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Localization of type III procollagen aminopeptide antigenicity in hepatocytes from cirrhotic human liver *

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Summary. Immunolocalization of type III procollagen (pro III) in normal and cirrhotic human liver was studied using rabbit antiserum specific for bovine type III procollagen aminopeptide. The material examined was deparaffinized, trypsin-treated hepatic tissue sections from 28 autopsy cases, including 19 cirrhotic and 9 normal liver donors. Immunostaining, performed by the unlabeled peroxidase-antiperoxidase antibody technique demonstrated that extracellular matrices corresponding to perisinusoidal reticulin, collagen in periportal areas, and blood vessel walls were the common sites of pro III antigenicity in both normal and cirrhotic liver. Moreover, in the cirrhotic liver, the fibrous septa of pseudolobules, and cytoplasm of hepatocytes and sinusoidal cells were positive when stained for pro III peptide. The differential counts of pro III positive cells in cirrhotic liver, however, revealed that the average ratio of these hepatocytes to sinusoidal cells was 25 to 1, indicating complete dominance of hepatocytes with respect to stainability for pro III peptide compared to sinusoidal cells. In hepatocellular carcinomas coexisting with cirrhosis, neoplastic cells also displayed pro III antigenicity.

These data suggest that hepatocytes of cirrhotic liver and hepatocellular carcinoma cells play a significant role in type III collagen synthesis in vivo.

Key words: Hepatic cirrhosis – Hepatocyte – Type III procollagen – Immunolocalization

Introduction

Chemical analyses have demonstrated a marked increase in collagen types I and III in cirrhotic human liver (Rojkind and Martinez-Palmo 1976; Seyer

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et al. 1977). Localization of both types of collagen in periportal and lobular areas of the cirrhotic liver has been reported using immunofluorescence (Gay et al. 1975; Nowack et al. 1976). However, it is not clear which type of cell is responsible for the overproduction of the interstitial collagen in cirrhosis. Hepatocytes and three types of sinusoidal cells (endothelial, Kupffer's, and fat-storing cell) are now considered to be likely candidates (Berman and Foidart 1980; Martinez-Hernandez 1984).

It is well known that collagen types I and III are synthesized in the form of a precursor (procollagen), which possesses an extra peptide segment at both ends of the molecule. Each additional peptide has distinct antigenic properties which are excised from the procollagen en block by specific peptidase during or after secretion of the proteins. Antibodies directed towards the determinants of these precursor specific portions can label procollagen before secretion and during extracellular processing in the interstitium.

Immunohistochemistry, however, has not been able to localize procollagen to a specific type of cell when studying tissue sections, although a variety of cells have been found to contain procollagen in cell culture (Gay et al. 1976; Müller et al. 1977; Sakakibara et al. 1982). Recently, successful immunohistochemical demonstration of various viral antigens (Huang 1975; Johnson et al. 1980), cytoskeletal proteins (Miettinen et al. 1984; Thomas et al. 1984), and immunoglobulin (Qualman and Keren 1979) in formalin-fixed, paraffin-embedded tissue specimens has been achieved by pretreatment with proteolytic enzymes such as trypsin, pronase, or pepsin.

We report here that trypsin treatment of paraffin embedded liver sections for unlabeled immunoperoxidase staining made it possible to visualize pro III peptide possessed by certain cells. The results obtained indicate that hepatocyte cytoplasm from cirrhotic human liver and hepatocellular carcinoma cells are major sites of pro III antigenicity, as is the extracellular fibrous matrix.

Materials and methods

Materials. Formalin-fixed, paraffin-embedded hepatic tissue blocks from 28 autopsy cases were collected to examine the localization of type III procollagen (pro III). Nineteen of the 28 cases had liver cirrhosis and of these 19, 6 had concurrent hepatocellular carcinoma. The remaining 9 cases had non-cirrhotic livers which were judged to be almost normal upon dissection.

Immunological reagents. The primary antibodies used for the immunostaining were rabbit antiserum specific for the precursor aminoterminal peptide (Col 1–3) of bovine type III procollagen. A commercially distributed radioimmunoassay kit for this peptide segment (Lots D-1003, D-1011, L-1010, Hoechst AG Radiochemisches Laboratorium, Marburg Germany) was used. Antibodies were produced according to the established method of Rohde et al. (1979) and rendered monospecific for the immunizing peptide by immunoabsorption (Nowack et al. 1976). The purified antibodies were dissolved in 0.5% normal rabbit serum and lyophilized (Rohde et al. 1979). It has previously been shown that the aminoterminal peptide of bovine type III procollagen is highly cross-reactive with the corresponding human materials.

After reconstruction with distilled water, antiserum was used in dilutions as high as 1:5,000 to 1:10,000 with phosphate buffered saline to eliminate non-specific staining. The second set of antibodies used were affinity-purified 7-S fractions of goat antisera to rabbit γ-globulin from 3 different laboratories (Cappel Labs, Miles Labs, Mercia-Brocades). Horse radish peroxi-

dase-rabbit anti-horse radish peroxidase soluble complexes used were obtained from Cappel Laboratories.

Immunohistochemistry (PAP method). For immunohistochemical staining, 4 μm-thick paraffin sections were extended on slide glasses which had been precoated with 0.1% Neoprene (polychloroprene, Ohken Shoji, Japan) in toluene. This was done in order to produce a firm attachment of the sections to the glass to prevent the digestive action of the trypsin (Hondo et al. 1982). After deparaffinization, tissue sections were treated with 0.25% trypsin (GIBCO) at 37° C for 60 min. The trypsin-treated sections were incubated at 4° C overnight with the primary antiserum diluted as mentioned above. The second antibodies and PAP complex, diluted 1:20 and 1:50, respectively, were sequentially applied to the sections for 30 min at room temperature. The bound PAP complex was visualized by incubation in a solution consisting of 5 mg of 3,3′-diaminobenzidine in 10 ml of Tris-HCl at pH 7.6 to which 0.1 ml of hydrogen peroxide was added over 2 to 5 min. The stained sections were dehydrated and mounted in synthetic resin without counterstaining.

To confirm antibody specificity, an immunoabsorption test was performed. Bovine pro III peptide was added to aliquots of diluted primary antiserum (60 ng/μl) and incubated at 4° C overnight. At the end of the incubation, samples were centrifuged at 2,000 × g for 30 min at 4° C and the supernatant serum was retained for the test. Further confirmation of the staining specificity was carried out using substitution of the primary antiserum with immunoglobulin from non-immunized rabbits and an application of PAP staining for pro III in deparaffinized, trypsin-treated tissue sections of normal human spleen and kidney obtained from autopsy cases. In these tissues, distinct patterns of localization of type III collagen and procollagen have previously been established using immunofluorescence (Becker et al. 1976; Timpl et al. 1973).

Quantitation of pro III positive cells in cirrhotic liver sections. To determine the cell type playing a major role in type III collagen synthesis, differential counts of pro III positive cells were performed in liver sections from 5 cases with cirrhosis. The numbers of stained cells per field were differentially counted using a light microscope with a 40-power objective and a 10-power ocular piece (Olympus BH-2). The mean values (±s.d.) of the number of stained cells from 5 different fields were calculated.

Results

Eighteen of the 19 cases with hepatic cirrhosis were males, ranging in age from 44 to 74 (average age; 54). Six of the 19 cases had associated hepatocellular carcinoma and 5 were positive for the HBs antigen in sera. Nine control cases with nearly normal livers consisted of 5 males and 4 females ranging in age from 0 to 84. In this group, the diseases which caused death were the following; pulmonary tuberculosis, pyelonephritis, subarachnoid haemorrhage, meningioma, hypopharyngeal carcinoma, pulmonary carcinoma, endocarditis fibrosa aortae (chronic rheumatic heart disease), diaphragmatic hernia, and subdural hematoma.

Localization of pro III peptide in the normal kidney and spleen

Control sections of normal spleen showed localization of pro III peptide to the wall of blood vessels, reticulin networks of red pulp, and dense stroma composing trabeculae (Fig. 1 A). In the normal kidney, staining was noted in the interstitium among tubules and Bowman's capsules of the glomeruli, but not inside the glomeruli (Fig. 1 B). These results are in accord with those described previously on the localization of collagen types I and III and procollagens using an immunofluorescence technique.

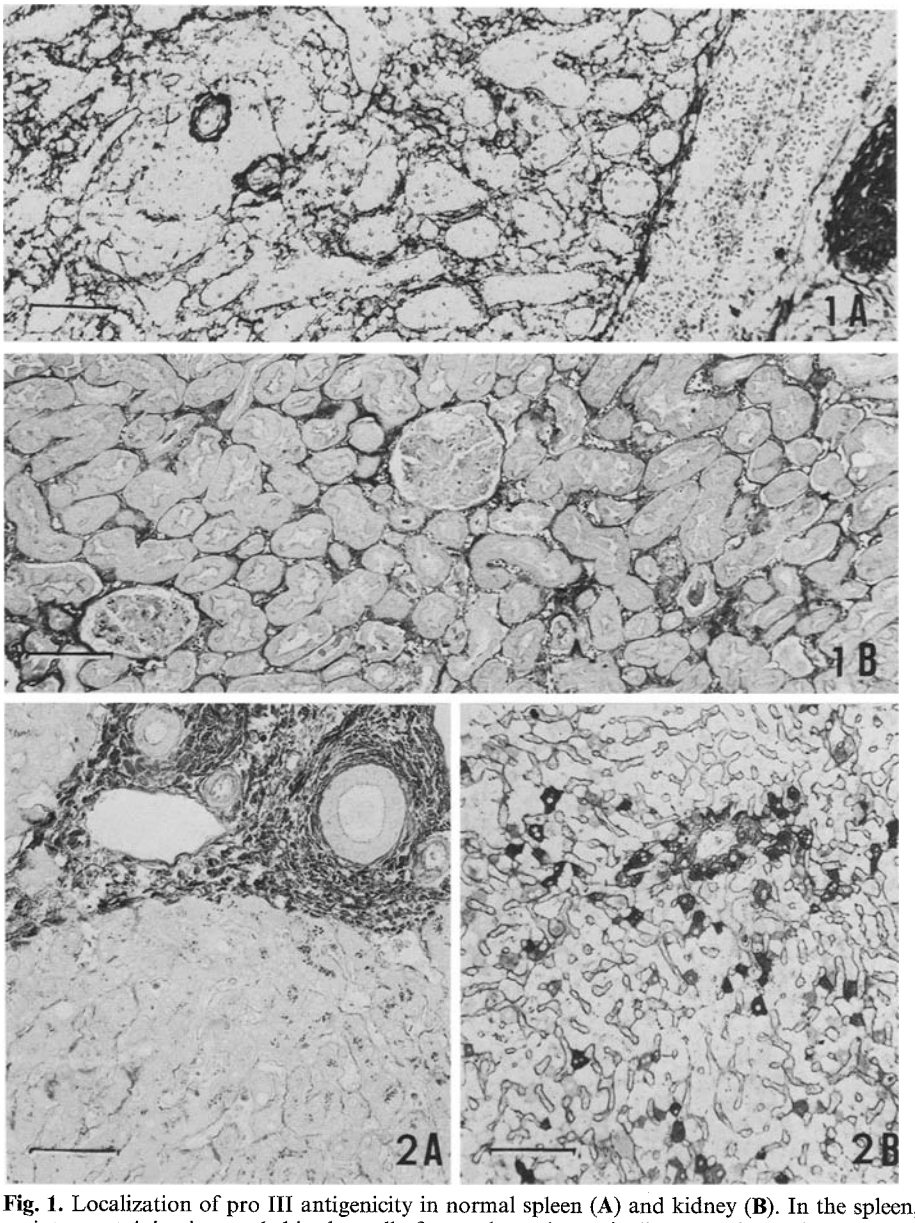

Fig. 1. Localization of pro III antigenicity in normal spleen (**A**) and kidney (**B**). In the spleen, an intense staining is revealed in the wall of central arteries, reticulin networks, and trabeculae. In the kidney, staining is noted in the interstitium, but not inside the glomeruli. Scales equal 100 μm (×112)

Fig. 2 A, B. Localization of pro III antigenicity in non-cirrhotic control livers. In the normal liver, periportal stroma, and perisinusoidal reticulin are stained, but neither hepatocyte or sinusoidal cell are positive (**A**). In contrast, the liver showing chronic hepatitis feature displays an intense reaction for pro III in the hepatocytic cytoplasm and perisinusoidal reticulin (**B**). Scales for **A** and **B** equal 50 μm (×224)

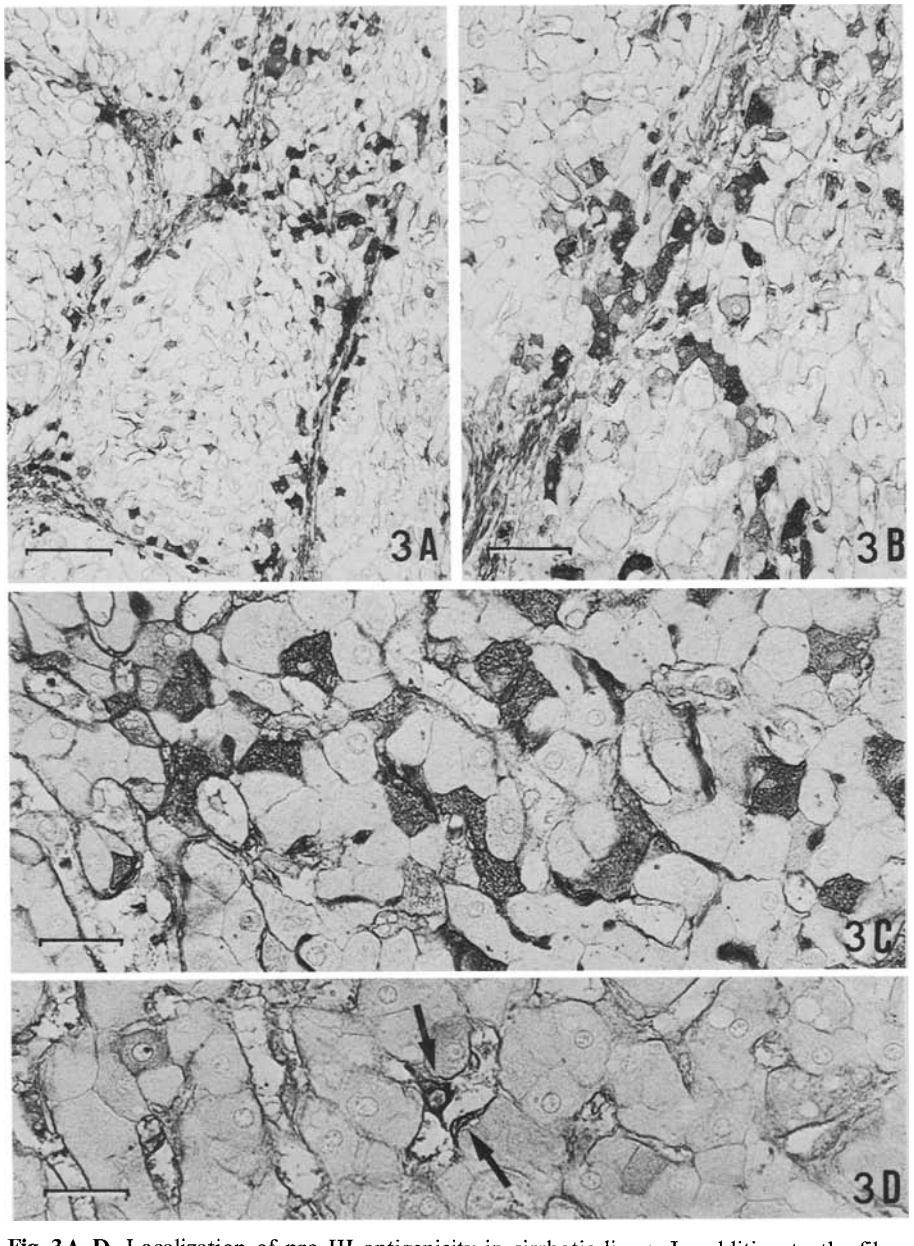

Fig. 3A–D. Localization of pro III antigenicity in cirrhotic livers. In addition to the fibrous structures corresponding to periportal stroma, fibrous septa among pseudo-lobules, and perisinusoidal reticulin, hepatocytic cytoplasm is positively stained for pro III (**A**, **B**, **C**). Scales for **A**, **B**, and **C** equal 100, 50, and 25 μm, respectively. **D** shows sinusoidal cells also stained for pro III. Scale equals 25 μm (× 448)

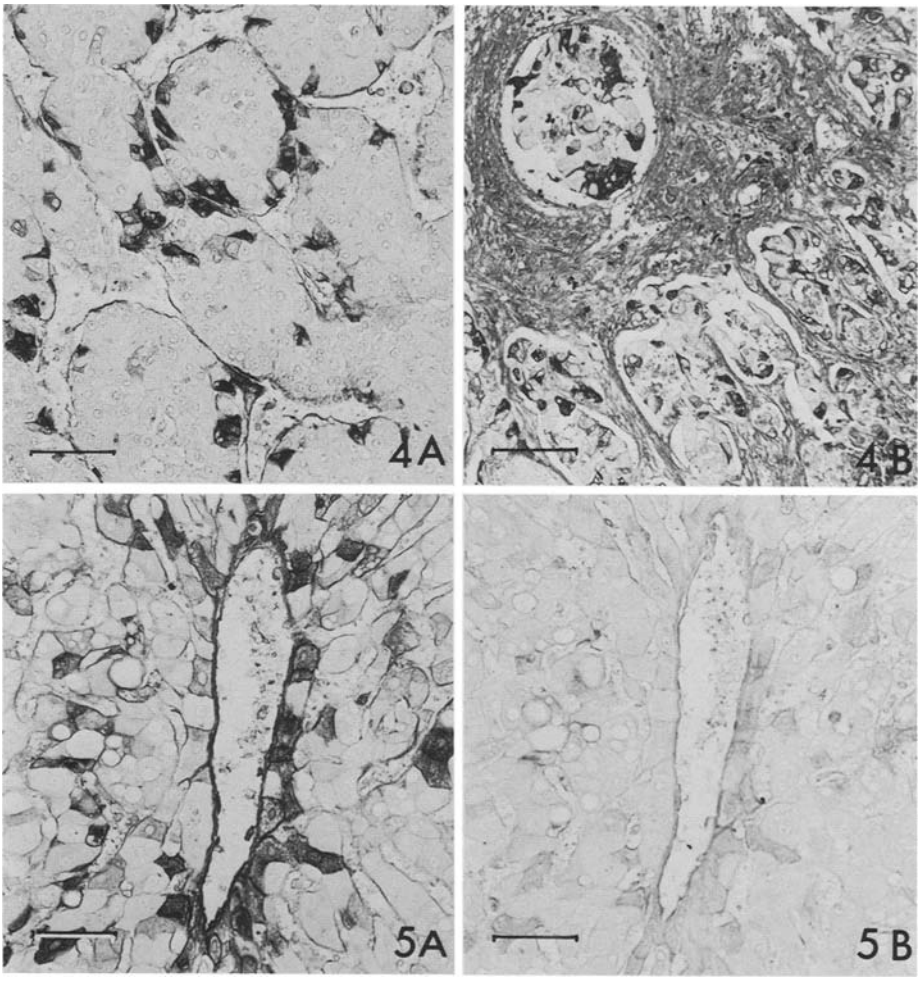

Fig. 4A, B. Localization of pro III antigenicity in hepatocellular carcinomas. In hepatocellular carcinoma growing in islet-forming pattern (**A**), the cytoplasm of neoplastic cells and the membranous zone surrounding tumour islets are stained. **B** shows a less-differentiated hepatocellular carcinoma in which tumour cells also display pro III antigenicity. Scales for **A** and **B** equal 50 μm (× 224)

Fig. 5A, B. Confirmation of the specificity of the primary antiserum by immunoabsorption test. When anti-pro III antiserum is used as primary antiserum, staining is present in the hepatocytic cytoplasm, perisinusoidal reticulin, and the wall of the central vein (**A**). Substitution of primary antiserum with antiserum absorbed with pro III peptide greatly reduces the intensity of the staining of the section adjacent to **A** (**B**). Scales for **A** and **B** equal 50 μm (x224)

Localization of pro III peptide in non-cirrhotic livers

The 9 control livers showed a weak but distinct staining reaction for perisinusoidal reticulin, periportal stroma and blood vessel walls. However, with one exception, no cellular elements were stained (Fig. 2A). This exceptional case, that of a subdural hematoma, disclosed prominent staining in perisi-

nusoidal reticulin and hepatocytes for pro III peptide (Fig. 2B). This case had received a blood transfusion (1,200 ml) 3 months prior to death and the liver was found to be in an early stage of chronic hepatitis.

Localization of pro III peptide in the cirrhotic liver

All 19 cases of hepatic cirrhosis demonstrated positive staining reaction for pro III peptide in extracellular fibrous structures corresponding to perisinusoidal reticulin, fibrous septa of pseudo-lobules involving portal triads, and vascular walls. With only one exception, they also displayed prominent cytoplasmic staining of hepatocytes in a granular fashion. The stained hepatocytes were randomly distributed in the pseudo-lobules, occasionally showing a tendency to gather along fibrous septa or around central veins. As a rule, the density of stained cells differed in different pseudo-lobules (Fig. 3A to 3C). In 5 active cases whose livers had scarcely undergone any autolytic changes, sinusoidal cells also stained for pro III, although less frequently and with less intensity (Fig. 3D). Few fibroblast-like cells in the stroma could be identified as positive. In the 6 cases of hepatocellular carcinomas coexisting with cirrhosis, many neoplastic cells exhibited distinct cytoplasmic staining, irrespective of the histological type of tumour (Fig. 4A and 4B). Immunoabsorption of the primary antiserum with immunizing antigen markedly reduced the staining reaction for this peptide (Fig. 5A and 5B). Substitution of the primary antiserum with non-immunized rabbit immunoglobulin led to a diffuse non-specific coloring of the specimens. Immunological reagents from different lots and/or different manufacturers always gave consistent results.

Differential counts of pro III positive cells in the cirrhotic liver sections

The liver sections of 5 cases with cirrhosis demonstrated well-preserved fine structure. In these cases, both hepatocytes and sinusoidal cells were found to be immunocytochemically positive for pro III peptide. Differential counts of stained cells in the liver sections revealed that the number of stained hepatocytes was 18 to 33 times greater than the number of stained sinusoidal cells. The average ratio of these hepatocytes to sinusoidal cells was 25 to 1 (Table 1).

Table 1. Differential counts of pro III positive cells in the cirrhotic liver sections. The numbers of stained cells per field were differentially counted using a light microscope with a 40-power objective and a 10-power ocular piece. Values are the mean ±s.d. of the number of stained cells from 5 different fields

Autopsy Number	Hepatocytes	Sinusoidal cells	H/S[a] ratio
312	87.5±13.0	4.6±2.5	19/1
350	64.0±18.3	2.0±1.1	32/1
30241	98.8±21.7	3.0±1.8	33/1
30282	54.4±16.0	3.0±0.6	18/1
30405	89.2±15.1	3.4±1.0	26/1

[a] Hepatocyte/Sinusoidal cell

Discussion

The present study was undertaken to determine the localization and distribution of type III procollagen in cirrhotic human liver. Although the primary antiserum against type III procollagen aminopeptide used could not be proven to be free of any natural antibodies reacting with normal tissue constituents, the specificity of the staining was confirmed by an immunoabsorption test and the distinct pattern of localization of the antigen. By using highly diluted antiserum, the possibility that an immunologically cross-reactive substance to the peptide would give a positive immunocytochemical reaction was minimized, although not absolutely eliminated. It is known that the affinity of cross-reactive antibodies is generally lower than that of specific antibodies, and in high dilution, only high affinity antibodies are revealed. Apart from these immunological methods, the specificity of localization was confirmed by the consistency of the results obtained with different preparations of primary and secondary antibodies. The similarity of the distribution patterns of stained hepatocytes among the cirrhotic livers and the significant differences between the stainability of hepatocytes from normal and cirrhotic livers is further evidence supporting our findings.

It was not unexpected that trypsin digestion would make it possible to identify procollagen situated inside cells, since procollagen types I and III and their terminal peptides are highly resistant to the digestive action of trypsin (Timpl et al. 1973). However, other genuine proteins are not resistant, thus pretreatment with trypsin can be expected to enhance the antigenic exposure of the specimen. This simple method for the visualization of procollagen-laden cells in paraffin-embedded tissue sections may contribute to the determination of the cell types which participate in collagen synthesis, as well as elucidating the pathogenesis of other fibrotic diseases, such as lung fibrosis, myelofibrosis, scleroderma, or scirrhous type carcinomas.

The data demonstrate that the antigenicity of pro III peptide is present not only in the extracellular matrix, but also in the cytoplasm of hepatocytes and sinusoidal cells of cirrhotic human livers. These types of cells are known to be the collagen secretors of the hepatic parenchyma. However, it is worth noting that there was a significant difference between the stainability of hepatocytes of normal livers and those of cirrhotic livers; nearly all of the cirrhotic livers examined possessed pro III positive hepatocytes (18 of 19, 95%). On the other hand, only 1 of 9 control livers was found to have pro III positive hepatocytes. This case, in which prominent hepatocytic cytoplasmic staining was revealed, had the histopathological features of chronic hepatitis. Moreover, in cirrhotic liver sections, differential counts of pro III positive cells disclosed that the average ratio of these hepatocytes to sinusoidal cells was 25 to 1, indicating complete dominance of the hepatocytes with respect to stainability for pro III peptide compared to the sinusoidal cells. Based on these results, we can conclude that the major part of type III collagen synthesis in hepatic cirrhosis is due to the hepatocytes.

Localization of antigenicity of pro III peptide in the cytoplasm of the

cells of hepatocellular carcinoma coexisting with cirrhosis is a new finding. Recent radioimmunoassay studies determined that the serum levels of pro-III peptide were markedly elevated in the sera of patients with acute and chronic hepatitis (Nakano et al. 1983) liver fibrosis and cirrhosis (Maruyama et al. 1984) and hepatocellular carcinoma (Igarashi et al. 1983). The present results provided evidence that the origin of the peptide detected in the sera of patients with these hepatic diseases is most likely the neoplastic or non-neoplastic hepatocyte. It remains unclear as to why the level of hepatocyte pro III synthesis is increased in these conditions of the liver. We can only speculate that an agent/agents which acts/act commonly on hepatocytes as stimulators for pro III synthesis are present in these cases.

Finally, there is the problem of which cell type is responsible for the overproduction of interstitial collagen in cirrhosis. According to the chemical analysis (Seyer et al. 1977), the interstitial collagen solubilized from cirrhotic human livers was 75% type I and 25% type III collagen. Recent data obtained in culture (Hata et al. 1980; Sakakibara et al. 1982; Smith and Niles 1980) and in in situ hybridization of type I collagen mRNA with hepatocytes (Saber et al. 1983) have indicated that hepatocytes have the capacity to synthesize type I collagen. It is therefore possible that hepatocytes from cirrhotic liver tissue can produce type I collagen in vivo as well. In order to prove this, further demonstration of the presence of type I procollagen in hepatocytes from cirrhotic liver is necessary. This, together with the present results, will make it possible to answer this problem definitively. By that time, however, the concept of cirrhosis may have changed somewhat.

Addendum

After this article was completed, using monoclonal antibodies against the helical determinant of human type III collagen prepared by Prof. Akira Ooshima, Wakayama Prefectural Medical College, we could confirm the localization of type III procollagen in hepatocytic cytoplasm from cirrhotic human livers.

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