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Tenascin and type IV collagen expression in liver cell dysplasia and in hepatocellular carcinoma

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Summary. The extracellular matrix (ECM) located in and around tumors is different from normal organ stroma, and there is evidence that it is critically involved in carcinogenesis and malignant growth. Whereas an abnormal composition of ECM in hepatocellular carcinomas (HCC's) has previously been demonstrated, not much is known so far with respect to putative HCC precursor lesions. We have, therefore, systematically analyzed the immunohistochemical reactivity for two major ECM components, tenascin and type IV collagen, in three types of liver cell dysplasia (LCD), and compared the findings with patterns observed in HCC's of different types and grades. Tenascin reactivity was generally stronger in HCC's than in cirrhosis. In cirrhotic nodules harboring areas of LCD, tenascin expression was significantly lower in small cell LCD than in large cell LCD. Type IV collagen reactivity in and around HCC's decreased as a function of a lower differentiation grade. In both groups of cirrhosis, i.e. with or without HCC, cirrhotic nodules occupied by the small cell variant of LCD exhibited a significantly lower type IV collagen reactivity than those with large cell LCD or simple regenerative cells. Taken together these findings suggest that, similar to adenomatous hyperplasia, small cell LCD is characterized by an abnormal tenascin and type IV collagen expression, thus reflecting the defective ECM pattern observed in HCC's.

Key words: Liver cell dysplasia, Hepatocellular carcinoma, Tenascin, Type IV collagen, Extracellular matrix

Introduction

The stroma and its extracellular matrix (ECM) located in and around tumors is different from the normal organ stroma, and there is strong evidence that it is critically involved in carcinogenesis and malignant growth. Derangements of cell-cell adhesion, of cell-ECM adhesion, and of cell-matrix anchorage, together with a change of ECM protein turnover, are crucial events in tumor progression, and are involved in invasion and metastasis. Similar to other malignant neoplasms, hepatocellular carcinomas (HCC's) exhibit an ECM different from normal hepatic parenchyma both with respect to morphologic structure and composition. HCC's have been consistently reported to synthesize collagenase IV and to be characterized by a decreased and discontinuous type IV collagen complement of the basal membranes (Grigioni et al., 1991; Patriarca et al., 1993). Tenascin is another ECM component which has recently been demonstrated to be abnormally expressed in HCC's (Yamada et al., 1992). Tenascin is a large ECM glycoprotein which, among other roles, is functional in epithelial-mesenchymal interfaces of several organs (Chiquet-Ehrismann et al., 1986; Erickson, 1989; Ekblom and Aufderheide, 1989). It has been shown that tenascin expression is higher in malignant tumors as compared with normal adult tissues, whereas other investigations suggested a prominent role for tenascin in stromal alterations associated with both, benign and malignant prostatic epithelial growth processes (Ibrahim et al., 1993). Therefore, tenascin expression may be predominant at sites of active tissue remodeling, and it would be of particular interest to know whether an altered expression would already occur in precancerous lesions. Recently, it has been demonstrated that ECM proteins, including tenascin, showed a discontinuous immunoreactivity in atypical macroregenerative nodules of liver, lesions suggested to represent HCC precursors (Patriarca et al., 1994).

In the present immunohistochemical study, we systematically analyzed the patterns of type IV collagen and tenascin reactivity in an other type of putative precursor lesion, liver cell dysplasia (LCD), in comparison with liver cirrhosis and HCC's of different types and grades.

Materials and methods

Material for histology

Tissue specimens of 36 Northern Chinese patients

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with liver cirrhosis and HCC, and 66 liver biopsies from patients of the same region showing cirrhosis not associated with HCC were retrospectively analyzed. The selection criterion for these cases was the presence of LCD within cirrhotic nodules, LCD being thus identified in a set of 1748 liver biopsies observed between 1957 and 1988 in a Chinese center (Zhao et al., 1994a). The material analyzed did not contain foci of atypical adenomatous hyperplasia (Nakanuma et al., 1993) or nodular borderline lesions (Ferrell et al., 1993), respectively.

For light microscopy, samples were fixed in 10% formaldehyde solution, dehydrated, and embedded in paraffin. Fixation before processing was less than 30 hours throughout. Sections were stained with hematoxylin and eosin.

Immunohistochemistry

For the immunohistochemical detection of type IV collagen (Col IV) and tenascin, mouse monoclonal antibodies were used (Dako). Dilutions of antisera were 1:200 for anti-Col IV and 1:25 for anti-tenascin. The sections were digested with 0.1% protease for 10 min at 37 °C and preincubated with 1% bovine serum albumin for blocking non-specific binding. Incubation with antibodies was at 4 °C for 20 hours. All sections were treated with the same batches of enzyme, antibodies and reagents, and only one preparation of substrate solution (New Fuchsin) was used. Immunostaining was executed by use of a modified APAAP procedure (Dako manual book; Zhao et al., 1994a). Counterstaining employed hematoxylin, and sections were mounted with aquadex.

Morphological classification of hepatic fibrosis, cirrhosis, LCD and HCC

The term, hepatic fibrosis, was employed for these biopsies showing septal fibrosis with or without nodular change. The highest grades of fibrosis are associated with cirrhotic change and, therefore, biopsies with fully established cirrhosis are included in this category. For grading of fibrosis, a system originally proposed by Knodell and coworkers (Knodell et al., 1981) was used with some modifications (Schmid et al., 1994). In this procedure, grade 0 denotes no fibrosis, grade 1, mild fibrosis (portal tracts only), 2, portal tract fibrosis plus incomplete septa, 3, septa bridging portal-portal, 4, septa bridging portal-central and/or focal incomplete cirrhosis, 5, diffuse complete and/or focal complete cirrhosis, and 6, diffuse complete cirrhosis.

Separately from fibrosis, biopsies exhibiting cirrhotic change (i.e., fibrosis grades 5 and 6) were classified as either micronodular or macronodular, employing criteria previously published (Anthony et al., 1978).

Features used to define LCD in contrast to normal or regenerating hepatocytes have previously been reported by our group (Zhao et al., 1994a). In brief, simple regenerating liver cells/hepatocytes (SRLC) are characterized as hepatocytes being smaller than normal parenchymal liver cells (NLC), but whose nuclear size is apparently normal. The main difference between SRLC and NLC is the typical arrangement of the former, in that SRLC have the tendency to form clusters within cirrhotic nodules, where they usually occur in peripheral parts and are then easily detectable due to nuclear crowding. Large liver cell dysplasia (LLCD) occurs in two variants, i.e. LLCD with nuclear hyperchromasia (LLCDe; Anthony, 1976; Zhao et al., 1994a) and LLCD with nuclear hypochromasia (LLCDo). In LLCDo, dysplastic hepatocytes are large cells with large nucleus and one or several prominent nucleoli. This cell type frequently exhibits an abundant, eosinophilic or clear cytoplasm. LLCDe has some features in common with LLCDo, but its cells usually exhibit a strongly eosinophilic cytoplasm and markedly polymorphous and hyperchromatic nuclei. Cells of both LLCDo and LLCDe frequently form clusters and occupy a part of a nodule, but they may form entire nodules. In small liver cell dysplasia (SLCD; Watanabe et al.., 1988), both the cell size and the nuclear size are clearly smaller than in cells of LLCD. In contrast to SRLC cells, the cytoplasm is usually basophilic, and the nuclei are hyperchromatic. Cells of SLCD are, in most instances, located in peripheral parts of cirrhotic nodules, where they can form clusters, but they sometimes form entire nodules. Ductular proliferations occurring in chronic fibrosing liver disease were indicated as either present or absent.

HCC's were classified using published guidelines (Gibson and Sobin, 1978; Nakashima and Kojiro, 1987). Trabecular, pseudoglandular, compact, scirrhous (sclerosing) and sarcomatoid types were distinguished. Grading of HCC's was performed according to Edmondson and Steiner (four grades; Edmondson and Steiner, 1954).

Assessment of tenascin and type IV collagen immunoreactivity

Tenascin immunoreactivity was graded as 0 (absent), 1+ (weak), 2+ (moderate) or 3+ (strong). In HCC's three reactive sites were separately graded, i.e. a peritrabecular area (including all epithelial formations found in a given HCC), a perivascular area (extracellular matrix around tumor vessels), and a peritumoral area, comprising liver tissue adjacent to the periphery of tumor nodules.

In cirrhosis with or without LCD, nodular centers and the nodule periphery were separately graded.

Col IV immunoreactivity was graded as 0 (absent), 1+ (weak), 2+ (moderate) or 3+ (strong). In HCC's, six reactive sites were separately analyzed for Col IV immunoreactivity: 1) tumor center, i.e. Col IV reaction product located around trabecular or other epithelial structures in the central half of tumor nodules; 2) tumor periphery: as with 1), but in the peripheral half of tumor nodules; 3) interface, i.e. immunoreactivity within the narrow zone of extracellular matrix interposed between

Table 1. Hepatocellular carcinomas (HCC): grading of tenascin immunoreactivity.

	No.	PERITRABECULAR REACTIVITY	PERIVASCULAR REACTIVITY	PERITUMORAL REACTIVITY
A. HCC types				
Trabecular	22	1.4±0.2	0.8±0.2	0.5±0.1
Pseudoglandular	5	2.6±0.4	2.0±0.4	0.8±0.4
Compact	5	1.6±0.4	1.2±0.4	0.8±0.4
Scirrhous	2	0.5±0.7	2.5±0.7	0.0
Sarcomatoid	2	2.0±0.7	1.0±0.7	1.0±0.6
*B. HCC grades**				
G2	10	1.3±0.3	1.0±0.3	0.5±02
G3	17	1.6±0.2	1.1±0.2	0.4±0.2
G4	9	1.7±0.3	1.2±0.3	1.0±0.2

*: differentiation grading according to Edmonson ans Steiner (1954). Values are indicated as mean±SD. For tenascin grading and calculation of values, see Materials and methods.

the tumor's periphery and adjacent liver tissue; 4) peritumoral, i.e. tenascin immunoreactivity around the tumor, but remote from the interface proper; 5) periarterial, i.e. around the walls of small arteries within HCC's; and 6) perivenous, i.e. reactivity around tumor veins.

For cirrhosis with or without LCD, a more simple stratification of Col IV immunoreactivity localization was used, in that the grade of staining was assessed in nodule centers (inner half), nodule periphery (outer half) and in the perinodular tissue as a whole.

For HCC's, five evaluation areas covering approximately 500 µm each were randomly chosen within a given reactive site. The grades of immunostaining estimated in these five areas were then summed up, and mean values based on the grouping used were calculated.

For the cirrhotic tissue, eight nodules from each case were evaluated per reactive site (see above), grades thus obtained summed up to a score, and mean values determined.

Statistical analysis

Values are indicated as means±SD. Kruskal-Wallis one-way analysis and Mann-Whitney's U test were employed. Wilcoxon's rank test and correlation R analysis were applied for the two groups of cirrhosis, and among the groupings as defined in the study.

Results

Histopathologic composition of material

22/36 evaluable HCC cases showed a trabecular growth pattern, followed in frequency by pseudoglandular (acinar) and compact groups, whereas scirrhous and sarcomatoid HCC's formed the minority (Table 1). No grade 1 HCC according to the Edmondson and Steiner classification was found. Most HCC's were either grade 2 or 3, but 9 cases exhibited at least foci with grade 4 (Table 1).

As seen in Table 4, micronodular cirrhosis was represented about twice as frequent as macronodular cirrhosis in the present material. Foci of simple

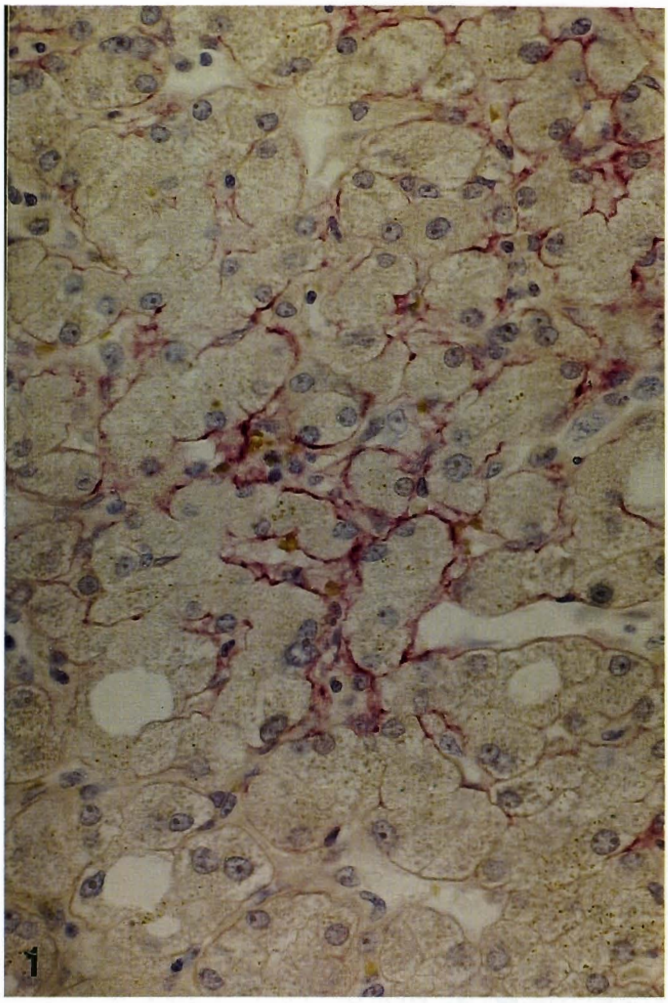

Fig. 1. Hepatic parenchyma from a cirrhotic nodule harboring LCD. Tenascin immunoreactivity is focal and in a perisinusoidal pattern, forming thin bands along hepatocyte plates. Tenascin immunostain, APAAP method. x 440

regenerating liver cells and of LCD were found both in cirrhotic livers with or without HCC, and the four types of lesions (SRLC, LLCDo, LLCDe and SLCD) were seen to occur together in one nodule in some instances.

Based on the definitions given above, foci of SRLC, LLCDo, LLCDe and SLCD were observed in cirrhotic

Table 2. Hepatocelluar carcinomas (HCC): grading of type IV collagen immunoreacitivity.

	No.	TUMOR CENTER	TUMOR PERIPHERY	INTERFACE ZONE	PERITUMORAL ZONE	PERIARTERIAL ZONE	PERIVENOUS ZONE
A. HCC type							
Trabecular	22	2.1±0.1	1.9±0.1	1.6±0.1	1.9±0.1	0.2±0.1	1.5±0.2
Pseudoglandular	5	2.6±0.3	2.2±0.3	2.2±0.3	2.2±0.4	1.4±0.2	2.6±0.3
Compact	5	2.0±0.3	1.6±0.3	2.4±0.3	2.0±0.4	2.0±0.2	2.4±0.4
Scirrhous	2	0.5±0.6	1.5±0.6	1.5±0.6	1.5±0.6	1.0±0.4	2.5±0.7
Sarcomatoid	2	1.0±0.6	1.5±0.6	3.0±0.6	2.0±0.6	0.5±0.4	3.0±0.7
*B. HCC grades**							
G2	10	2.4±0.2	2.0±0.2	2.1±0.2	2.4±0.2	0.6±0.2	1.9±0.3
G3	17	2.0±0.2	2.0±0.1	1.7±0.2	2.0±0.2	0.3±0.1	1.9±0.2
G4	9	1.5±0.3	1.3±0.2	1.8±0.3	1.3±0.2	0.4±0.2	1.8±0.3

*: differentiation grades according to Edmondson and Steiner (1954). Values are indicated as mean±SD. For type IV collagen grading and calculation of values, see Materials and methods

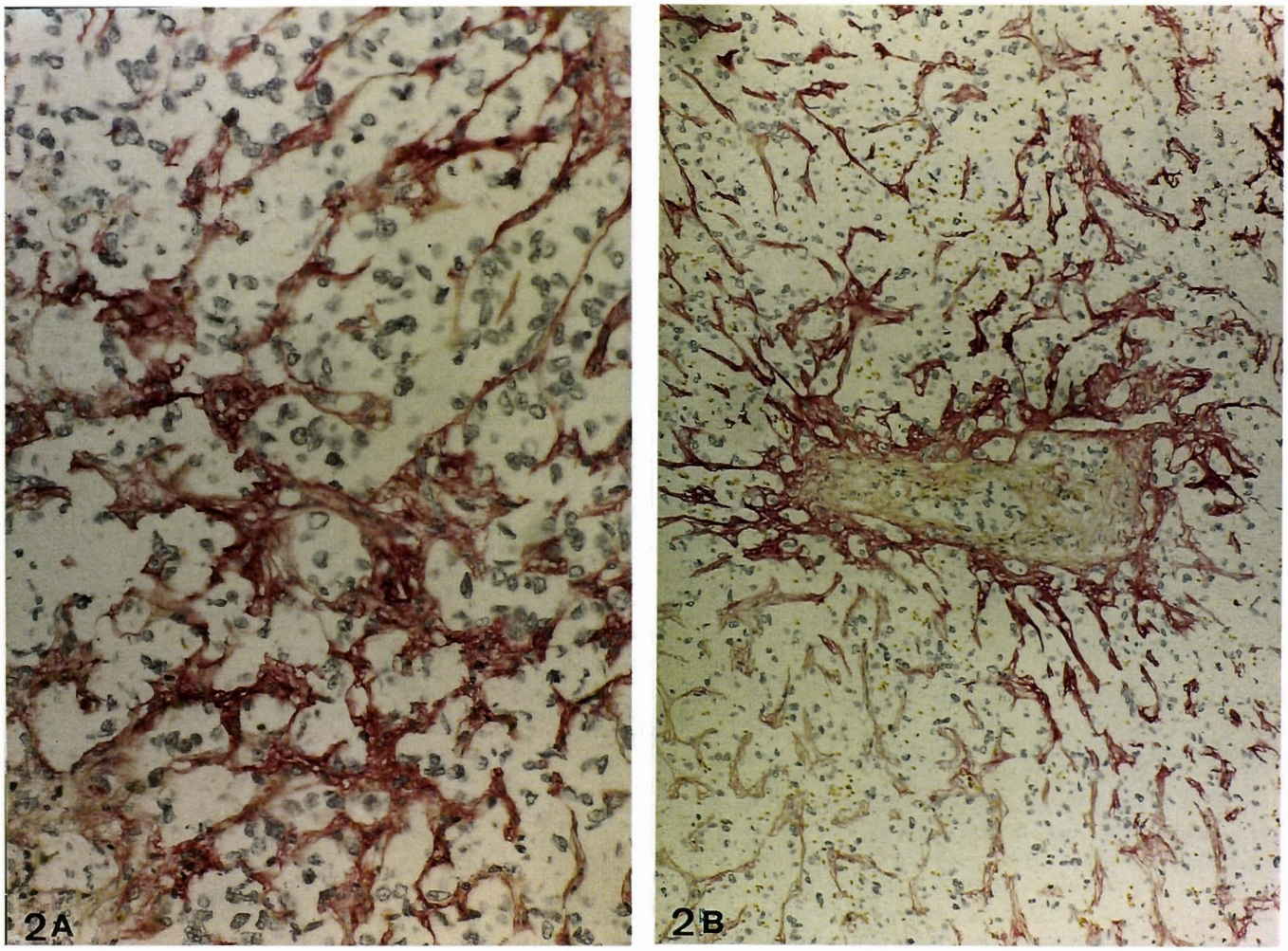

Fig. 2. A. Hepatocellular carcinoma, peritrabecular pattern of tenascin expression. Note that reactivity is focally accentuated, whereas it is defective elsewhere. **B.** Tenascin expression in hepatocellular carcinoma. In this situation, reaction product is pronounced in parts of the tumor facing the stroma, i.e. close to the invasion front, where active tissue remodelling takes place. Tenascin immunostain, APAAP method. A, x 250; B, x 175

livers without HCC with a frequency of 33.3%, 13.8%, 2.7% and 22.2% respectively, whereas their frequencies in livers with HCC were 25.7%, 30.3%, 6.0%, and 39.4%, respectively.

Qualitative assessment of tenascin and type IV collagen immunoreactivity

In the parenchyma of cirrhotic nodules, tenascin immunoreactivity was generally weak, and visualized in the form of a focal linear reaction product following the contours of the hepatic sinusoids (Fig. 1). Sometimes, only a short segment of sinusoid wall was stained, whereas, less frequently, tenascin immunoreactivity completely enveloped small groups of hepatocytes. A stronger tenascin reactivity was observed in septa situated between nodules chiefly at the border between parenchyma and septal connective tissue. Similar patterns of staining were observed in cirrhosis with or without HCC. In HCC's tenascin was chiefly expressed in a peritrabecular pattern, an irregular an partly defective reaction product being visible along the trabecular periphery (Fig. 2A). Tenascin reactivity was also noted around tumoral blood vessels and, mainly in case of expanding tumors («pushing lesions»; Baer et al., 1989), tenascin was variably expressed within the connective tissue interface representing the border area between the tumor's periphery and adjacent liver tissue (Fig. 2B). It appeared that the intensity of tenascin immunostaining was highest in a narrow zone of stroma immediately outside the tumor border, then decreasing as a function of the distance from the tumor.

Similar to tenascin, Col IV immunoreactivity was registered in cirrhosis both, in the central and peripheral parts of cirrhotic nodules. Expectedly, staining closely followed the sinusoidal contours, but was generally stronger than staining for tenascin (Fig. 3).

In HCC's several distinct patterns of Col IV immunostaining could be worked out.

This collagen was frequently strongly expressed along intratumoral, sinusoid-like blood vessels, but in contrast to cirrhotic nodules, this peritrabecular staining was clearly stronger, and the bands of reaction product were broader (Fig. 4A). In part of the tumors, a distinct interface strongly positive for Col IV was found, clearly marking the tumors; invasion front (Fig. 4B). Apparently, this pattern co-localizes with that seen with tenascin in part of the cases (see above). A third pattern of Col IV staining consisted of a strong positivity around larger intratumoral blood vessels, and a fourth, usually less pronounced involved the connective tissue around HCC's, i.e. the part being situated outside the more strongly staining interface area.

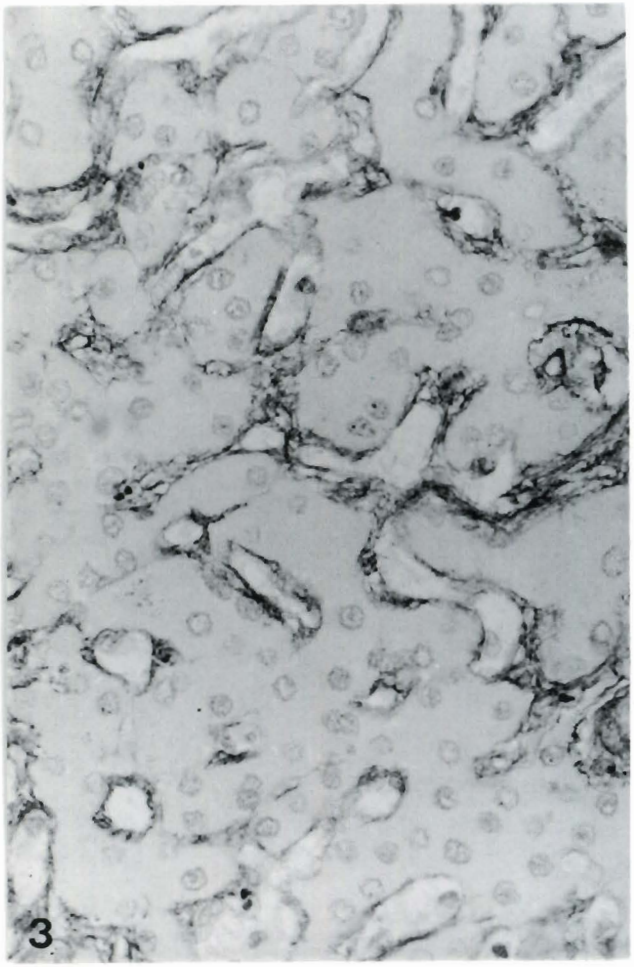

Fig. 3. Liver parenchyma from a cirrhotic nodule showing strong perisinusoidal reactivity for type IV collagen. Note that reaction product is not strictly linear, but rather a perisinusoidal meshwork of variable thickness. Type IV collagen immunostain, APAAP method. x 440

Table 3. Liver cirrhosis/fibrosis with or without liver cell dysplasia (LCD) associated with hepatocellular carcinoma (HCC): grading of tenascin immunoreactivity

	NODULE CENTER	No.	NODULE PERIPHERY	No.
A. Cirrhosis				
Macronodular	3.0±1.3	12	6.1±1.3	12
Micronodular	6.0±0.9	21	9.2±1.0	21
B. Fibrosis				
Grade 3	3.8±1.5	10	7.4±1.6	10
Grade 4	5.6±1.9	6	8.0±2.0	6
Grade 5	5.5±.1.1	17	8.9±1.2	17
Grade 6	4.6±2.7	3	8.6±2.9	3
C. LCD/SRLC				
LLCDe	11.2±1.4	4	11.3±2.4	3
SLCD	3.2±1.0	8	8.9±0.9	20
SRLC	7.2±0.7	15	8.8±1.3	10
D. Ductular proliferations				
Absent	4.8±1.0	19	7.8±1.1	19
Present	5.1±1.1	17	8.8±1.2	17

Values are indicated as mean±SD. *: fibrosis grading, see Materials and methods; LCD: liver cell dysplasia; LLCDe: large liver cell dysplasia with hyperchromatic nuclei; SLCD: small liver cell dysplasia; SRLC: simple regenerative liver cells.

Grading of tenascin and type IV collagen immunoreactivity in hepatocellular carcinomas

Cumulated grading of tenascin immunoreactivity in HCC's of different types and differentiation grades is listed in Table 1. The data support the qualitative impression that strong, i.e. grade 3 reactivity is unusual in these tumors, irrespective of the structure stained with a given tumor. For all histologic types of HCC analyzed (all containing trabecula to variable degrees, this pattern being pure in 22 cases) it turned out that the overall grade of peritrabecular tenascin reactivity was significantly higher than the grade of peritumoral reactivity ($p<0.0001$). Perivascular tenascin reactivity (tumoral vessels) was significantly higher than peritumoral reaction ($p<0.017$). It appears, therefore, that immunoreactive tenascin is preferentially deposited at intratumoral epitheliomesenchymal interfaces, but less at the peripheral interface between the tumor and the peritumoral tissue.

For Col IV, a more complex stratification of potential reactive zones in and around HCC's was employed (Table 2). The least overall degree of Col IV reactivity was found around tumor arteries, being significantly less than all other categories ($p<0.0001$). Peritumoral Col IV grade decreased as a function of tumor differentiation grade (Table 2; $p= 0.047$), the difference between Edmondson-Steiner grades 2 and 4 being significant ($p= 0.021$).

This observation may indicate that increased invasiveness of less differentiated HCC's is associated with poorer Col IV deposition in the tissue adjacent to the tumor. Overall, there was a trend for HCC's with a morphology deviating from the «classical types» (i.e., scirrhous and sarcomatoid forms) to contain less immunostainable Col IV within the tumor mass itself,

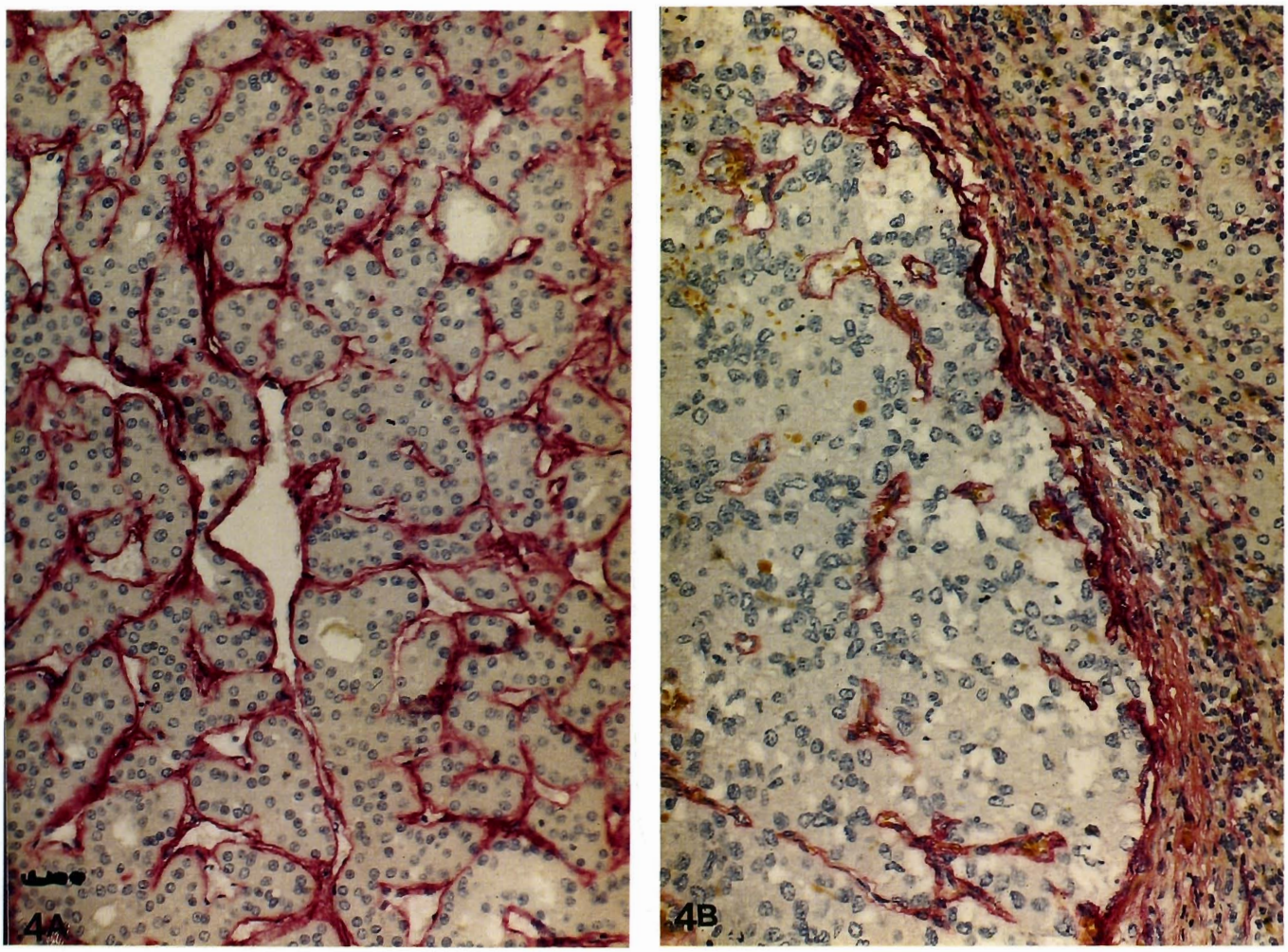

Fig. 4. A. Peritrabecular type IV collagen reactivity in hepatocellular carcinoma. Reaction product is limited to the narrow space lining the carcinoma cell plates. **B.** Type IV collagen reactivity in hepatocellular carcinoma: interface and peritumoral pattern. Carcinoma tissue itself is poor in reactivity whereas there is a strong reaction at the border of the advancing front (interface), and also reactivity in adjacent compressed hepatic parenchyma. Type IV collagen immunostain, APAAP method. A, x 175; B, x 200

Table 4. Liver cirrhosis/fibrosis with of without liver cell dysplasia (LCD) associated with hepatocellular carcinoma (HCC): grading of type IV collagen immunoreactivity.

	NODULE CENTER	No.	NODULE PERIPHERY	No.	PERINODULAR	No.
A. Cirrhosis						
Macronodular	10.0±1.7	12	9.6±1.7	12	13.9±1.8	12
Micronodular	15.6±1.3	21	13.3±1.3	21	16.1±1.4	21
*B. Fibrosis**						
Grade 3	12.3±2.0	10	9.6±1.7	10	15.4±2.0	10
Grade 4	9.1±2.6	6	9.8±2.2	6	13.3±2.6	6
Grade 5	16.7±1.5	17	15.1±1.3	17	17.3±1.5	17
Grade 6	15.6±3.7	3	7.3±3.1	3	12.6±3.7	3
C. LCD/SRLC/NLC						
NLC	9.0±2.2	7	8.1±2.2	6		
LLCDe	12.2±2.9	4	13.6±1.8	9		
LLCDo	20.0±4.1	2	22.0±3.9	2		
SLCD	12.9±1.5	14	11.2±1.4	14		
SRLC	19.6±1.9	9	12.2±2.4	5		
D. Ductular proliferations						
Absent	10.2±1.2	19	10.1±1.3	19	11.8±1.1	19
Present	18.5±1.3	17	14.2±1.3	17	20.0±1.2	17

values are indicated as mean±SD. *: fibrosis grading, see Materials and methods. LCD: liver cell dysplasia; LLCDe: large liver cell dysplasia with hyperchromatic nuclei; LLCDo: large liver cell dysplasia with hypochromatic nuclei; SLCD: small liver cell dysplasia; SRLC: simple regenerative liver cells; NLC: normal liver cells.

whereas no difference was found with respect to the tumor interface or other locations (Table 2).

Grading of tenascin and type IV collagen immunoreactivity in liver fibrosis/cirrhosis associated with HCC

Data for cumulated grades of tenascin and Col IV are compiled in Tables 3, 4. For both micro- and macronodular liver cirrhosis, the cumulated grade of tenascin staining was significantly higher in peripheral parts of cirrhotic nodules than in the nodular center ($p<0.0001$). A similar phenomenon was found in liver fibrosis with incipient remodeling of the lobular architecture, indicating that tenascin was more expressed at peripheral sites where active remodeling is taking place. For central or peripheral parts of deformed lobules in liver fibrosis, tenascin reactivity did not vary as a function of the degree of fibrosis.

Tenascin reactivity was also stronger in the peripheral zones of nodules containing LCD or clusters of regenerating hepatocytes (Table 3). Overall tenascin reactivity was significantly lower in SLCD than in the large cell variant ($p=0.01$), and lower than in areas populated by simple regenerative hepatocytes within cirrhotic nodules ($p=0.016$). Therefore, areas dominated by SLCD are clearly different from all other parenchymal components with respect to tenascin expression.

The overall grade of Col IV reactivity was significantly higher in micronodular than in macronodular cirrhosis (Table 4; $p=0.016$), and was higher in septal fibrosis alone than in macronodular cirrhosis ($p=0.02$). In comparison with cirrhotic nodules consisting of non-atypical regenerative hepatocytes (SRLC), nodules with a large fraction of SLCD exhibited a lower Col IV grade ($p= 0.016$).

Table 5. Liver cirrhosis/fibrosis with or without liver cell dysplasia (LCD) not associated with hepatocellular carcinoma (HCC): grading of tenascin immunoreactivity

	NODULE CENTER	No.	NODULE PERIPHERY	No.
A. Cirrhosis				
Macronodular	5.0±0.7	11	4.6±0.7	11
Micronodular	6.0±0.9	32	1.3±0.4	32
Mixed	1.2±0.7	11	2.1±0.7	11
*B. Fibrosis**				
Grade 3	1.7±1.4	4	0.5±1.3	4
Grade 4	2.6±0.6	18	1.5±0.6	18
Grade 5	1.7±0.5	26	2.6±0.5	26
Grade 6	2.1±0.6	18	2.8±0.6	18
C. LCD/SRLC				
LLCDe	1.0±1.5	2	4.2±0.8	7
LLCDo		0	4.5±1.1	4
SLCD	4.0±0.9	5	3.6±0.5	14
SRLC	3.7±0.3	31	2.5±0.5	20
D. Ductular proliferations				
Absent	2.2±0.4	46	2.5±0.4	47
Present	1.6±0.6	19	1.7±0.6	19

Values are indicated as mean±SD. *: fibrosis grading, see Materials and methods; LCD: liver cell dysplasia; LLCDe: large liver cell dysplasia with hyperchromatic nuclei; LLCDo: large liver dysplasia with hypochromatic nuclei; SLCD: small liver cell dysplasia; SRLC: simple regenerative liver cells.

Grading of tenascin and type IV collagen immunoreactivity in liver fibrosis cirrhosis not associated with HCC

Data for cumulated grades of the two ECM proteins

Table 6. Liver cirrhosis/fibrosis with or without liver cell dysplasia (LCD) not associated with hepatocellular carcinoma (HCC): grading of type IV collagen immunoreactivity.

	NODULE CENTER	No.	NODULE PERIPHERY	No.	PERINODULAR	No.
A. Cirrhosis						
Macronodular	14.8±2.4	11	11.4±1.9	11	17.9±1.9	11
Micronodular	9.5±1.4	32	8.0±1.1	32	13.2±1.1	32
Mixed	8.1±2.4	11	4.5±1.9	11	11.0±1.9	11
*B. Fibrosis**						
Grade 3	6.0±4.0	4	7.7±3.4	4	7.2±3.3	4
Grade 4	10.8±1.8	18	8.7±1.6	18	13.0±1.5	18
Grade 5	8.5±1.5	26	7.4±1.3	26	13.3±1.3	26
Grade 6	13.8±1.8	18	8.6±1.6	18	15.2±1.5	18
C. LCD/SRLC/NLC						
NLC	4.5±1.5	14	3.7±1.4	16		
LLCDe		0	9.5±4.1	2		
LLCDo	15.2±1.8	9	12.0±1.6	12		
SLCD	6.6±3.2	3	8.1±1.6	13		
SRLC	15.2±1.1	31	10.8±1.3	19		
D. Ductular proliferations						
Absent	10.8±1.2	47	8.4±0.9	47	12.1±0.9	47
Present	9.5±1.8	19	7.3±1.5	19	16.4±1.4	19

Values are indicated as mean±SD. *: fibrosis grading, see Materials and methods. LCD: liver cell dysplasia; LLCDe: large liver cell dysplasia with hyperchromatic nuclei; LLCDo: large liver cell dysplasia with hypochromatic nuclei; SLCD: small liver cell dysplasia; SRLC: simple regenerative liver cells; NLC: normal liver cells.

are listed in Tables 5, 6. In macronodular cirrhosis, there was no difference in tenascin grade between nodule center and periphery, whereas in micronodular cirrhosis, peripheral parts had a higher grade. Peripheral parts of nodules occupied by LLCD exhibited a tenascin grade similar to that of macronodular cirrhosis without LCD. In the group of cirrhosis without HCC, there was no significant difference between SRLC and SLCD with respect to tenascin grading. Overall Col IV grading was significantly higher in macronodular than in micronodular cirrhosis, for peripheral central, and perinodular areas. As in the cirrhosis group associated with HCC, nodules harboring SLCD showed a significantly lower Col IV grade than nodules chiefly consisting of SRLC ($p=0.01$).

Discussion

Two groups of hepatic lesions are currently considered as potential precursors of hepatocellular carcinoma (HCC), i.e. atypical nodular lesions and liver cell dysplasia (LCD). The former group is characterized by grossly recognizable nodules showing hepatocellular atypia and abnormal architecture, and is termed atypical adenomatous hyperplasia (AAH; macroregenerative nodules type II; Nakanuma et al., 1993; Hytiroglou et al., 1995) or borderline lesions (Ferrell et al., 1993), even though a generally accepted nomenclature does not yet exist. AAH is thought to represent a pathway for human hepatocarcinogenesis. In contrast, the significance of LCD as a precursor for the development of HCC is still debated (Watanabe et al., 1988; Zhao et al., 1994a), albeit small dysplastic foci may coexist with HCC (Nieburgs et al., 1965; Furuya et al., 1988; Wada et al., 1988; Nakanuma et al., 1993) and the small cell variant of LCD has been shown to be immunoreactive for the p53 protein (Zhao et al., 1994b).

Whereas macroregenerative nodules with atypia form grossly visible and bulging lesions, LCD is visualized as clusters of atypical hepatocytes, in some instances however involving cirrhotic nodules in their entirety (Zhao et al., 1994a). It is not known whether the apparent replacement of regenerative hepatocytes in nodules by dysplastic cells is related to either a progressive transformation of hepatocytes, or to overgrowth of a nodule's cell population by a new cell lineage. The finding of p53 protein overexpression in an LCD subtype (Zhao et al., 1994b) may support the latter mechanism.

Normal and abnormal epithelial cell proliferation is strongly dependent on the cells' interaction with components of the extracellular matrix (ECM). Therefore, the remodeling and cell replacement process supposed to occur in nodules harboring LCD may be associated with alterations of the composition and structure of ECM at these sites.

Here we can demonstrate that there are considerable differences between cirrhotic nodules, nodules containing LCD, and HCC's, with respect to both, tenascin and type IV collagen distribution. Tenascin reactivity was generally weak in cirrhotic nodules not showing foci of LCD, irrespective of the absence or presence of HCC. Tenascin was visualized in the form of a focal linear reaction product along the contours of sinusoids. A more important tenascin expression was noted in septa situated between cirrhotic nodules, thus confirming previous findings obtained in a rat model of hepatic fibrosis (Van Eyken et al., 1992). The overall

pattern of tenascin reactivity was similar in nodules with or without LCD. Therefore, even though liver cirrhosis is associated with an increase of ECM in the perisinusoidal space and with capillarization (Schaffner and Popper, 1963; Schuppan, 1990), and activated Ito cells/perisinusoidal myofibroblasts have been shown to be engaged in tenascin production in this situation (Schwögler et al., 1992; Van Eyken et al., 1992), tenascin appears to be preferentially localized at sites of increased remodeling, i.e. at the parenchyma-fibrosis interface, as previously reported (Van Eyken et al., 1992).

In contrast to cirrhotic nodules, tenascin reactivity was generally stronger in HCC's, usually forming a band of reaction product along the trabecules, but also being layed down around tumor blood vessels, and expressed at the tumor-liver interface. In a previous report on HCC's, tenascin had been found in the capsule and lobular septa, but not in the sinusoidal walls of the tumors (Yamada et al., 1992).

Grading of tenascin reactivity in cirrhotic nodules with or without large areas of LCD uncovered differences with respect to the amount and distribution of this ECM component. Irrespective of the size of cirrhotic nodules, the cumulated grade of tenascin staining was significantly higher in peripheral parts of nodules, i.e. at the interface between nodular parenchyma and adjacent fibrotic tisue. This finding suggest that, in liver cirrhosis, tenascin expression prevails at sites of active tissue remodeling, underlining the role of this protein in extracellular matrix organisation and in epithelial-mesenchymal interactions (Chiquet-Ehrismann et al., 1986; Ekblom and Aufderheide, 1989; Erikson, 1989; Tiitta et al., 1994; Vollmer, 1994). A similar phenomenon was observed in liver biopsies showing fibrosis, but only incipient nodular change, reflecting an earlier phase of cirrhogenesis. The grade of tenascin reactivity was higher in parenchymal areas facing fibrosis, whereas in liver lobules with a still preserved architecture, there was no difference of tenascin staining grade with respect to peripheral or central lobular areas. The finding of a higher tenascin expression at the nodular periphery or border was also in evidence for nodules occupied by LCD.

Interestingly, however, overall tenascin reactivity was significantly lower in areas of small liver cell dysplasia than in areas predominantly consisting of large liver cell dysplasia or regenerative hepatocytes. The tenascin immunophenotype of small cell liver dysplasia therefore seems to reflect a pattern of defective ECM formation previously reported for poorly differentiated hepatocellular carcinomas (Grigioni et al., 1987; Donato et al., 1989; Terada and Nakanuma, 1991; Patriarca et al., 1993). In a former investigation we showed that nuclear features of small cell liver dysplasia closely reflected those of poorly differentiated hepatocellular carcinomas, whereas karyometric features of large liver cell dysplasia were close to those of well-differentiated hepatocellular carcinomas (Zhao et al., 1994a). Furthermore, the small cell variant was shown to be the only LCD being immunoreactive for the p53 protein (Zhao et al., 1994b). Therefore, the distinct phenotype of small cell liver dysplasia is also in evidence with respect to a major ECM component, similar to an another potential precursor lesion, atypical macroregenerative nodules, where a defective ECM has previously been demonstrated (Patriarca et al., 1994).

For type IV collagen expression in and around HCC's, peritumoral reactivity decreased as a function of the differentiation grade, the difference between Edmondson and Steiner grades 2 and 4 being clearly significant. This finding suggests that an increased invasiveness of less differentiated HCC's is associated with poorer type IV collagen deposition in peripheral stromal parts and in the invaded tissue adjacent to the tumor. In addition, there was a trend for HCC's with a morphology deviating from the «classical» types (i.e. scirrhous and sarcomatoid variants) to contain less stainable type IV collagen within the tumor itself, whereas no difference was observed with respect to the tumor interface. A relationship between HCC differentiation and the type IV collagen status in the ECM of these tumors has previously found, with differentiated tumors showing a relatively intact basement membrane and poorly differentiated HCC's exhibiting a defective basement membrane (Grigioni et al., 1987; Donato et al., 1989; Patriarca et al., 1993). As the immunoreactivity for type IV collagen was, however, stronger in HCC's than in normal tissue, it has been concluded that the sinusoid-like vessels of these neoplasms have the features of capillaries (Terada and Nakanuma, 1991), previously been shown by use of electron microscopy (Haratake and Scheuer, 1990). An aberrant composition of ECM, mainly in the perivascular and peritumoral compartment of HCC's, therefore also refers to type IV collagen. Hence, it was of interest to test whether, similar to tenascin, an abnormal phenotype of type IV collagen expression would already occur at the level of potential precursor lesions. Previous ultrastructural comparisons of sinusoids in HCC, adenomatous hyperplasia, and fetal liver have shown that incomplete basement membrane formation and some reduction in sieve plates occurred in adenomatous hyperplasia, but these changes were less expressed than in HCC (Haratake et al., 1992). Furthermore, macroregenerative nodules, which are currently suggested to make part of the spectrum of borderline lesions, disclosed a discontinuous immunoreactivity for ECM proteins, including type IV collagen, laminin, fibronectin and tenascin (Patriarca et al., 1994). Here we can demonstrate that the overall grade of type IV collagen immunoreactivity was significantly higher in micronodular than in macronodular cirrhosis associated with HCC, whereas the reverse result was obtained in the group not associated with HCC. In both cirrhosis groups, nodules harboring small cell liver dysplasia exhibited a significantly lower type IV collagen

reactivity than nodules showing large cell dysplasia or no dysplasia. Taken together these findings suggest that, similar to adenomatous hyperplasia, liver cell dysplasia of the small cell type has an abnormal ECM with respect to two major proteins involved, i.e. tenascin and type IV collagen. The ECM immunophenotype of small cell dysplasia of the liver appears to mimick a pattern occurring in HCC's of low differentiation, thus further underlining a relationship between this type of dysplasia and HCC's as previously shown with regard to karyometric features and p53 expression (Zhao et al., 1994a,b).

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