Distribution Pattern of Liver Matrix Proteins, Fibronectin and Type I Collagen, in DAB-Induced Hepatoma of Rat

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Katsukura, Y., Abe, N., Watabe, N. and Tsuchiya, T. Distribution Pattern of Liver Matrix Proteins, Fibronectin and Type I Collagen, in DAB-Induced Hepatoma of Rat. Tohoku J. exp. Med., 1985, 146 (4), 405-417 —— Specific antibodies to fibronectin and type I collagen in rat livers were used to demonstrate these matrix proteins with direct immunoperoxidase method in paraffin sections of normal liver, DAB-induced hepatoma and CCl4-induced fibrotic liver. In normal livers, the immunoreactive products of both matrix proteins were found in the periportal regions, while the hepatocytes and most of the interstitial matrix remained unstained. The specimens obtained from DAB-treated liver showed a more intense reaction with fibronectin antibody in the perisinusoidal space including proliferated cholangiolar cells, as compared to no reaction with type I collagen antibody. In CCl4-fibrotic liver, apparent reactions were also found for both matrix proteins in the periportal interstitium and in progressing fibrotic area with severe fatty metamorphosis. These findings suggest that these matrix proteins have an advantage in the attempt to distinguish different patterns of neoplastic alteration in experimental rat livers. —— fibronectin; type I collagen; DAB-hepatoma; CCl4-fibrosis; matrix protein

A normal liver structure is based on the result of the orderly distribution of hepatocytes and of the intercellular matrix. In the liver injury, a fibrosis results in capsular thickening, enlargement of portal tracts and marked collagen deposition in exchange of Disse’s spaces, leading to severe restriction in blood and hepatocytes (Grimaud and Borojevic 1977). In such pathological conditions, type I, III, and IV collagens are increased. Ratio of the type I to type III collagen may reflect the degree of chronicity of the liver disease and may represent an index of stability of collagen deposits in the liver (Rojkind and Dunn 1979; Grimaud et al. 1980). On the other hand, an increase in type IV collagen induce capillarization of sinusoids as the morphological change (Grimaud and Borojevic...
Basal laminae usually contain type IV, V collagen and structural glycoproteins such as fibronectin, laminin (Timpl et al. 1979; Labat-Robert et al. 1981) as well as some proteoglycans like chondroitin sulphate proteoglycans (Katsukura et al. 1980, 1983). The normal distribution pattern of matrix macromolecules is upset in a variety of pathological conditions. It changes also as a result of aging process (Robert and Robert 1973; Katsukura et al. 1980). Some of the most conspicuous modifications of the intercellular matrix are observed in malignant tumors and result from at least two mechanisms. The first mechanism depends on the secretion of enzymes capable of attacking intercellular matrix. The second depends on the perturbation of the program of biosynthesis of macromolecules of the intercellular matrix (Hornebeck et al. 1980; Woolley et al. 1980). Although their relative importance may vary from one type of tumor to another, both mechanisms play a role in the modifications of normal tissue structure induced by the malignant process (Labat-Robert et al. 1981). The interaction between the cell membrane and the intercellular matrix is a prerequisite for normal differentiation and histogenesis. The modified tissue pattern invariably accompanied by malignancy can be considered to result from loss of this specific cell-cell and cell-matrix interactions (Mautner and Hynes 1977; Vaheri and Mosher 1978; Yamada and Olden 1978).

Fibronectin was shown to recognize specifically collagen molecules of several types as well as glycosaminoglycans (Engvall and Ruoslahti 1977; Engvall et al. 1978; Ruoslahti et al. 1981; Katsukura et al. 1983) and to be involved in cell-cell recognition phenomena (Labat-Robert et al. 1981). The majority of transformed cells in culture has been reported to have lost their surface bound fibronectin (Vaheri and Mosher 1978; Yamada and Olden 1978).

The objective of the present study is to investigate the distribution pattern of fibronectin and type I collagen in the liver matrix accompanied by cell migration of DAB-induced hepatocarcinoma in rat.

**Materials and Methods**

*Experimental animals and tissue preparations*

**DAB-hepatoma**: Both sexes of Wistar strain rats, 8 weeks of age, were fed commercial pellets containing 0.06% DAB (p-dimethylaminoazobenzene). Rats were sacrificed for autopsies at about 20 to 50 weeks from the beginning of the experiment. Ten livers with hepatic tumors after 20 weeks application of DAB were examined for the early stage of carcinogenesis, of which 6 tumor masses were 1 to 3 mm in diameter and the others were smaller.

**CCl₄-fibrosis**: Subcutaneous injections of a 1:1 mixture (0.4 ml) of CCl₄ (carbon tetrachloride) and olive oil to normal rats (8 weeks of age) for 10 to 15 weeks, were performed. Severe fatty metamorphosis were produced with the necrotic foci of liver cells and the perportal fibrosis. They were examined in comparison with DAB-hepatoma.

For immunohistochemical examination, liver specimens were fixed in FP-solution at 4°C overnight and embedded in paraffin (i.e., composed of saturated solution of picric acid
Matrix Proteins in DAB-Hepatoma

75 ml and formalin 25 ml). Tissue sections were subjected to direct immunoperoxidase techniques.

Staining procedures

Immunohistochemistry. The antiserum for type I collagen was obtained from rabbits immunized with rat type I collagen (Calbiochem-Behring Corp.). The crude immune serum was purified by DEAE-cellulose chromatography equilibrated with 0.01 M phosphate buffer at pH 7.8. Cross-reacting antiserum was eliminated by adsorption after repeated reciprocal passages on the different collagen types, rat type III, IV (Calbiochem-Behring Corp.) or fibronectin bound to CNBr-activated sepharose. Final mono-specificity of this antiserum was described in the previous paper (Abe et al. in press). This gave a single precipitin line against the respective rat type I collagen. The antiserum for fibronectin was purchased from Collaborative Res. Inc. The specificity of this fibronectin antiserum was evaluated for cross reactions with rat fibronectin (rat fibronectin, Calbiochem-Behring Corp) (Katsukura et al. 1983).

Direct immunoperoxidase staining for fibronectin or type I collagen was performed as follows. The thin sections were kept on glass slides at 37°C overnight, deparaffinized and washed in 2 changes of 0.05 M Tris-saline, pH 7.6, for 5 min, then incubated in the peroxidase-labeled anti-human fibronectin and anti-rat type I collagen antisera for 25 min at a 1/20 and 1/60 dilution, respectively. The antibodies used for the direct immunoperoxidase method were rabbit anti-human fibronectin and rabbit anti-type I collagen HRPO conjugates developed by Nakane and Kawai (1974) (HRPO, horseradish peroxidase, Type VI, Sigma Co., Ltd.). Three washes with Tris-saline at 4°C were carried out for 30 min, followed by immersion in 0.05% 3, 3'-diaminobenzidine tetrahydrochloride, Sigma Co., Ltd. After washing in 3 changes of Tris-saline buffer for 5 min and where necessary, the cell nuclei were stained with Mayer's hematoxylin.

For the control, the following experiments were carried out instead of the antisera; (a) with normal rabbit serum labeled with HRPO and (b) with anti-fibronectin and anti-type I collagen antisera which were adsorbed with the same antigens.

Electron microscopy. The fresh liver specimens were immersion-fixed in 3% glutaraldehyde and postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.2, for 2 hr, respectively. Tissues were dehydrated in acetone and embedded in epoxy resin.

Results

After the administration of 0.06% DAB for 20 to 50 weeks, most livers in the test animals showed severe morphological changes accompanied by the neoplastic alteration. Those were numerous small basophilic and cholangiolar cells with a poorly developed cytoplasm in the peripheral area adjacent to the bile ducts and in the middle portion of lobuli along the sinusoids.

In the untreated control, all rats revealed no morphological changes throughout the liver tissue. The specific immunoperoxidase reactions for fibronectin were observed in the vicinity of the central and the portal tracts (Fig. 1). Occasionally, a faintly positive reaction of fibronectin was observed along the bile canaliculi of the peripheral hepatocytes.

Remarkable immunohistochemical findings as the sequential changes during the hepato-biliary carcinogenesis caused by DAB ingestion were obtained in the fibronectin distribution. The earlier appearance of this matrix protein was recognized in the peripheral region of necrotic foci, in the periportal area and in
the sinusoids accompanied with proliferating cholangiolar cells after 20 week’s feeding of DAB (Fig. 2). The proliferation of bile duct cells associated with the increased deposits of fibronectin at the portal area and the sinusoids appeared in the livers of rats on the later stage after 30 to 50 week’s feeding of DAB (Fig. 3 and 4).

On the other hand, the immunoperoxidase reaction for type I collagen was restricted in the peripheral areas of the central and portal tracts of both normal and DAB-ingested rats (Fig. 5). The proliferating zones of bile duct cells showed no immunoreaction for type I collagen except in small amounts in the vicinity of necrotic foci (Fig. 6). In the later stage, migration of the proliferating cholangiolar cells was observed along the sinusoids, but the immunoreactive products for type I collagen were not manifested as compared to fibronectin distribution (Figs. 7 and 8).

Electron microscopic observations of the relevant area showed apparent proliferating cells and Kupffer cells adjacent to the increased collagen bundles in the periportal interstitium (Fig. 9).

On the other hand, the distribution patterns of fibronectin and type I collagen in the primary stage of the liver fibrosis caused by CCl₄ administration were as follows; Fibronectin was first distributed in the periportal and perisinusoidal spaces and followed by type I collagen being deposited on the relevant fibronectin distribution (Figs. 10 and 11).

**DISCUSSION**

We have examined a large number of liver sections from DAB-ingested rats showing varying degrees of hepatocarcinogenesis and found a few remarkable differences from the normal staining patterns in fibronectin and somewhat type I collagen distribution. The intensity of the fibronectin immunoreactivity in DAB-hepatoma was increased in the perisinusoidal sites and the portal area, which suggested an increased deposition of this matrix protein.

The antibody to type I collagen showed no distinct perisinusoidal staining in DAB-hepatoma, which was particularly positive in the border regions between liver lobules and Glisson’s capsules. The lack of staining in the sinusoidal spaces including neoplastic cells by antibody to type I collagen may be due to masking or a decrease in the amount of antigen. Therefore, in this experimental condition type I collagen had no relation to cell migrating pathway, although electron microscopic observations of the relevant area showed apparent proliferating cells and Kupffer cells adjacent to the increased collagen bundles in the periportal interstitium.

Concerning the immunoreactivity to type I collagen in liver fibrosis caused by CCl₄ intoxication, fibronectin was first distributed in the periportal and perisinusoidal spaces and followed by this type of collagen being deposited on the relevant fibronectin distribution at the primary stage of the fibrosing process (Abe
et al. in press).

This finding is supported from the report that type I and III collagens appear to be the main components of the fibrotic connective matrix in the enlarged portal spaces and of the Disse's reticulin framework (Grimaud et al. 1980). The origin of fibronectin during tissue damages and inflammations such as DAB or CCl4 intoxication is not fully established yet (Jensen et al. 1983; Abe et al. in press). Evidences are accumulating that fibronectin, in factor XIIIa mediated complex with fibrin laid down during the early phases of tissue injury and repair, is plasma-derived (Clark et al. 1982; Katsukura et al. 1983). This is supported by the report that the human fibronectin injected into mice is insolubilized in tissue in vivo (Oh et al. 1981). Fibronectin is a part of the fibrinous network that constitutes the early granulation tissue, and may be of essential importance for the subsequent migration and proliferation of matrix-producing mesenchymal cells (Kurkinen et al. 1980; Ishida and Tanaka 1982).

In solid human tumors, the modified fibronectin patterns were observed in various tissue carcinomas. In sarcomas, fibronectin disappeared from the membranes of transformed cells and decreased in intensity in the intercellular matrix (Labat-Robert et al. 1981).

In the present study, the proliferating cholangiolar cells were demonstrated to migrate into the distributed fibronectin in the perisinusoidal spaces. Basically, the cholangiolar cells in normal state may synthesize and may secrete this matrix protein underneath their basement membrane. Therefore, the proliferating cholangiolar cells may be invasive dominantly into the fibronectin deposits area rather than non-fibronectin distributed sites. Type I collagen has no association with these migrating pathway in this study, since type I collagen is surrounded by fibrillar type III collagen in the tissue degeneration (Grimaud et al. 1980).

The matrix proteins such as fibronectin and several types of collagen have an advantage in the attempt to distinguish different patterns of neoplastic alterations in the rat livers. Such pattern identification should make it possible to establish a correlation between the quality of liver fibrosis and tumorigenesis.

References


illustrations follow
Figs. 1-4. Direct immunoperoxidase stainings for the localization of fibronectin in different stages of DAB-hepatoma.

Fig. 1. Weakly positive reaction of fibronectin around the central veins (F-arrows). No reaction products are visible in hepatocytes and sinusoids. A 30 week old control rat. \( \times 180 \)

Fig. 2. Twenty weeks after the beginning of 0.06% DAB application. Conspicious fibronectin deposits appear in the sinusoids (F-arrows). Hepatocytes in this stage show various cytological features. P: portal vein. \( \times 240 \)

Fig. 3. Thirty weeks after the beginning of 0.06% DAB application. Intense reactions of fibronectin in the portal tracts and sinusoids including tumor cells (arrow heads) are recognized. \( \times 180 \)

Fig. 4. Magnification of Fig. 3 shows tumor cells in the fibronectin distributed sites in the sinusoid (small arrows show moderate reaction for fibronectin antibody). \( \times 420 \).
Matrix Proteins in DAB-Hepatoma
Figs. 5-8. Direct immunoperoxidase stainings for the localization of type I collagen in different stages of DAB-hepatoma.

Fig. 5. Moderate amounts of the reaction products for type I collagen are observed around the central vein. Hepatocytes and sinusoids show no reaction products. A 30 week old control rat. ×180

Fig. 6. Thirty weeks after the beginning of 0.06% DAB application. Numerous proliferating cholangiolar cells (asterisk-white bars) with poorly developed cytoplasm are seen in the region of the portal tracts and necrotic foci (N). Tumor cells (proliferating cholangiolar cells) in this stage are oval, round or spindle-shaped. Immunoreactive products are only observed weakly in the portal area. ×240

Figs. 7 and 8. Fifty weeks after DAB application. Proliferating cholangiolar cells are observed along the sinusoids (asterisk-white bars). No immunoreactive products are recognized in the sinusoid including tumor cells. ×240
Fig. 9. Electron micrograph of 30 weeks application of DAB. Proliferating cholangiolar cells are visible around the portal vein. Nucleus in endothelium (E) shows deep invaginations of the nuclear envelope. C, collagen bundles; T, cholangiolar tumor cells; P, Kupffer cell. \( \times 3,800 \)

Figs. 10 and 11. Immunoreactions for fibronectin and type I collagen at 15 weeks application of \( \text{CCl}_4 \). Immunoreactive products are observed in the portal area, respectively. Fig. 10. for fibronectin, Fig. 11. for type I collagen. \( \times 70 \)