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# Carcinoma-associated perisinusoidal laminin may signal tumour cell metastasis to the liver

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**Summary.** The perisinusoidal space of the liver shows extensive modulation of the extracellular matrix in response to various pathological conditions. We studied perisinusoidal laminin expression immunohistochemically using polyclonal and monoclonal antibodies in 110 human liver specimens obtained at autopsy. In normal adult liver the perisinusoidal spaces contained only minimal amounts of immunoreactive laminin. In 86% of patients dying from cancer with liver metastasis, however, a distinct increase in the amount of perisinusoidal laminin could be demonstrated. The perisinusoidal space also contained laminin in cancer patients without liver metastasis. In 3 cases of leukaemia sinusoids were laminin negative. In cirrhosis and chronic passive congestion there was, as expected, laminin immunoreactivity in the perisinusoidal space. The results obtained using polyclonal antibodies against laminin were confirmed using chain-specific monoclonal antibodies against B2 laminin. In an ex vivo assay, viable tumour cells (Panc-1 and clone A) were found to bind with remarkable specificity to frozen sections of liver tissue containing perisinusoidal laminin as opposed to liver tissues without laminin. We suggest that this perisinusoidal laminin may directly on indirectly mediate tumour cell metastasis to the liver.

**Key words:** Laminin – Liver metastasis – Perisinusoidal – Homing – Attachment

## Introduction

The perisinusoidal space of the normal liver is lined by hepatocytes and Ito cells on one side and endothelial cells on the other. The endothelial cell lining is fenestrated and in most species has no formed basement membrane (Burkel and Low 1966; Wisse 1977; Rojkind and Perez-Tamayo 1983). Under certain pathological conditions such as cirrhosis, extracellular matrix constituents, including laminin, become deposited in the perisinusoidal space (Bianchi et al. 1984; Martinez-Hernandez 1985). Untrastructurally, a basement membrane can be demonstrated in these livers accounting for the so-called capillarization (Schaffner and Popper 1963). Discrete transcripts of genes encoding various extracellular matrix components, including laminin B1, can be detected in the perisinusoidal cells of the normal human liver, but the hybridization signal was significantly increased during fibrillogenesis (Miliani et al. 1989, 1990). An acute development of basement membranes in the perisinusoidal space has been described following focal hepatic injury (Cossel 1966). An increased amount of laminin has been demonstrated in such conditions (Ogawa et al. 1986). Thus, laminin-producing perisinusoidal cells in the liver appear to have the capacity to increase laminin synthesis in response to a variety of non-specific stimuli.

Laminin in its classical form is composed of three chains, A, B1 and B2, with three short arms (36, 36, and 48 nm) and one long arm (77 nm) (reviewed by Beck et al. 1990). However, structural variants have been described, some of which are tissue specific. Thus, S-laminin (synaptic laminin), a 190 kDa B1 homologue is enriched at the neuromuscular junction, although it is also present in the glomeruli and some blood vessels (Hunter et al. 1989). Merosin (M), a 300 kDa laminin A related chain, is exclusively present in the basement membranes of trophoblast, striated muscle and Schwann cells (Ehrig et al. 1990). Recently, we reported a transient expression of B1, B2, S and M laminin in the perisinusoidal space during post-natal rat liver development and following hepatectomy (Wewer et al. 1992). These experimental data and the molecular biology reports on the up-regulation of genes for extracellular matrix molecules in fibrotic livers (Miliani et al. 1989, 1990), prompted us to explore whether tumour metastasis to the liver is accompanied by restructuring of liver sinusoids and whether the perisinusoidal spaces contain more immunodetectable laminin under these conditions.

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In the present study we demonstrate an increased expression of laminin in the perisinusoidal space of the liver in patients with cancer. We further demonstrate that this laminin seems to contain B2 subunit. In an attempt to elucidate the biological function of this laminin we established an ex vivo assay in which tumour cells were incubated with frozen sections of liver specimens with and without persinusoidal laminin. Our results indicate that perisinusoidal laminin may modify the microenvironment facilitating tumour cell metastasis to the liver.

## Materials and methods

Samples of liver tissue were obtained from autopsies at the Frederiksborg Amts Sygehus, Hillerød or at the Rigshospitalet, Copenhagen, Denmark. Post-mortem times were 12–24 h in most cases. Liver specimens from 110 cases were examined with ages ranging from 14 to 86 years. At least two samples from the non-capsular region were examined from each case. Liver metastases, if present, were also examined. Tissue specimens were routinely fixed in 10% buffered formalin. Furthermore, for optimal immunostaining with the monoclonal antibodies, additional samples were fixed in 96% ethanol/acetic acid (99:1) as described previously (Wewer et al. 1992). The tissue specimens were embedded in paraffin using standard techniques, and the blocks stored at 4° C until sectioning. For the ex vivo assay tissue specimens were snap-frozen in liquid nitrogen and stored at −80° C until further use. Diagnoses were made on the basis of histological features and the clinical information available. The 110 cases comprised: 67 cases of cancer, of which 42 had macroscopic liver metastases; 3 cases of leukaemia; 11 cases of liver cirrhosis; 4 cases of chronic passive congestion; and 25 cases dying from various other reasons, most often cardiovascular diseases. More specifically the 3 cases of leukaemia comprised 2 cases of chronic myeloid leukaemia and 1 case of chronic lymphocytic leukaemia. The 67 cases of cancer comprised: 20 lung carcinomas (13 squamous cell carcinomas, 6 small cell carcinomas, 1 adenocarcinoma); 8 colon/rectum adenocarcinomas, 6 breast carcinomas (invasive ductal carcinoma – not otherwise specified), 5 pancreatic adenocarcinomas, 5 hepatocellular carcinomas, 4 renal pelvic carcinomas (transitional cell carcinomas), 3 gastric adenocarcinomas, 3 oesophageal squamous cell carcinomas, 2 bladder carcinomas (transitional cell carcinomas), 2 endometrial adenocarcinomas, 1 thyroid carcinoma (follicular carcinoma), 1 ovarian cancer (serous cystadenocarcinoma), 1 melanoma, 3 fibrosarcomas, and 3 tumours of unknown origin.

Two human carcinoma cell lines were used: Panc-1, derived from the exocrine pancreas (Lieber et al. 1975) and clone A, a colon carcinoma (Danecker et al. 1989). Cells were grown in Eagle's MEM with 10% fetal bovine serum (culture medium) in Nunc flasks (Nunc, Naperville, Ill., USA). Cell suspensions for the ex vivo assays were prepared as follows. Cultured cells were trypsinized and resuspended as a single cell suspension in culture medium and allowed to recover in a test tube at 37° C for 45 min. The cell suspensions were gently shaken at 10-min intervals. After this period cells were pelleted, resuspended at a concentration of 100000 cells/ml in culture medium and seeded immediately onto the tissue sections. More than 95% of the cells were found to be viable by the dye exclusion test.

We used the following antibodies: (1) polyclonal rabbit antiserum against rat laminin purified from the L2 tumour (Wewer 1982; Engvall et al. 1983); (2) affinity-purified polyclonal anti-rat laminin antibodies (Albrechtsen et al. 1981); (3) polyclonal antiserum against human laminin (Wewer et al. 1983); and (4) chain-specific monoclonal antibody 2E8 ($IgG_1$) recognizing human laminin B2 chain (Engvall et al. 1986), kindly provided by Dr. Eva Engvall (La Jolla, Calif., USA). A series of other chain-specific monoclonal antibodies (against A, B1, S, M chain of laminin) used on rat livers before (Wewer et al. 1992) were also tested. However, since we were unable to obtain consistent immunohistochemical staining results on liver tissue sections derived from autopsies, these results are not included.

Freshly cut sections were deparaffinized, rehydrated and treated with 10% hydrogen peroxide in methanol for 10 min at room temperature to abolish endogenous peroxidase. Enzymatic pretreatment of the sections was carried out to unmask laminin epitopes. Formalin-fixed tissue specimens were pretreated with 4 mg/ml pepsin (P-7000, Sigma, St. Louis, Mo., USA) in 0.01 M hydrochloric acid for 1–2 h at 37° C. Tissue specimens fixed in ethanol/acetic acid were pretreated with 2 μg/ml protease (P-5147, Sigma) in 0.05 M TRIS-HCl, pH 7.4 for 5 min at room temperature. The unlabelled peroxidase-antiperoxidase technique (Sternberger 1979) was used for the rabbit antisera. The primary antiserum was diluted 1:500 and the affinity purified antibodies to 5 μg/ml in 0.05 M TRIS-HCl, pH 7.4 and incubated with the sections overnight at 4° C in moist chamber. The secondary and tertiary antibodies (Z196, Z113) were obtained from Dako (Copenhagen, Denmark) and used in a 1:50 dilution and incubated with the sections for 30 min each. The peroxidase was finally visualized by staining with 3.3′-diaminobenzidine tetrahydrochloride (Sigma), followed by 1 min of haematoxylin counterstaining. Washing (3 × 10 min) between the different steps was carried out in 0.05 M TRIS-HCl, pH 7.4 containing 0.25 M sodium chloride. The monoclonal antibody (culture supernatant) was diluted 1:2 and the secondary antibody consisted of peroxidase-conjugated rabbit anti-mouse immunoglobulins (P161, Dako). For controls preimmune rabbit or mouse serum and IgG were used instead of the primary antibody. Furthermore, inhibition assays were performed in which purified rat laminin was added in increasing amounts to the primary antibody solution used for incubation. For statistical analysis the Student's t-test was used.

Frozen sections (8 μm thick) were mounted on Lab Trek Chamber Slide (two-well; Nunc) air-dried for about 5 min, and pre-incubated with PBS (phosphate-buffered saline, 137 mM sodium chloride, 1.7 mM potassium chloride, 8.1 mM sodium hydrogen phosphate, 1.5 mM potassium dihydrogen phosphate, pH 7.2) followed by culture medium for 30 min. This pre-incubation step was essential to minimize non-specific attachment. All incubations were carried out at 37° C in 5% carbon dioxide. Aliquots of 100000 cells in 1 ml culture medium were placed over the tissue section. The sections were rinced with PBS after 1, 2, 3, 4 and 5 h of incubation, fixed and stained with haematoxylin and eosin. All assays were carried out in quadruplicate. The number of cells adhered and spread per "unit" (2000 $\mu m^2$) was counted. For statistical analysis the two-sample rank sum (Mann-Whitney) test was used.

## Results

The results are summarized in Table 1. In the normal human liver only very small amounts of perisinusoidal laminin immunoreactivity could be demonstrated (Fig. 1). However, laminin was demonstrated in the perisinusoidal space of the liver in 70 of 110 autopsy cases. As expected, all cases of cirrhosis ($n=11$) and chronic passive congestion ($n=4$) showed perisinusoidal laminin immunoreactivity. In the liver with macroscopic liver metastatic, perisinusoidal laminin was seen in 86% (Fig. 2). However, even in cancer patients without liver metastasis the perisinusoidal space contained laminin in 78% cases. Three cases of leukaemia showed no increased perisinusoidal laminin immunoreactivity. Among 25 patients dying from other reasons, mostly cardiovascular diseases, only 3 had increased laminin expression in the perisinusoidal space.

The pattern of laminin immunostaining is detailed

**Table 1.** Perisinusoidal laminin immunostaining in liver tissue specimens from 110 patients

| Diagnosis | No | Laminin-positive |
|---|---|---|
| Cancer[a] | 67 | 78% (52 cases) |
| Leukaemia | 3 | 0% (0 cases) |
| Cirrhosis | 11 | 100% (11 cases) |
| Congestion | 4 | 100% (4 cases) |
| Other diseases | 25 | 12% (3 cases) |
| Total | 110 | (70 cases) |

The expression of perisinusoidal laminin in patients with cancer (78%) is significantly higher than in patients dying from other reasons (12%) ($P < 0.001$).

[a] Of patients with liver metastasis (42 of the 67 patients) 86% expressed perisinusoidal laminin

in Fig. 3. A distinct linear immunostaining reactivity was located along the sinusoids. Immunoreactive laminin was distributed equally in the three Rappaport zones of the acinus. However, not all perisinusoidal spaces contained laminin. Cytoplasmic laminin immunoreactivity was occasionally noted in the perisinusoidal cells. In general, the blood vessels in the portal tract were stained more intensely for laminin than the central vein or the perisinusoidal space. Polyclonal antiserum to rat laminin, affinity-purified antibodies to rat laminin and polyclonal antiserum to human laminin showed the same immunostaining pattern. The presence of perisinusoidal laminin in patients with cancer was also verified using chain-specific monoclonal antibodies against laminin B2 (not shown). Controls in which specific antiserum was replaced with pre-immune serum showed no staining. Absorption of the antiserum with purified rat laminin diminished or abolished the immunoreactivity.

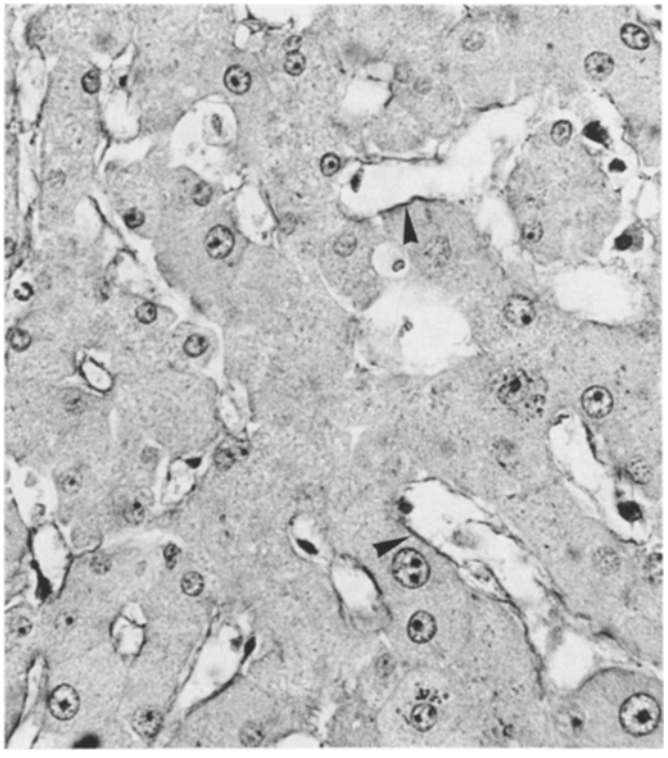

**Fig. 1.** Normal human liver. Formalin-fixed liver sections were immunostained with polyclonal antiserum to laminin. Note that subtle amount of laminin can be seen along the sinusoids (*arrowheads*). PAP technique, antibody serum 1:500, counterstained with haematoxylin, ×300

An ex vivo assay was designed in which viable tumour cells (Panc-1 and clone A) were seeded onto frozen sections of liver tissue. As demonstrated in Table 2 and Fig. 4, these tumour cells appeared to adhere and spread

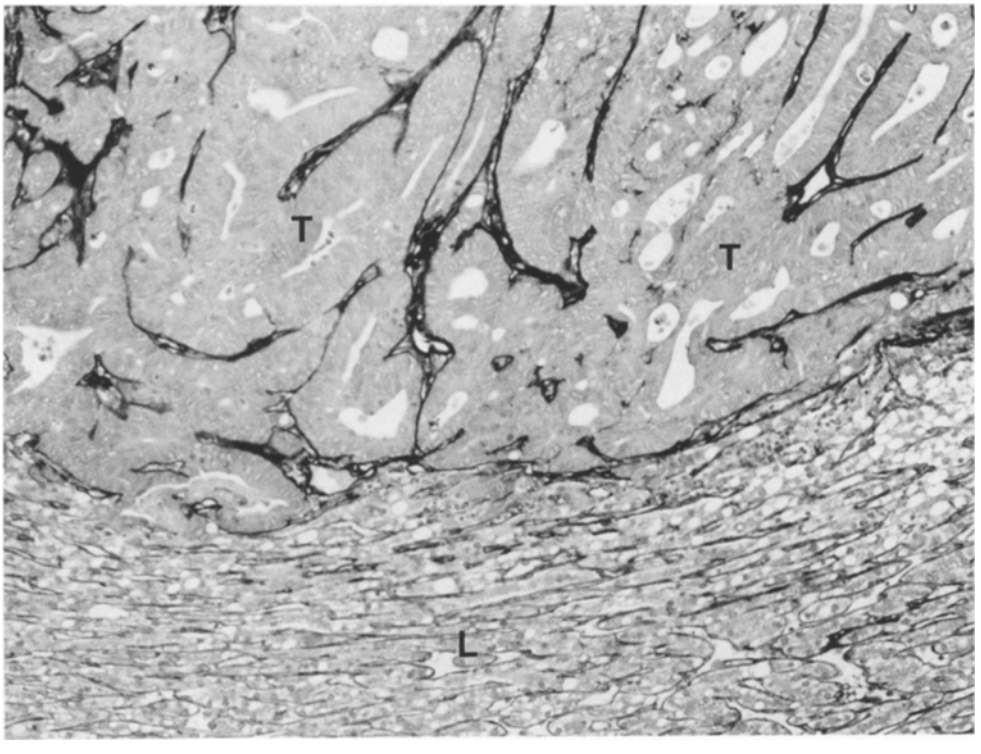


**Fig. 2.** Tumour metastasis in the liver. In this case a breast carcinoma (*T*) expressed laminin-positive basement membrane-like structures around the tumour islands. Note that perisinusoidal laminin can be seen in the surrounding liver tissue (*L*). PAP technique, antiserum dilution 1:500, counterstained with haematoxylin, ×132

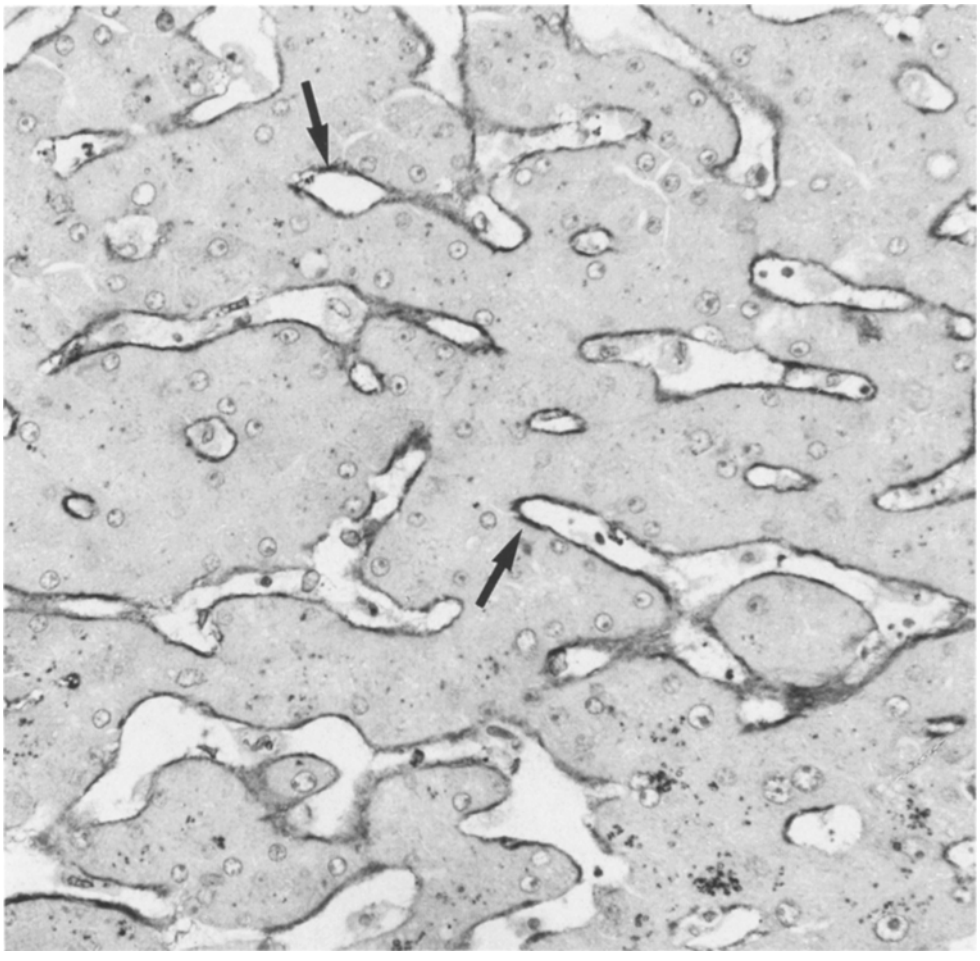

**Fig. 3.** Perisinusoidal laminin in the liver tissue of a patient who died from gastric cancer. Formalin-fixed tissue liver sections were immunostained with polyclonal antiserum to laminin. Note intense laminin immunoreactivity along the sinusoids (*arrows*). PAP technique, antiserum dilution 1:500, counterstained with haematoxylin, ×350

**Table 2.** Tumour cells (clone A and Panc-1) seeded onto frozen sections of liver tissue from patients with or wihout cancer (plus and minus perisinusoidal laminin)

| | Liver tissue with perisinusoidal laminin | Liver tissue without perisinusoidal laminin |
|---|---|---|
| | Number of cells per unit | Number of cells per unit |
| Clone A | 92.3+9.2 | 51.2+12.7 |
| Panc-1 | 25.6+3.4 | 8.8+ 3.5 |

Using the two-sample rank sum (Mann-Whitney test), the difference between adherence and spreading to sections with perisinusoidal laminin is significantly higher than to sections without such laminin; for both cell lines $P<0.00001$

significantly better onto liver sections comprising perisinusoidal laminin as opposed to sections without such laminin.

## Discussion

A large variety of hepatic pathological conditions are accompanied by an increase in the extracellular matrix, most notably in the fibrotic diseases (reviewed by Rojkind and Perez-Tamayo 1983; Schuppan 1990). We now add to this list of diseases and report that accumulation of perisinusoidal laminin is seen in patients with cancer. Thus perisinusoidal laminin had accumulated in the liver tissue in 86% of 42 patients dying from cancer with liver metastasis.

There is some discrepancy in the literature regarding the presence of laminin in the perisinusoidal space of the normal liver. Some laboratories have reported the presence of distinct amounts of perisinusoidal laminin (Abrahamson and Caufield 1985; Maher et al. 1988; Griffith et al. 1991), but most laboratories reported that the laminin immunoreactivity ranged from negative/negligible to weakly positive (Hahn et al. 1980; Bianchi et al. 1984; Martinez-Hernandez et al. 1984, 1991; Albrechtsen et al. 1988; Senior et al. 1988; Xu et al. 1989; Abdel-Aziz 1990; Reif et al. 1990; Rescan et al. 1990). Our results obtained in rats are concordant with the latter reports (Wewer et al. 1992). Among several possible explanations for these reported differences, two should be mentioned: one relates to differences in sampling of the liver tissue specimens for investigation; the other relates to the anti-laminin antibodies used. It is of paramount importance to note that perisinusoidal laminin is nearly always present in the subcapsular area. In the deeper portions of the liver only subtle amounts

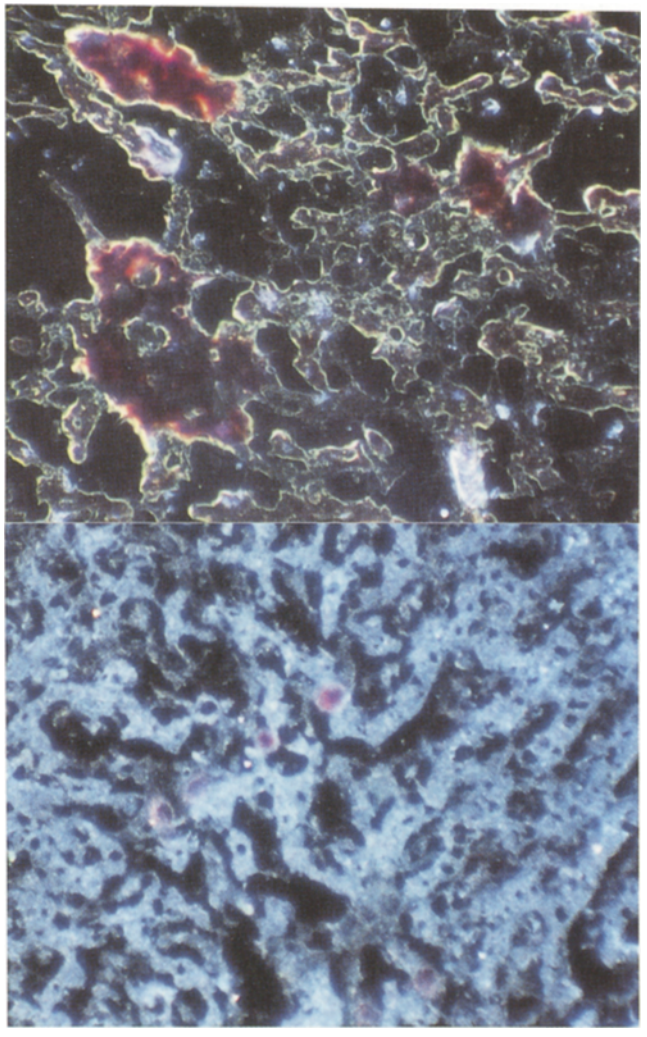

**Fig. 4.** Ex vivo assay in which viable tumour cells (Panc-1) have been seeded on to frozen sections of liver tissue with (**A**) or without (**B**) perisinusoidal laminin. Note that the presence of perisinusoidal laminin appeared significantly to stimulate tumour cell attachment and spreading. Interference filter, × 200

of perisinusoidal laminin are detected in human liver, as demonstrated in Fig. 1. The basement membranes of the biliary ducts and the blood vessels, on the other hand, express consistently strong laminin immunoreactivity and thus serve as a built-in positive control.

The exact specificity of the antibodies is important. The polyclonal antibodies that show even minimal cross-reactivity with fibronectin, type IV collagen or heparan sulphate proteoglycan will inevitably show perisinusoidal staining, because these constituents are normally present at this location (Hahn et al. 1980; Geerts et al. 1986; Burt et al. 1990). To rule out such a false-positive reaction, the use of monoclonal antibodies against laminin is of great value. In a previous study on the rat liver (Wewer et al. 1992) we used a panel of chain-specific monoclonal antibodies against A, B1, B2, S and M laminin and found little or no perisinusoidal laminin immunoreactivity in the normal adult rat liver. In the present study we also applied a well-characterized B2 laminin monoclonal antibody and found, as expected, a positive basement membrane staining of the biliary ducts and the blood vessels. In contrast, only subtle amounts of B2 laminin immunoreactivity could be seen in the perisinusoidal space of the normal human liver.

Despite the ongoing debate about the normal liver, most authors agree that under certain circumstances the level of perisinusoidal laminin immunoreactivity increases dramatically (Hahn et al. 1980; Bianchi et al. 1984; Martinez-Hernandez 1985, 1991; Albrechtsen et al. 1988; Senior et al. 1988; Xu et al. 1989; Abdel-Aziz 1990; Rescan et al. 1990; Wewer et al. 1992). This was recently substantiated at the molecular level by Northern blot and by in situ hybridization studies using various laminin probes (Senior et al. 1988; Miliani et al. 1989; Reif et al. 1990; Rescan et al. 1990; Wewer et al. 1992). In the present study we have now demonstrated the presence of an increased accumulation of perisinusoidal laminin expression in patients with cancer.

The increased amount of perisinusoidal laminin in cancer patients is most likely produced by the perisinusoidal cells. Ito cells can become "activated" and attain phenotypic characteristics of transitional cells or myofibroblasts. Cytoskeletal markers such as alpha smooth muscle actin and desmin appear in such activated perisinusoidal cells (Schmitt-Graf et al. 1991). Interestingly, these authors also noted an increased number of anti-alpha smooth muscle actin-positive cells in the non-neoplastic liver tissue adjacent to malignant lesions. The exact mechanism by which perisinusoidal cells increase their synthesis of extracellular matrix components is not known, but recent evidence strongly suggests a role for cytokines (polypeptide growth factors) such as transforming growth factor beta (reviewed by Sappino et al. 1990). Thus, the finding in the present study of laminin in the perisinusoidal space in patients with cancer could be explained by the influence of growth factors produced either locally in the liver or by the tumour cells.

On the basis of the data in the literature it would appear that laminin is produced in the liver. We cannot, however, totally exclude the possibility that perisinusoidal laminin may in part originate from the serum through gaps in the endothelial lining. The concentration of laminin has been measured radioimmunologically in patients with malignancies and found to be significantly elevated in patients with carcinoma and leukaemia but not in patients suffering from sarcomas or lymphomas (Rochlitz et al. 1987). We studied immunohistochemically a series of liver specimens from primary rat yolk sac tumour with high laminin serum concentration and found no increased accumulation of perisinusoidal laminin (unpublished) results). Thus, serum derivation of the hepatic perisinusoidal laminin seems unlikely.

The putative role of perisinusoidal laminin in facilitating tumour cell adhesion was addressed by using an ex vivo assay, in which viable tumour cells were seeded onto frozen sections of liver tissue. The principle of the ex vivo homing assay was originally developed by Stamper and Woodruff (1976). This method has been extensively used to study mechanisms involved in traffic-

king of normal leucocytes and lymphoid malignancies (reviewed by Butcher 1990). Interestingly, lymphomas that bound well to high endothelial venules (in frozen sections of lymphoid tissue) in an ex vivo assay spread early and produced generalized lymphadenopathy when injected into syngeneic recipients (Bargatze 1987; Sher et al. 1988). Conversely, lymphoma incapable of binding in the ex vivo assay only produced local tumour masses at the site of injection. These experiments documented the fact that the ex vivo assay may accurately predict the homing of the lymphomas in vivo. In the present study we developed an ex vivo assay in which we plated viable tumour cells onto frozen sections of liver specimens with or without perisinusoidal laminin. We observed that tumour cells (Panc-1 and clone A) preferentially adhered and spread to frozen sections of liver tissue containing perisinusoidal laminin as opposed to sections without such laminin. These results indicate that laminin could mediate liver metastasis, facilitating directly the adhesion of tumour cells or indirectly by affecting the endothelial cells, and thus provide a basis for homing of the tumour cells to the liver. However, since laminin occurs in livers of cancer patients both with and without liver metastases, it would be unwise to suggest that the only function of perisinusoidal laminin is to facilitate liver metastasis. Other possible functions for this laminin and the exact reasons for the appearance of laminin in the perisinusoidal spaces remain to be explored.

In conclusion, we have demonstrated a distinct increase of perisinusoidal laminin in patients with cancer. Using an ex vivo assay we present evidence that this laminin may directly or indirectly be involved in tumour cell homing to the liver.

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