Stansted, UK). They were cut on a Reichert Ultratome (Jung, Wien, Austria) and examined without further staining on a Siemens Elmiskoop IA electron microscope (Siemens, Karlsruhe, Germany).

Western Blotting

Western blot analysis was performed for CD4, PECAM-1, and tenascin in samples obtained at 11, 12, 14, 18, and 21 weeks of gestation. Tissue preparation varied according to the antigen tested.

As previously described, analysis for CD4 and PECAM-1, tissue pieces weighing 10 to 50 mg were homogenized with a Dounce homogenizer in ice-cold 1 M/L sodium carbonate buffer, pH 8.2, containing the following protease inhibitors: 0.25 mg/mL pepstatin, 1.25 mg/mL trypsin, 1.25 mg/mL antipain, 0.25 mg/mL aprotinin, and 0.5 mM/L phenylmethylsulfonyl fluoride. All reagents were from Sigma. The resulting homogenates were centrifuged for 15 minutes at 10,000 x g at +4°C. Pellets were resuspended in 0.1 M/L Tris HCl, pH 6.8. Reducing buffer containing sodium dodecyl sulfate and dithiothreitol (lane marker reducing buffer; Pierce, Rockford, IL) was finally added at 1:5 dilution.

For tenascin analysis, tissue preparation was performed according to methods previously described. Briefly, tissue pieces weighing 10 to 50 mg were homogenized with a Dounce homogenizer in CAPS buffer, pH 11, containing 200 mM/L 3-cyclohexylamino)-1-propane sulfonic acid (Sigma), 150 mM/L NaCl, 1 mM/L EDTA, and the same antiproteases as described above. Homogenates were incubated in this buffer for 15 minutes at +4°C and neutralized with 2 M/L NaH2PO4.

After appropriate preparation, samples of approximately 100 μg protein per lane were applied to either a 6% or a 9% polyacrylamide slab gel. After electrophoretic separation, proteins were blotted overnight onto a nitrocellulose sheet (Schleicher and Schuell, Feltbach, Switzerland). After the quenching of filters for 1 hour at room temperature with 5% nonfat dried milk diluted in PBS containing 0.1% Tween 20 (Merck), antigens were shown with an indirect immunoperoxidase technique. Filters were incubated for 1 hour at room temperature with the primary antibody diluted in PBS, washed for 15 minutes in PBS, and then incubated for 1 hour at room temperature with sheep-specific peroxidase-labeled antimouse Ig antibody (Amersham, Little Chalfont, UK) diluted 1:1,000 in PBS. After washing, peroxidase activity was shown by enhanced chemiluminescence (ECL Western blotting detection system; Amersham). Blots were finally exposed to autoradiography films.

RESULTS

The expression of the various markers studied was examined according to the gestational age. We particularly focused on the major stages in human liver development, as identified by previous embryologic studies.

The first stage corresponds to the period ranging from 5 to 7 weeks of gestation. At this stage, the hepatic diverticulum, formed by 4 weeks of gestation, is in close contact with the septum transversum. It consists of hepatoblasts arranged in thick, anastomosed cords separated by irregular vascular spaces lined by flattened endothelial cells and containing intravascular blood cells, mostly of the erythroid lineage (Fig la). At the periphery of the hepatic diverticulum, growing cords of hepatoblasts invade the mesenchymal of the septum transversum and progressively surrounds its capillaries. In the 7-week-old embryos, the very first islands of hepatic hematopoiesis are found in close contact with hepatoblasts, consisting mainly of erythroblasts. At this time of development, intrahepatic capillary vessels cannot be properly classified as sinusoids. However, for reason of clarity, we will nevertheless use the term sinusoid to refer to the capillary vessels located between the cords of hepatoblasts.

The second stage corresponds to the period ranging from 8 to 10 weeks of gestation, during which the definitive vascular architecture of the fetal liver becomes established. Afferent portal veins surrounded by a mesenchymal tissue and limited by the ductal plate are observed within the fetal liver. Hepatic cords are separated by sinusoid-like vessels containing he-

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**Fig 3. Expression of the functional markers CD4 and CD14 according to the gestational age. At 8 weeks of gestation (a), CD4 is restricted to large and irregular isolated cells likely to correspond to Kupffer cells (arrows) and is not detected on adjacent sinusoidal endothelial cells (arrowheads). At 15 weeks of gestation (b), CD4 is readily detected along the sinusoidal lining (arrows). Ultrastructural examination (c) shows the expression of CD4 by a sinusoidal endothelial cell (arrows) separated from the adjacent hepatocyte (H) by the space of Disse (D). At 20 weeks of gestation (d), CD4 is faintly detected on the sinusoidal endothelial lining (arrows). (e, b, and d) Immunoperoxidase with nuclear staining by Mayer's hematoxylin was used. Original magnifications (a) ×200, (b) ×150, and (d) ×180. (c) Electron microscopy and immunoperoxidase without staining were used. Original magnification ×17,000; scale bar = 0.5 μm.**
Fig 4.
matopoietic islands (Fig 1b). Some efferent vessels, in the form of terminal suprahepatic veins, are identifiable. Hematopoietic islands mainly consist of erythroid clusters accompanied by large, multinucleated cells of the megakaryocyte lineage.

The third stage corresponds to the period ranging from 10 to 12 weeks of gestation, which corresponds to the onset of the maximum intensity of hematopoesis. The last stage corresponds to the last trimester of gestation, during which fetal hematopoesis progressively stops.

**Immunohistochemical Results**

The immunohistochemical results are summarized in Table 2.

*Expression of PECAM-1, CD34 Protein, and 1F10 Antigen According to the Gestational Age*

From 5 to 7 weeks of gestation, both the capillary vessels of the septum transversum and the intrahepatic sinusoids were positive for PECAM-1 (Fig 2a), the CD34 protein (Fig 2b), and the 1F10 antigen. A localized decrease in the apparent expression level was frequently observed for PECAM-1 (Fig 2c) and the 1F10 antigen (Fig 2d) on the part of the large vessels of the septum transversum closer to the hepatic diverticulum. At 8 and 9 weeks of gestation, PECAM-1, CD34 (Fig 2e), and the 1F10 antigen were strongly expressed by the endothelial lining of portal vessels. Their apparent expression levels were heterogeneous on the endothelial lining of sinusoid vessels. These markers remained readily detected on the sinusoidal endothelial cells located at the contact with portal spaces (Fig 2e). At a distance from portal spaces, the expression was faint or undetectable (Fig 2e). From 10 weeks of gestation onwards, the distribution of PECAM-1 (Fig 2f), CD34, and 1F10 antigen was restricted to portal vessels. No expression was detected on sinusoidal endothelial cells by both light microscopic and ultrastructural examination. In addition to its expression on endothelial cells, PECAM-1 was also detected on the megakaryocytes present within the hematopoietic nests from 10 weeks of gestation. In contrast, we did not detect any CD34+ hematopoietic progenitor cells in our samples.

*Expression of P-Selectin (CD62-P) and DAF According to the Gestational Age*

At 5, 6, and 7 weeks of gestation, for which only paraffin-embedded material was available, the markers of adult liver sinusoidal endothelial cells CD4, CD14, CD32, and ICAM-1 could not be detected in our technical conditions.

At 8 weeks of gestation, no expression of CD4, CD14, CD32, and ICAM-1 was detected on the endothelial lining of liver sinusoids. In contrast, CD4 (Fig 3a) and ICAM-1 were detected on large macrophages bulging into the sinusoidal lumen, likely to correspond to fetal Kupffer cells. At 10 weeks of gestation, CD4, CD32, and ICAM-1 were first detected on the sinusoidal lining of fetal liver. However, at this stage, their distribution was irregular and discontinuous along the sinusoidal wall. The apparent expression level of CD4, CD32, and ICAM-1 progressively increased on sinusoidal endothelial cells from 10 to 12 weeks of gestation. The distribution of these markers along the sinusoidal wall was continuous after 12 weeks of gestation (Fig 3b). Ultrastructural examination confirmed that fetal sinusoidal endothelial cells, along with Kupffer cells, expressed the CD4 molecule, with a distribution restricted to their luminal surface (Fig 3c). The receptor for the lipopolysaccharide-binding protein (CD14) was not detected on sinusoidal endothelial cells until 20 weeks of gestation (Fig 3d). It remained faintly detected from 20 weeks of gestation onwards.

*Expression of the Markers Specific for Adult Liver Sinusoidal Endothelial Cells According to the Gestational Age*

At 5, 6, and 7 weeks of gestation, laminin was strongly expressed by the basal lamina in portal spaces and was more faintly detected in the perisinusoidal matrix (Fig 4a). Tenascin was undetectable along sinusoids but was detectable in the mesenchyme of the septum transversum (Fig 4b). At 8 and 9 weeks of gestation, laminin was detected in
Fig 5.
very low amounts in the perisinusoidal matrix, in which it formed thin and discontinuous deposits. In contrast, laminin remained strongly detected in the epithelial and vascular basal lamina of portal tracts. Tenascin remained undetectable along sinusoids, whereas it was readily detected in the mesenchyme surrounding the portal veins and the centrilobular veins. From 10 weeks of gestation onwards, laminin was restricted to the basal lamina of portal tracts and was undetectable in the perisinusoidal matrix (Fig 4c). The presence of tenascin was faintly detectable along sinusoids for the first time at 12 weeks of gestation (Fig 4d). Ultrastructural examination confirmed its deposition in the perisinusoidal matrix (Fig 4e). The amount of tenascin progressively increased along sinusoids until 15 weeks of gestation (Fig 4f). After this date, its apparent level of expression was comparable to that observed in the adult perisinusoidal matrix. Simultaneously, tenascin progressively disappeared from the mesenchyme surrounding the portal and centrilobular vessels (Fig 4f).

Expression of the Extracellular Matrix Components Fibronectin and Thrombospondin According to the Gestational Age

At 5, 6, and 7 weeks of gestation, the expression of fibronectin and thrombospondin could not be tested in our technical conditions. At 8 and 9 weeks of gestation, fibronectin was present in both the perisinusoidal matrix (Fig 5a) and the mesenchyme of portal tracts. At this stage, thrombospondin was detected only in megakaryocytes and was not observed in the subendothelial matrix of intrahepatic sinusoid vessels (Fig 5b).

From 10 weeks of gestation onwards, the amounts of fibronectin (Fig 5c) and thrombospondin (Fig 5d and e) present in the perisinusoidal matrix progressively increased until 20 weeks of gestation. The apparent level of expression of fibronectin remained unchanged until birth, whereas that of thrombospondin progressively decreased from 35 weeks of gestation onwards. In the immediate perinatal period, as in the adult liver, only scattered deposits of thrombospondin were present in the perisinusoidal matrix (Fig 5f).

Western Blotting

Liver samples from various developmental stages (11, 12, 15, 18, and 21 weeks of gestation) were probed by Western blotting for the expression of PECAM-1, CD4, and tenascin (Fig 6). PECAM-1 was detected as two bands of approximately 90 and 110 kD. The apparent level of detection of the bands did not change significantly during the development. This is in accordance with our immunohistochemical study, which showed no change in the pattern of expression of PECAM-1 from 11 weeks of gestation onwards. CD4 migrated as a single band of 55 kD at all stages. The reactive band was weak at weeks 11, 12, and 14 of development; was readily detected at 18 weeks; and was strongly detected at 21 weeks. This is in accordance with our immunohistochemical results, which showed the progressive increase in CD4 expression along sinusoids after 10 weeks of gestation. Antitenascin antibody detected two high molecular weight proteins of approximately 210 and 260 kD at all developmental stages studied. The apparent level of the two bands was the same at 11 and 21 weeks of gestation. This might indicate that the progressive deposition of tenascin along sinusoids, observed in our study from 12 weeks of gestation, is counterbalanced by the simultaneous disappearance of this extracellular matrix protein from the hepatic mesenchyme.

DISCUSSION

Our study shows that intrahepatic capillary vessels undergo a marked structural remodeling early in the course of liver organogenesis between 5 and 12 weeks of gestation. During this period, intrahepatic capillary vessels progressively lose the expression of several markers expressed by embryonic endothelial cells and retained by most adult microvascular endothelial cells, such as the cell-cell endothelial adhesion molecule PECAM-1 and the sialomucin CD34. In the same time, the composition of the adjacent subendothelial matrix progressively shifts from that of a typical vascular basement membrane, rich in laminin and devoid of tenascin, to that typical of the adult perisinusoidal matrix, nearly devoid of laminin and rich in tenascin. This period of structural differentiation corresponds to the establishment of the definitive vascular architecture of the liver and to the onset of intrahepatic hematopoiesis, which begins by 7 weeks of gestation and reaches its maximum intensity by 12 weeks of gestation. It is interesting to note that the overall structure of intrahepatic sinusoids at 12 weeks of gestation is very close to that of the sinuses of the hematopoietic bone marrow. Like fetal hepatic sinusoids, adult bone marrow sinuses are lined by a fenestrated and discontinuous endothelial lining, and lack any detectable expression of PECAM-1, and are devoid of organized basement membrane containing laminin. On the basis of studies performed in the mouse, it is also probable, although not yet definitely established in humans, that the endothelium of adult bone marrow si-
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nuses, in contrast to that of intramedullary arterioles, lacks the CD34 protein. The absence of PECAM-1 and CD34 protein in fetal sinusoids and bone marrow sinuses suggests that their presence is not necessary for the exit of blood cells. This is in contrast with the role played by these molecules in the transendothelial migration of leukocytes in other organs and at sites of inflammation.33-35

The deposition of tenascin in the perisinusoidal matrix of the liver might also be interpreted in the line of the existence of a specific hematopoietic microenvironment in the fetal liver. This is suggested by the recent demonstration that, in the adult human bone marrow, tenasin,33 along with other extracellular matrix components such as fibronectin and thrombospondin, contributes to create the microenvironment necessary to support hematopoiesis. The commitment of tenasin in the formation of the hematopoietic environment in the fetal liver is further suggested by the late onset of the synthesis and deposition of this protein in the perisinusoidal matrix, which coincides with the onset of the maximum hematopoietic activity of the fetal liver. An alternative hypothesis for the role of tenasin in the fetal liver would be that, as in other organs,36 this extracellular matrix component plays a morphogenetic role during liver organogenesis. It is known that tenasin is involved in epithelial-mesenchymal interactions within several developing organs, such as the gut37 and the kidney.3839 In the developing liver, its location at the interface between hepatoblasts and endothelial cells might suggest a comparable role. However, several arguments make this hypothesis unlikely. (1) Our analysis of tenasin in fetal liver by using Western blotting techniques did not show any change in the characteristics of this molecule with time, in contrast to the situation observed in the developing gut37 and kidney.38 (2) The onset of tenasin deposition, detected in our study at 12 weeks of gestation, occurs after the completion of the first phase of structural differentiation of the wall of intrahepatic capillary vessels week. It is therefore unlikely that tenasin plays a major role in this process.

Most of the characteristics acquired by the intrahepatic parenchymal capillary vessels during the early phase of liver organogenesis and likely to play a role in the hematopoietic function of the fetal liver are retained after the decline of the hematopoietic activity of the liver in the prenatal period and persist during the whole postnatal life. The only significant change observed in our study is the decrease in the apparent expression level of thrombospondin, which begins by 35 weeks of gestation, in correlation with the onset of the progressive decrease of the hematopoietic activity of the fetal liver. It might therefore be speculated that some of the more striking characteristics of adult liver sinusoids, such as the absence of endothelial expression of PECAM-1 and CD34, the near absence of laminin, and the abundance of tenasin in the subendothelial matrix, record the past fetal hematopoietic function of the liver. In this context, it is interesting to recall that the adult liver remains able to support active hematopoiesis, as shown by the occurrence of hepatic myeloid metaplasia in conditions associated with bone marrow myelofibrosis.4041

In addition to the development of characters likely to be associated to the hematopoietic function of fetal liver, fetal sinusoidal endothelial cells also progressively acquire the functional characteristics of their adult counterparts. Our study indicates that this process of functional differentiation initiates immediately after the completion of the process of structural differentiation that takes place from 5 to 10 weeks of gestation. The most specific feature of adult sinusoidal endothelial cells is the expression of the CD4 protein,1342 which is first detected in our study at 10 weeks of gestation. The expression level of CD4 progressively increases with time, as shown by our immunohistochemical and Western blotting results. It would be interesting to determine whether the induction of CD4 expression by liver sinusoidal endothelial cells is associated with the induction of the regulating transcriptional factors identified for this molecule in T-lymphocyte precursors.5447 The induction of expression of CD4 on fetal sinusoidal endothelial cells is accompanied by the expression of several other functional markers of adult sinusoidal endothelial cells, such as ICAM-1 and the receptors II for the Fc fragment of IgG. In our study, all these proteins can be detected as soon as 10 weeks of gestation along liver sinusoids, and their expression increases with time. In contrast, another functional marker of adult sinusoidal

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Fig 6. Western blotting analysis of fetal liver samples. Protein immunoblots of fetal liver extracts at 11, 12, 14, 18, and 21 weeks of gestation (lanes 1 through 5) were performed with anti-PECAM-1 (CD31), anti-CD4 (CD4), or antitenascin (TN) antibodies. Equal amounts of protein from each extract were blotted and probed with specific antisera. The apparent expression level of PECAM-1 (CD31), detected as two bands of approximately 90 and 110 kD, is comparable at all stages of liver development. CD4 migrates as a single band of 55 kD. Its expression increases markedly from 12 weeks of gestation onwards. Tenascin (TN) is detected as two bands of approximately 210 and 260 kD. No qualitative change is observed between the different stages of liver development. The apparent expression level is comparable from 12 to 21 weeks of gestation.
endothelial cells, the lipopolysaccharide-binding protein receptor (CD14), is detected only at 20 weeks of gestation and its expression increases only in the immediate prenatal period. In this respect, our results are therefore in keeping with the results of flow cytometry studies showing the very low number of CD14+ cells in the human fetal liver. This might concur with the late functional development of the gut, which is the main source of lipopolysaccharide found in the portal blood circulating in liver sinusoids. Our study also shows that the lack of expression of P-selectin and DAF that characterizes adult sinusoidal endothelial cells occurs early in the process of differentiation of liver sinusoids. In our study, in accordance with previous studies, these two proteins are not detectable in sinusoidal endothelial cells as soon as 8 weeks of gestation. However, in the absence of data about the earlier stages of liver organogenesis, we could not determine whether the lack of P-selectin and DAF by fetal sinusoidal endothelial cells is due to an early repression or to an absence of induction of their expression.

The molecular events involved in the process of fetal sinusoidal differentiation are poorly known. A direct role of the specific liver microenvironment in inducing and maintaining the sinusoidal pattern of differentiation of endothelial cells is suggested by experimental studies. However, the corresponding inductive factors and their cellular sources remain to be characterized. A role for hepatocyte growth factor (HGF) is suggested by a recent experimental study showing that transgenic mice lacking HGF expression present liver abnormalities characterized by the dilatation of intrahepatic sinusoid vessels associated with an atrophy of hepatocyte cords.

In conclusion, our study provides an in situ analysis of the process of differentiation of fetal liver sinusoidal endothelial cells. Two coordinated events, involving both sinusoidal endothelial cells and their adjacent subendothelial matrix, take place: (1) an early structural differentiation from 5 to 12 weeks of gestation that likely contributes to make liver sinusoids adapted to the hematopoietic function of fetal liver and (2) a later functional differentiation from 10 weeks of gestation onwards resulting in the acquisition of the differentiated characteristics of adult liver sinusoidal endothelial cells that makes them adapted to the specific functions and microenvironment of the adult liver tissue.

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