Ultrastructural localization of integrin subunits α3 and α6 in capillarized sinusoids of the human cirrhotic liver

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Summary. Normal liver sinusoids are not lined by a basement membrane (BM). In contrast, in the course of development of liver cirrhosis, a structured BM is formed de novo in the space of Disse. This BM contributes to the inhibition of the metabolic function of the liver but the pathogenic background of the formation of this perisinusoidal BM is still unclear. Integrins of the β1-class are generally essential for BM stability and some of them (such as α2β1, α3β1 and α6β1) appear de novo in the perisinusoidal space of the cirrhotic liver. Their cellular distribution in capillarized sinusoids as well as the correlation between their cellular distribution and the formation of the microvascular BM in the cirrhotic liver has not been shown at the ultrastructural level. In the present work we aimed to clarify this issue. We focused on integrins α3β1 and α6β1 and localised them ultrastructurally in human cirrhotic liver microvessels using postembedding immunogold which allows the ultrastructural localization of antigens with high resolution in the tissue. The newly formed basement membrane of capillarized sinusoids was visualized by means of fixation with addition of tannic acid, which enables the visualization of structures of the extracellular matrix with the highest resolution. Also, we carried out laminin detection using postembedding immunogold. Our results show that both α3β1 and α6β1 are expressed on the surface of both hepatocytes and endothelial cells, i.e. on both sides of the newly formed basement membrane. This latter shows zones of higher density both in close proximity to the endothelial and to the hepatocytic surfaces which resemble laminae densae. We propose that hepatocytes and endothelial cells may, therefore, by expressing such integrins, contribute to the formation of this pathological BM in the microvessels of the human cirrhotic liver. On stellate cells, which are major producers of BM components, both integrins α3β1 and α6β1 were also localized.

Key words: Integrins, Basement membrane, Human cirrhotic liver, Postembedding immunogold, Silver enhancement

Introduction

One of the unique features of the hepatic perisinusoidal matrix is that although it contains basement membrane components, such as type IV collagen and perlecan (Hahn et al., 1980; Murdoch et al., 1994), there is no structured basement membrane (Schaffner and Popper, 1963). However, in the course of liver cirrhosis, a structured basement membrane develops in sinusoids (Schaffner and Popper, 1963). This pathological basement membrane contributes to the inhibition of the metabolic function of the liver (Martinez-Hernandez and Martinez, 1991). The pathogenic background of basement membrane formation in the hepatic perisinusoidal space is still unclear. It is known that basement membrane components in a free solution in vitro can self-assemble and thus polymerize to form a basement membrane (Yurchenco, 1994). On the other hand, it has been well demonstrated that in vivo (Kreidberg et al., 1996; Di Persio et al., 1997; Brakebusch et al., 2000; Raghavan et al., 2000) β1-class integrins are necessary for basement membrane formation in the hepatic perisinusoidal space which resemble laminae densae. We propose that hepatocytes and endothelial cells may, therefore, by expressing such integrins, contribute to the formation of this pathological BM in the microvessels of the human cirrhotic liver.
the cirrhotic basement membrane in capillarized sinusoids. In this context, the cellular localization of such integrins may help to clarify which of the cells adjacent to the newly formed basement membrane are involved in its formation and stability. The study of the ultrastructural localization of these integrin subunits expressed de novo in capillarized sinusoids is particularly interesting from the point of view of basement membrane formation in contrast, for example, to other subunits, such as \( \beta 1 \) or \( \alpha 1 \), which are expressed both in normal and in diseased liver (Couvlerard et al., 1993). This knowledge would contribute considerably to our understanding of the molecular mechanisms which regulate de novo formation of the perisinusoidal basement membrane in liver cirrhosis in humans. In the present study, we focused on the integrins \( \alpha 3\beta 1 \) and \( \alpha 6\beta 1 \) and aimed to correlate their ultrastructural distribution in human liver capillarized sinusoids with the newly formed basement membrane in capillarized sinusoids. This latter was visualized by means of fixation with addition of tannic acid which enables visualization of basement membranes (and extracellular matrix in general) with high resolution in ultrathin sections (Herken and Barrach, 1985; Quondamatteo et al., 1998). Also, laminin detection was carried out as a marker of sinusoidal capillarization (Hahn et al., 1980). Integrin as well as laminin distribution was studied by means of postembedding-gold immunohistochemistry which enables the detection of antigens in ultrathin sections with a higher resolution and higher sensitivity in comparison to pre-embedding techniques (Herken et al., 1987; Herken and Miosge, 1991).

Materials and methods

Tissue studied

Specimens from cirrhotic livers explanted from nine patients undergoing liver transplantation in the Department of Transplantation Surgery of the University of Goettingen were collected according to the Guidelines of the Ethics Committee of the Faculty of Medicine of the University in Goettingen. The cirrhosis was either due to alcoholism (n=2), was secondary to B-hepatitis virus infection (n=1), secondary to C-hepatitis virus infection (n=2), was a biliary cirrhosis (n=1), or was of unclear etiology (n=2).

During preparation, fragments for examination were taken from at least three different regions from each specimen for each fixation/embedding protocol. After appropriate fixation (see below) the tissue was embedded in epon for an optimal basement membrane visualization or in LR-Gold resin for the immunohistochemical detection of the integrin subunits and of laminin molecules.

Embedding in Epon

Liver specimens were obtained immediately after removal of the liver and cut into pieces (1 mm\(^3\)) in 0.1 M phosphate-buffered saline. The tissue was fixed for 90 min in a solution of 1% tannic acid + 1% glutaraldehyde in 0.1M Sörensen's buffer (pH=7.4). Tissue fragments were washed for 20 min in 0.15 M phosphate-buffered saline and subsequently treated with 1% osmium tetroxide in 0.15 M phosphate-buffered saline for 2 h. Specimens were then dehydrated in a graded ethanol series and embedded in epon. For orientation purposes, semithin sections were cut and stained with toluidine blue. Ultrathin sections were then cut with a Reichert-Jung ultramicrotome, collected on formvar-coated copper grids, stained with uranyl acetate for 10 min and with lead citrate for 7 min and examined with a Zeiss EM 109 or a LEO 906E electron microscope.

LR-Gold embedding

Liver specimens were obtained immediately after removal of the liver and cut into pieces (1 mm\(^3\)) in 0.1 M phosphate-buffered saline. The tissue was then fixed in 4% paraformaldehyde + 0.5% glutaraldehyde for 30 min. Thereafter, free aldehydes were washed out by incubating the tissue for 45 min with 10mM NH\(_4\)Cl at 4 °C, and the tissue was dehydrated in a graded ethanol series up to 70% ethanol. The tissue was then infiltrated with the hydrophilic resin LR-Gold (London Resin, Reading, UK) for 1 h at -25 °C, then with LR-Gold supplemented with 0.8% of the light-sensitive initiator benzil overnight at -25 °C in the dark. Polymerization was carried out at -25 °C for 24 h under the light of a 500W halogen lamp.

Anti-laminin antibodies and antibodies against integrin subunits \( \alpha 3 \) and \( \alpha 6 \)

Anti-laminin antibodies were purchased from Sigma (Munich, Germany). Anti-laminin antibodies were polyclonal, raised in rabbits using laminin-1 as antigen (\( \alpha 1, \beta 1, \gamma 1 \), Burgeson et al., 1994) and affinity purified. Antibodies against the integrin subunits \( \alpha 6 \) (GoH3 rat monoclonal antibody) and \( \alpha 3 \) (J143 mouse monoclonal antibody) were obtained from hybridoma supernatants (Kantor et al., 1987; Sonnenberg et al., 1987). These were concentrated by means of 45% ammonium sulfate (3.9 M) precipitation at 4 °C. The presence of immunoglobulins was ascertained by SDS-page electrophoresis before antibodies were used for the immunohistochemical detection of integrin subunits.

Postembedding gold immunohistochemistry

Light microscopy

Semithin sections (1 \( \mu \)m) were cut using a Reichert-Jung ultracut microtome, collected on microscope slides (Super Frost*/Plus, Menzel, Braunschweig, Germany) and dried for 2 h at 40 °C. The sections were rehydrated with double-distilled water and then treated with Lugol-
solution for 5 min. Thereafter, the slides were dipped in 4% sodium thiosulfate for 20 sec and washed for 2 min in double-distilled water. The sections were predigested with ficin (24 mg/ml in double-distilled water) for 15 minutes at room temperature and unspecific binding sites were blocked with 1% BSA-TBS for 10 min at room temperature. Sections were subsequently processed for the immunohistochemical detection of laminin molecules and of integrin subunits α3 and α6:

**Laminins.** Semithin sections were incubated with the primary antibody 1:10 in 0.05 M Tris-buffered saline (pH=7.4) for 90 min at room temperature. After having been washed in Tris-buffered saline, sections were incubated with a goat anti-rabbit antibody (Medac, Hamburg, Germany) 1:200 in 0.05 M Tris-buffered saline (pH=7.4), for 20 min at room temperature. The secondary antibody was coupled to 8 nm colloidal gold particles in our laboratory (De Mey et al., 1981). Colloidal gold particles of 8 nm were obtained by adding 1 ml of 1% tetrachloroauric acid to 106 ml of 2.2 mM sodium citrate.

**Integrin subunit α3.** Semithin sections were incubated with gold-labeled J143-antibody for 2 h at room temperature, diluted 1:100 in 0.05 M Tris-buffered saline (pH=7.4). Primary antibodies were directly coupled to 8 nm colloidal gold particles in our laboratory (De Mey et al., 1981).

**Integrin subunit α6.** Semithin sections were incubated with gold-labeled GoH3-antibody for 2 h at room temperature, diluted 1:100 in 0.05 M Tris-buffered saline (pH=7.4). Primary antibodies were directly coupled to 8 nm colloidal gold particles in our laboratory (De Mey et al., 1981).

Colloidal gold was visualized in semithin sections by silver enhancement. Following the antibody reactions, the sections were rinsed with 0.05 M Tris-buffered saline. In order to minimize subsequent loss of label, sections were post-fixed for 2 min in 2.5% glutaraldehyde and after extensive washing with double-distilled water, immunogold reactions were enhanced by incubating the sections for 5 to 10 min with the developing solution, freshly prepared prior to use (200 mg hydroquinone in 40 ml citrate buffer, pH=3.8 supplemented with 80 mg silver acetate in 40 ml double-distilled water). The development of the reactions was carried out in the dark in order to prevent non-specific precipitates of metallic silver. The reactions were stopped by washing the sections with 4% sodium thiosulfate for 5 min. After having been washed, the sections were dried at 40 °C, stained with toluidine blue and cover slipped. They were then examined under a Zeiss light microscope.

**Electron microscopy**

Ultrathin sections (70 nm) were cut from different regions of the cirrhotic nodules and collected on formvar-coated nickel grids. Sections were incubated with ficin (24 mg/ml in double-distilled water) for 15 min at room temperature and then washed with double-distilled water. Unspecific binding sites were blocked with 1% bovine serum albumin in 0.05 M Tris-buffered saline for 10 min. Thereafter, ultrathin sections were processed for the immunohistochemical detection of laminin and of the integrin subunits α3 and α6. Immunohistochemistry was performed as described above, but primary antibodies (and secondary for laminin detection) were coupled to gold particles of 16 nm instead of 8 nm. These were obtained by reducing 50 ml of 0.01% tetrachloroauric acid with 1 ml of 1% sodium citrate (De Mey et al., 1981; Slot and Geuze, 1981). After incubation with the antibody the sections were rinsed with 0.05 M Tris-buffered saline and stained with uranyl acetate for 10 min and lead citrate for 10 min at room temperature. They were then examined with a Zeiss EM 109 electron microscope.

**Control reactions**

To exclude unspecific binding of colloidal gold particles, semithin and ultrathin sections were incubated with pure gold solution under the same conditions as described above. Additional negative controls were carried out by incubating sections with a gold-labeled anti-rabbit antibody (Medac, Hamburg, Germany).

**Results**

**Ultrastructure**

Parenchymal microvessels of cirrhotic liver were lined with a continuous endothelium. Microvilli of hepatocytes were bent along the hepatocytic surface. A basement membrane positive for tannic acid (Figs. 1-3), was visible between endothelial cells and hepatocytes. The basement membrane of the capillarized sinusoids showed a clear lamina densa in close proximity to its endothelial side. Between this lamina densa and the membrane of the endothelial cells a thin lamina rara was visible. A further higher density of the tannic acid-positive material was visible near the hepatocytes, forming another lamina densa. This was separated from the hepatocytes by a narrow and irregular lamina rara. In the space between the two laminae densae filamentous basement membrane material strongly positive for tannic acid was found.

**Immunohistochemistry**

**Laminin localization**

After incubation of the semithin sections with anti-laminin antibodies (Fig. 4a), a clear continuous staining lining the parenchymal microvessels was seen. At the level of the basement membrane zones of bile ducts and the stromal blood vessels, staining was also seen. At the ultrastructural level (Fig. 4b), gold labeling for laminin was diffusely distributed throughout the basement membrane.
membrane of capillarized sinusoids, as well as in other basement membranes (e.g. bile ducts).

Alpha-3 integrin localization

After incubation of the semithin sections of cirrhotic human liver with anti-α3 antibodies (Fig. 5a), a clear continuous staining lining the capillarized sinusoids was observed. The basement membrane zones of bile ducts and of stromal blood vessels located within fibrotic septa were also clearly stained. Moreover, there was weaker staining in mesenchymal cells located in the fibrotic septa.

At the ultrastructural level, human cirrhotic liver...
Fig. 5. a. Postembedding immunogold for the detection of the α3 integrin subunit on a semithin section of cirrhotic human liver parenchyme. Capillarized sinusoids are lined by positive immunostaining (arrows). h: hepatocytes. x 1000. b. Cirrhotic human liver. Ultrastructural localization of the α3 integrin subunit by means of postembedding immunogold in capillarized sinusoids. Positive staining is present both on hepatocytic (black arrows) and on the endothelial (open arrows) surfaces. h: hepatocytes; ec: endothelial cell; cs: lumen of the capillarized sinusoid; rbc: red blood cell. x 11000

Fig. 6. Cirrhotic human liver. Ultrastructural localization of the α3 integrin subunit by means of postembedding immunogold in capillarized sinusoids. Positive staining (arrows) is present at the surface of endothelial cells (ec). cs: lumen of the capillarized sinusoid; rbc: red blood cell. x 15000.

Fig. 7. Stellate cell (sc) of cirrhotic human liver after ultrastructural localization of the α3 integrin subunit. Positive staining (arrows) is present on the stellate cell (sc) surface. x 5250

Fig. 8. a. Postembedding immunogold for the detection of the α6 integrin subunit on a semithin section of cirrhotic human liver parenchyme. Capillarized sinusoids are lined by positive immunostaining (arrows). h: hepatocytes. x 1000. b. Cirrhotic human liver. Ultrastructural localization of the α6 integrin subunit by means of postembedding immunogold in capillarized sinusoids. Positive staining is present on the hepatocytic (black arrows) surface. h: hepatocytes; ec: endothelial cell; cs: lumen of the capillarized sinusoid. x 15000

Fig. 9. Cirrhotic human liver. Ultrastructural localization of the α6 integrin subunit by means of postembedding immunogold in capillarized sinusoids. Positive staining is present on endothelial (black arrows) and on hepatocytic (open arrow) surfaces. h: hepatocytes; ec: endothelial cell; cs: lumen of the capillarized sinusoid. x 15000

Fig. 10. Stellate cell prolongation (**) of cirrhotic human liver after ultrastructural localization of the α6 integrin subunit. Positive staining (black arrows) is present along the stellate cell surface. h: hepatocyte; ec: endothelial cell. x 13000
parenchymal microvessels showed staining for α3-integrin which was located along the extracytoplasmic side of the hepatocytic cell surface (Fig. 5b), as well as over the abluminal cell surface of the microvascular endothelium (Fig. 6). The pattern was often focal. The surfaces of stellate cells were also stained (Fig. 7).

Alpha-6 integrin localization.

After incubation of the semithin sections with anti-α6 antibodies (Fig. 8a) the pattern of the immunohistochemical staining did not differ from that of α3 staining. Also, in the case of α6, staining was seen to line intraparenchymal microvessels as well as the basement membrane zones of all bile ducts and stromal blood vessels. Staining was also seen on mesenchymal cells located in the fibrotic septa.

Ultrastructurally, in the perisinusoidal space of human capillarized sinusoids gold labeling was observed along the extracytoplasmic side of the hepatocytic (Fig. 8b) as well as of the endothelial cell surfaces (Fig. 9). Labeling for α6 also occurred in a focal pattern, similar to that of α3. Gold labeling was also detected on stellate cell surfaces (Fig. 10).

Discussion

In the present work we have shown for the first time the ultrastructural distribution of integrin subunits α3 and α6 in capillarized sinusoids of cirrhotic human liver. The distribution of such integrin subunits in human liver cirrhosis has previously been studied at the light microscopic level (Volpes et al., 1991; Couvelard et al., 1993; Torimura et al., 1997). In contrast, their cellular distribution has never been shown at the ultrastructural level. However, due to the limited resolution of light microscopy, only an ultrastructural investigation would clarify the cellular distribution of such integrin subunits in cirrhotic liver sinusoids. We aimed to answer this question and, therefore, localized α3 and α6 integrin subunits ultrastructurally.

Here we have shown that in capillarized sinusoids of the cirrhotic human liver, α3 and α6 integrin subunits are located on the surface of endothelial cells, of hepatocytes and of stellate cells. The integrin subunit α3 was reported to be mainly located at the periphery of the cirrhotic nodules in frozen sections (Couvelard et al., 1993) but we did not note any particular topographic correlation. Probably, the specimens that we examined were of later stages and in the later stages of cirrhosis, α3 integrin molecules in the liver may increase. This is conceivable since α3β1 integrins are essential for basement membrane structure (Kreidberg et al., 1996; DiPersio et al., 1997; Sasaki et al., 1998; Aumailley et al., 2000; Brakebusch et al., 2000; Raghavan et al., 2000; Li et al., 2002). Therefore, the elucidation of the cellular distribution of α3 and α6 in sinusoids of the cirrhotic human liver (and therefore of α3β1 and α6β1) would be help to clarify which cells may contribute to the process of capillarization of the sinusoids, which is a key event in cirrhosis (Schaffner and Popper, 1963; Martinez-Hernandez and Martinez, 1991).

As shown here, in the capillarized sinusoids of human liver cirrhosis, integrin subunits α3 and α6 are present along both the membranes of hepatocytes and endothelial cells. This indicates that α3β1 and α6β1 are expressed both in hepatocytes and endothelial cells. This correlates with sinusoidal capillarization. In fact, we also show laminin molecules in the perisinusoidal space and presence of a basement membrane which are known markers of sinusoidal capillarization (Schaffner and Popper, 1963; Hahn et al., 1980).

In the newly formed pathological perisinusoidal basement membrane, structures which resemble laminae densae can be found both in the proximity of the hepatocytic and of the endothelial cell borders. This, together with laminin detection in the perisinusoidal space and with localization of α3β1 and α6β1 in hepatocytes and endothelial cells, indicates that in sinusoids of cirrhotic human liver the integrins α3β1 and α6β1 are expressed on both sides of the newly formed pathological basement membrane. One interesting
Integrins α3 and α6 in human cirrhotic liver

question is what the influence of these integrins on hepatocytes and on endothelial cells in the human cirrhotic liver may be.

It is possible that these β1-class integrins may somehow contribute to the organization of the newly formed pathological basement membrane which completely fills the space of Disse and separates the capillarized sinusoids from the hepatocytes. There is in fact much evidence that indicates β1-class integrins are essential for the formation of a structured and stabilised basement membrane (Kreidberg et al., 1996; DiPersio et al., 1997; Sasaki et al., 1998; Aumailley et al., 2000; Brakebusch et al., 2000; Raghavan et al., 2000; Li et al., 2002). Also, evidence that argues in favour of β1-class integrins as organisational factors for basement membrane structures at the cell surface has been provided (Fleischmajer et al., 1998a,b; Henry et al., 2001; Lohikagans et al., 2001). According to this view, β1-class integrins (possibly in concert with other receptors, e.g. dystroglycan or further, still unknown molecules) could catalyst the self-assembly of basement membrane components and stabilise the basement membranes formed. Our in vivo data from human liver cirrhosis, showing integrins α3ß1 and α6ß1, i.e. receptors for basement membrane components, at locations adjacent to the newly formed pathological basement membrane of sinusoids are consistent with this view.

It is also known that signalling mediated by β1-class integrins may also modulate the production of basement membrane components (Sasaki et al., 1998; Aumailley et al., 2000; Li et al., 2002). In the present work, we have demonstrated the presence of α3 and α6 integrin subunits in stellate cells in vivo for the first time. In this context, one can assume that the presence of α3ß1, α6ß1 (and possibly α6ß4) integrins on stellate cells may regulate a signalling pathway necessary for basement membrane production. Stellate cells are the main producers of extracellular matrix molecules and thus also of basement membrane components in liver cirrhosis (Reeves and Friedman, 2002). It has been shown that the integrin subunit α6 is expressed by stellate cells in culture (Carloni et al., 1996). Here we show this in vivo and we also show that stellate cells in vivo express the subunit α3.

In summary, in the present work, we have shown the ultrastructural distribution of integrin subunits α3 and α6 in cirrhotic human capillarized liver sinusoids for the first time on the surface of endothelial cells, of hepatocytes and of stellate cells. Our findings indicate that, in vivo, the expression of integrins α3ß1 and α6ß1 (and probably also α6ß4) correlate with basement membrane formation in capillarized human cirrhotic sinusoids. This suggests that the notion that β1-integrins take part in regulation of basement membrane formation may apply to the formation of the perisinusoidal basement membrane in human cirrhotic liver as well. In line with this, our findings also suggest that the suppression of the expression of such integrins could be a possible therapeutic approach towards inhibiting basement membrane formation in the cirrhotic liver in the future. This could contribute to an improvement of the metabolic function of the liver in the course of hepatic cirrhosis.

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805
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