Virchows Arch [Pathol Anat] (1986) 409:37–46

Virchows Archiv A

Immunolocalization of type III collagen and procollagen in cirrhotic human liver using monoclonal antibodies

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Summary. Immunolocalization of type III collagen and procollagen in cirrhotic human liver was studied using monoclonal antibody specific for the helical determinant of type III collagen extracted from human placenta. Deparaffinized, trypsin-treated cirrhotic liver sections from 8 autopsy cases were examined by the unlabeled peroxidase-antiperoxidase and immunofluorescence techniques. These techniques revealed the localization of this epitope shared by type III collagen and procollagen not only in the extracellular matrix of hepatocytes and sinusoidal cells but also in the cytoplasm. In hepatocellular carcinoma concurrent with cirrhosis, neoplastic cells were shown to react with this antibody as well.

These results are consistent with data obtained using antiserum specific for bovine type III procollagen aminopeptide which appeared in our previous report.

Key words: Hepatic cirrhosis – Type III collagen – Immunolocalization – Monoclonal antibody

Introduction

In a previous report, we demonstrated the localization of type III procollagen in cirrhotic human livers. It was found not only in the extracellular matrix but also in the cytoplasm of hepatocytes and sinusoidal cells, using an antiserum specific for bovine type III procollagen aminopeptide (Sakakibara et al. 1985). This commercially-distributed polyclonal antibody prepa-

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ration is, however, not absolutely specific for the immunizing peptide, despite its great convenience.

In the present study, we re-examined material including that used previously, utilizing monoclonal antibodies which react with the helical determinant of human type III collagen (Ooshima et al. submitted) using two diverse immunohistochemical techniques, immunofluorescence and unlabeled immunoperoxidase. We obtained identical results with respect to the localization of the epitope in formalin-fixed, paraffin-embedded liver sections from autopsy cases.

Materials and methods

Materials. Formalin-fixed, paraffin-embedded hepatic tissue sections from 10 autopsy cases were investigated. Eight of the 10 cases had liver cirrhosis and of these 8, 5 had concurrent hepatocellular carcinoma. The remaining 2 cases had non-cirrhotic livers which were normal at the microscopical level. In our previous study (Sakakibara et al. 1985), these 2 normal and the 3 cirrhotic livers (autopsy numbers 255, 312, and 350) were examined to localize type III procollagen aminopeptide (P-III-P). Paraffin-embedded tissue sections from a normal kidney of an autopsy case and the surgically-removed subcutaneous granulation were used as the antigen controls.

Antibody preparation. Anti-human type III collagen monoclonal antibody (mAb-III) was prepared as follows: Type III collagen was extracted from the human placenta according to an established method and digested with tryptin. The trypsic digests were used as antigens (100 μg) for the immunization of BALB/c mice subcutaneously with Freund's complete adjuvant. Three days after the fourth booster injection, the small lymphocytes removed from the spleen of the sensitized mice were fused with mouse myeloma P_3-NS 1-1-Ag 4-1 (NS-1) cells in the presence of polyethylene glycol. The resultant hybrid cells were selected as described by Köhler and Milstein (1975) and subsequently cloned by limiting dilution assay. The clone obtained yields an IgG with a gamma-1 heavy-chain and a kappa light chain which reacts with only α_1 (III) chain by immunoblotting. The antibodies were used at a protein concentration of 94 μg/ml (1:40) for immunofluorescence and 3.76 μg/ml (1:1,000) for the peroxidase-antiperoxidase method.

Pretreatment with trypsin. Prior to immunostaining, tissue sections were attached to a glass slide coated with 0.1% Neoprene (polychloroprene) in toluene. After deparaffinization in xylene and rehydration, the specimens were treated with 0.25% trypsin (GIBCO) at 37° C for 60 min. The sections were then washed in cold phosphate buffered saline (PBS) to stop the enzyme activity.

Peroxidase-antiperoxidase method (PAP). Following trypsin digestion, the sections were incubated overnight at 4° C with the primary antibodies diluted 1:1,000. Subsequent to this, the second antibodies (affinity-purified goat IgG to mouse IgG, Cappel Labs., lot 22334) diluted 1:20, the third antibodies (affinity-purified rabbit anti-goat IgG, Cappel Labs., lot 22406) diluted 1:20, and PAP complex (goat peroxidase-antiperoxidase complex, Cappel Labs.) diluted 1:50 were sequentially applied to the sections for 30 min each at room temperature. The reaction was developed for 2–5 min by incubation in Graham-Karnovsky's reagent, followed by dehydration and mounting in synthetic resin. Counterstaining was not performed unless specifically mentioned. Controls included substitution of PBS or non-immunized mouse serum for the primary antibodies and the parallel incubation of the sections of the kidney and granulation tissues.

Immunofluorescence. Deparaffinized, trypsin-treated specimens were incubated overnight at 4° C with the primary antibodies diluted 1:40. After three washes (5 min each), the sections were incubated with fluorescence-isothiocyanate (FITC)-conjugated sheep anti-mouse IgG

(Cappel Labs., lot 23533) for 30 min at room temperature. It was stated that this second antibody preparation was affinity-purified and not cross-reactive with human IgG. After 3 washes, the label was visualized with an OLYMPUS Model BHS-RFK microscope. The specificity was controlled as described above.

Results

Localization of type III collagen and procollagen in control sections of normal kidney, liver, and subcutaneous granulation tissue

In the normal kidney, the mAb-III specifically stained the interstitium and Bowman's capsules of the glomeruli, but not inside the glomeruli (Fig. 1 A). The parallel incubation of the normal liver with the same antibody preparation showed prominent immunoreaction in the portal triads, perisinusoidal reticulin, and the wall of the central veins, but no reaction with any cellular element (Fig. 1 B). These results are in accord with those described before on the localization of collagen type III using immunofluorescence (Furthmayr et al. 1982b; Timpl et al. 1973). In addition, we examined tissue sections of surgically-removed, post-traumatic granulation. Positive staining was revealed in the dense fibrous stroma, blood vessel walls and the cytoplasm of fibroblasts, as expected. The stained fibroblasts were located in the oedematous stroma of the limited areas of the specimen (FIg. 1 C). When the trypsin treatment was omitted, this pattern of cellular staining did not appear.

Localization of type III collagen and procollagen in cirrhotic liver

The results obtained from 8 cirrhotic and 2 normal liver specimens are presented in Table 1. Virtually all livers were reactive with mAb-III in the extracellular fibrous matrix, including the perisinusoidal reticulin and the wall of the central veins. Moreover, with only one exception, the hepatocyte cytoplasm of the cirrhotic livers showed the same reactivity as the interstitium. In general, the stained hepatocytes were randomly distributed in the pseudolobules, however, many were frequently found in the vicinity of fibrous septa (Fig. 2 A and B). In a few cases, sinusoidal cells reacted positively with no distinct distribution pattern (Fig. 2 C and D). In 3 of the 5 hepatocellular carcinomas occurring together with cirrhosis, the cytoplasm of the neoplastic cells were clearly stained (Fig. 2 E). Controls overlaid with normal mouse serum or PBS in place of the primary antibodies always produced a negative reaction.

Immunofluorescence results

A standard indirect fluorescence method revealed positive results in 3 of the 8 cirrhotic liver sections. The remaining 5 could not be stained. Using this method, the cytoplasm of hepatocytes and hepatocellular carcinoma cells were readily stained (Fig. 3 A–C), however, the cytoplasm of the sinu-

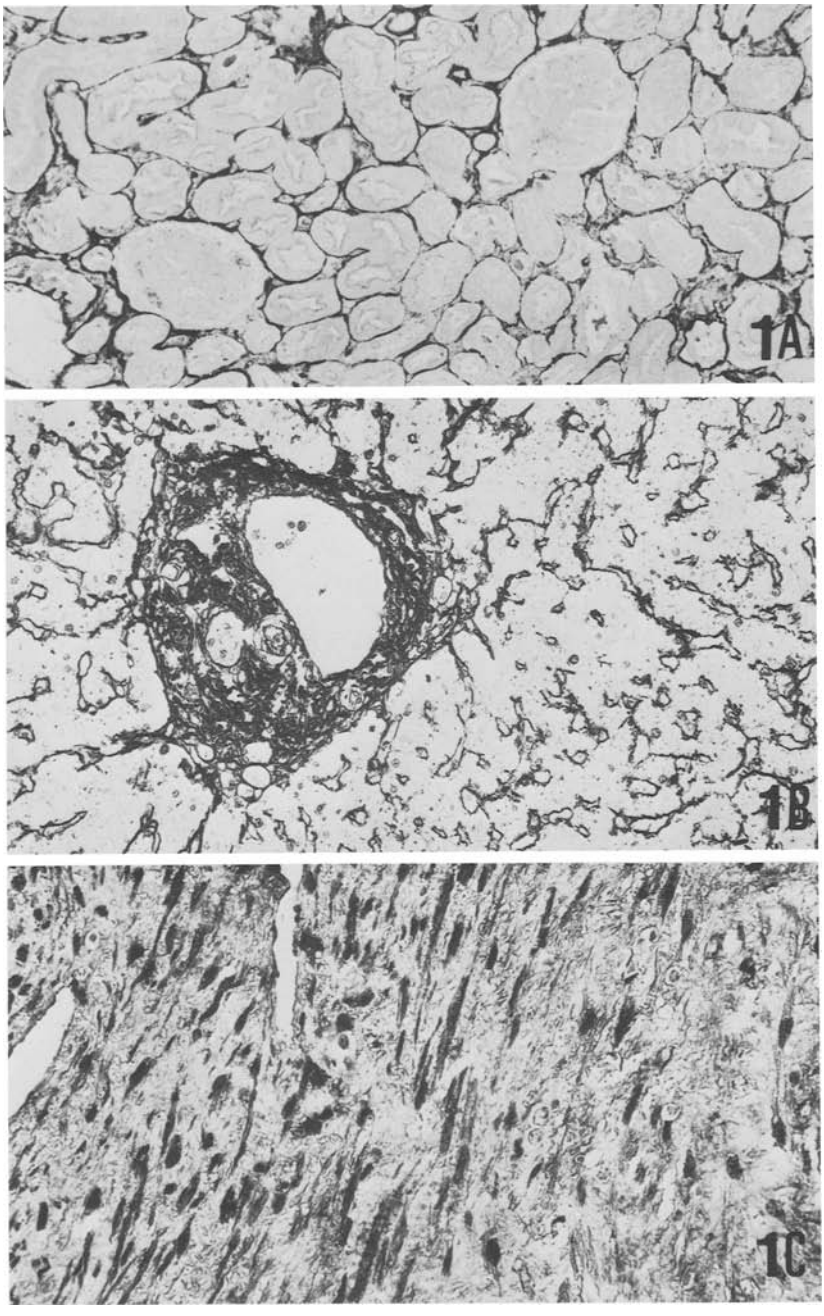


Fig. 1 A–C. PAP staining with mAb-III in control tissues. **A** Positive reaction is noted in the interstitium and Bowman's capsules of the glomeruli in the kidney (× 105). **B** Periportal connective tissues and perisinusoidal reticulin in the normal liver are prominently stained (× 210). **C** Precursor collagen can be seen in the cytoplasm of fibroblasts located in the granulation tissue. Counterstained with haematoxylin (× 420)

Table 1. Stainability[a] of intralobular cells with mAb-III

Autopsy number	Age/Sex	Pathological diagnosis	Stainability	
			Hepatocyte	Sinusoidal cell
Cirrhotic liver				
255	45 M	L.C[b] (nutritional type)	+	−
312	54 M	L.C (postnecrotic type)	+++	+
323	74 M	L.C (postnecrotic type)	++	−
350[c]	58 M	L.C (postnecrotic type)	+++	+
356[c]	55 M	L.C (postnecrotic type)	−	−
359[c]	63 M	L.C (postnecrotic type)	+	−
364[c]	48 M	L.C (postnecrotic type)	++	−
365[c]	55 M	L.C (postnecrotic type)	++	+
Normal liver				
344	57 F	hypopharyngeal cancer	−	−
347	54 F	pulmonary carcinoma	−	−

[a] The stainability was graded according to the ratio of reactive cells (− to +++)
[b] L.C indicates liver cirrhosis
[c] Cases with hepatocellular carcinoma

soidal cells was only slightly stained. The fibrous stroma were consistently stained to a moderate degree.

Comparison of the results obtained with mAb-III and anti-P-III-P

The results of staining a series of 8 cirrhotic and 2 normal hepatic tissues with mAb-III were identical to those obtained with anti-P-III-P with respect to the localization and the distribution pattern of the antigens. However, mAb-III produced a considerably more intense reaction in the extracellular matrix, especially, of the perisinusoidal reticulin in normal liver than did anti-P-III-P antiserum by the PAP method. mAb-III labels both collagen and procollagen, while anti-P-III-P is unable to react with collagen located extracellularly. The variation in reactivity is presumably attributable to the difference in the number of antigens each antibody preparation can react with.

Discussion

Using tissue sections, many investigators have demonstrated the co-distribution of collagen and procollagen in the extracellular matrix. However, the studies failed to reveal any procollagen contained within cells by immunofluorescence using anti-collagen antibodies in combination with procollagen specific antibodies (Furthmayr et al. 1982b; Wick and Timpl 1975).

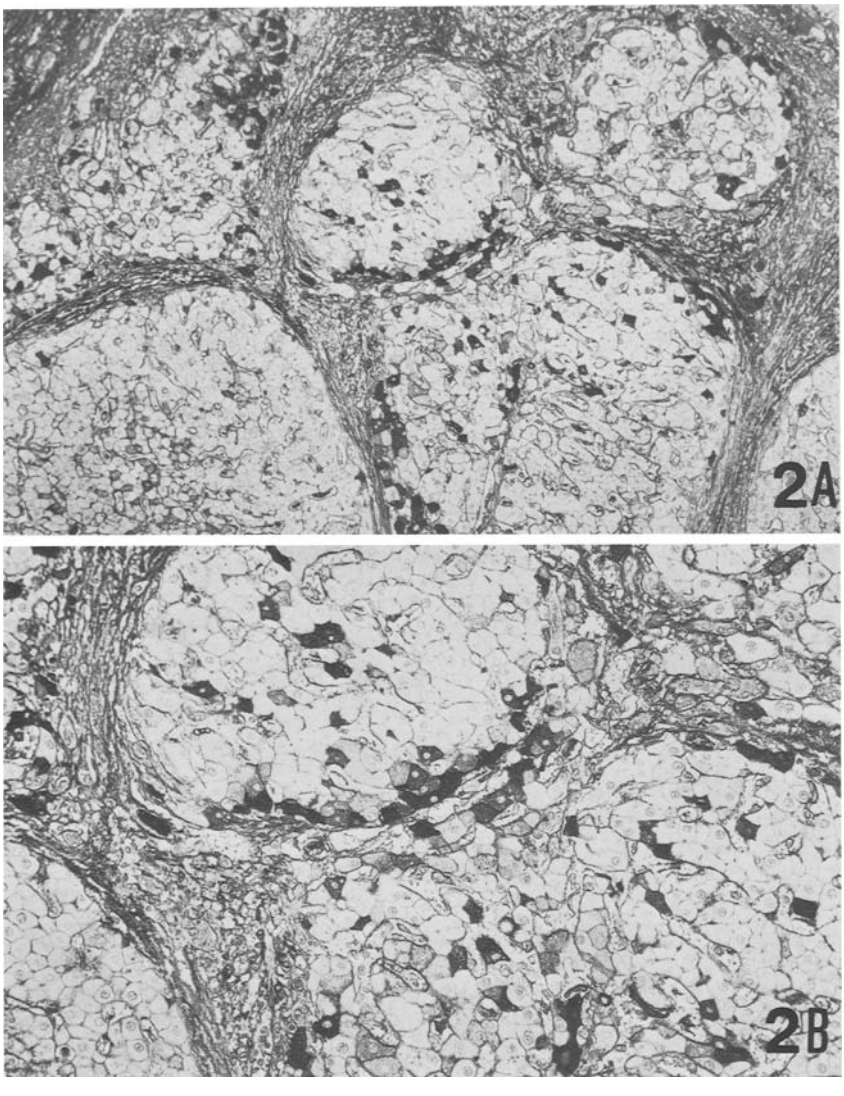
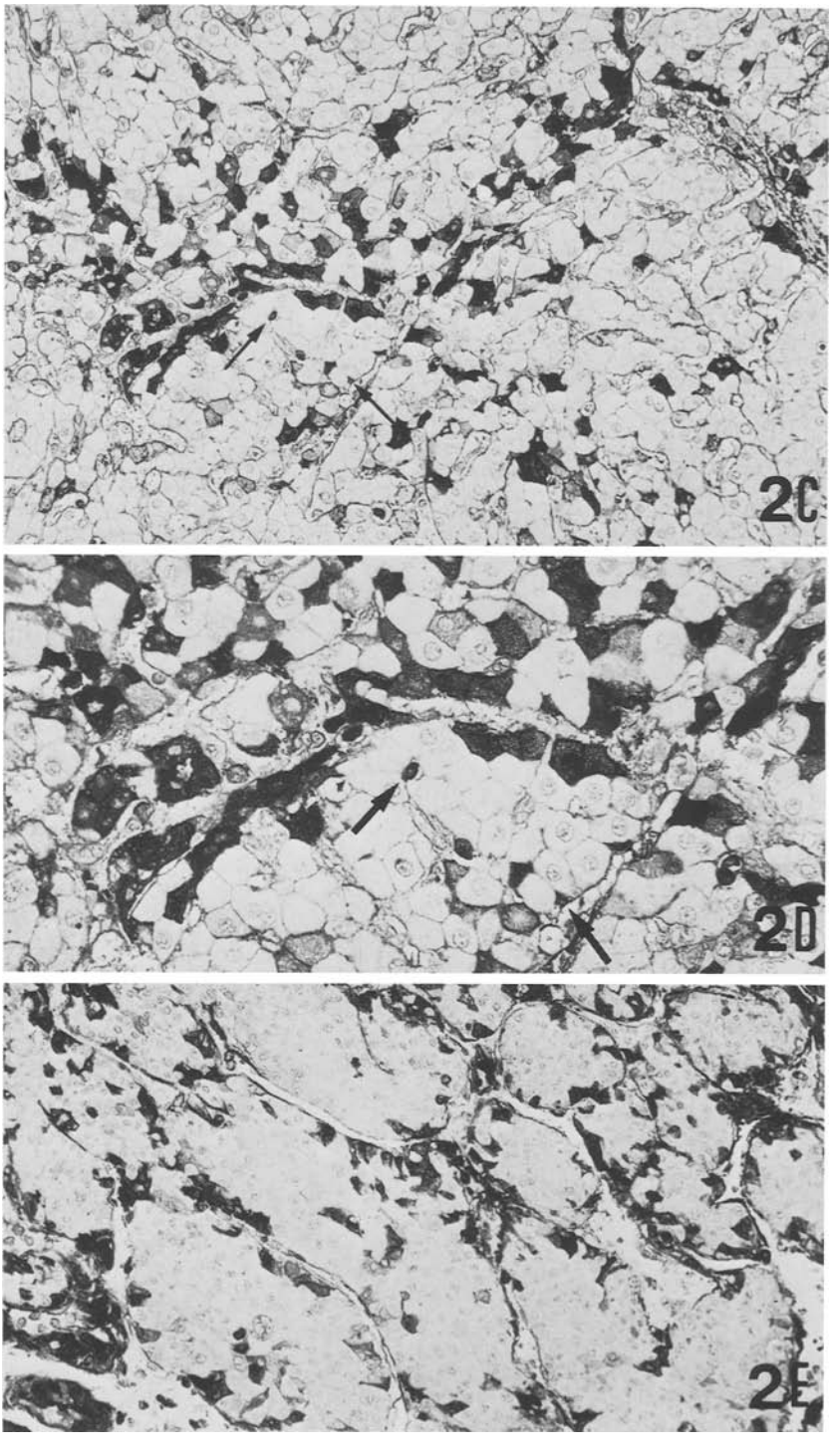


Fig. 2A–D. PAP staining with mAb-III in cirrhotic livers. **A** Marked staining is revealed in the intralobular cells, mainly the hepatocytes, as well as in the fibrous stroma (×105). **B** A higher magnification of a portion of **A**. Many stained hepatocytes are situated near the fibrous septa (×210). **C** In addition to hepaotcytes, a small number of sinusoidal cells (*arrows*) have clearly been stained (×210). **D** A higher magnification of **C** (×420). **E** Hepatocellular carcinoma cells growing in an islet-forming pattern are also reactive to mAb-III (×210)

A previously reported, we found that both intra- and extracellular procollagens were immunohistochemically detectable on paraffin-embedded tissue sections after pretreatment with trypsin. Since the antibody preparation used was a commercial anti-bovine procollagen III amino-peptide rabbit antiserum from a radioimmunoassay kit specific for this precursor peptide,


2C
2D
2E

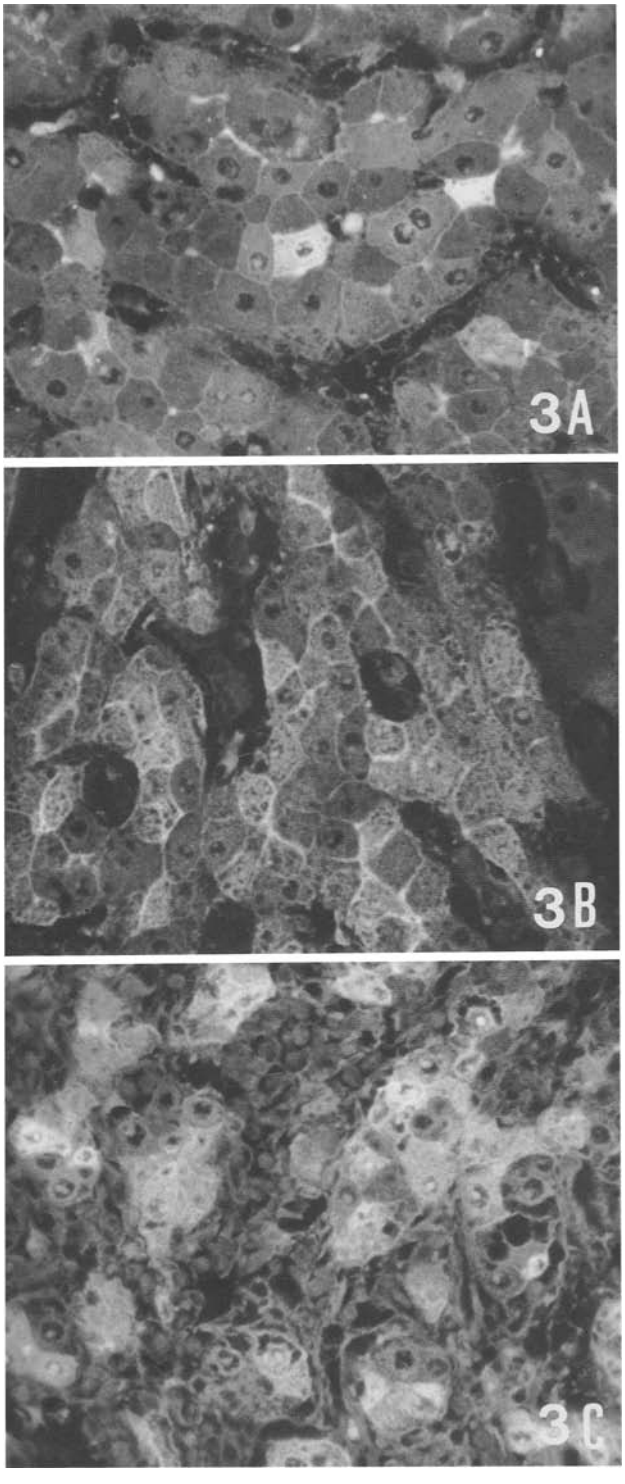


Fig. 3A–C. Immunofluorescence pictures of cirrhotic livers stained with mAb-III. **A** The two fluorescence-positive hepatocytes can be seen in the middle of the picture (×420). **B** In this field, hepatocytes are stained in a reticular or granular fashion. A strong reaction can be seen along the cell membrane (×420). **C** Hepatocellular carcinoma cells are also reactive with mAb-III (×420)

we felt it was necessary to confirm the results with the most specific reagent, monoclonal antibodies.

It is now known that there are three classes of antigenic determinants on the native collagen molecule which are common to both collagen and procollagen. Antibodies specific for these determinants can recognize collagen deposited in the extracellular matrix and procollagen before and after secretion from the producer cells. The monoclonal antibodies used in the present study were specific for one of these three determinants of human type III collagen which had been extracted from the placenta. The determinant is located in the triple helical body of the molecule and is resistant to various proteases, such as pepsin, trypsin, and pronase (Furthmayr 1982a).

The results obtained indicate that the localization of this epitope and the precursor specific aminoterminal peptide of type III procollagen, demonstrated with conventional rabbit antiserum, are identical. This makes the possibility negligible that our previous results were false positives due to contamination of non-specific antibodies, ie., antibodies from impurities in the antigen used in their production, natural antibodies to normal tissue constituents, or cross-reactive antibodies. In addition, immunofluorescence produced the same results, indicating that the positive staining exhibited by the peroxidase-antiperoxidase method was neither diffusion artifacts of the histochemical reagents nor a false positive reaction derived from endogeneous peroxidase. The controls in which the first antibody was replaced with normal mouse serum or PBS had only a diffuse faint background staining in all experiments. This assures that the positive staining was not due to non-specific antibodies contained in the polyclonal second and/or third antibody preparations, but rather to the monoclonal primary antibodies. When the post-traumatic subcutaneous granulation tissue was examined, a positive cytoplasmic reaction was revealed in the fibroblasts. This cellular staining did not occur without the pretreatment of the tissue sections with trypsin, therefore, there is little doubt that trypsin digesiton is effective for the visualization of procollagen-laden cells. Based on these observations, we confirm the specific localization of type III procollagen in cirrhotic human livers.

Recent biochemical data (Hata and Ninomiya 1984) has provided evidence that, even in the normal state, 80% of the collagen produced by the liver in adult rats is attributable to the hepatocytes. In our previous study, despite no precise quantitation of collagen-synthesizing activities, the differential counts of the procollagen-laden cells revealed a ratio 25 times higher for hepatocytes than sinusoidal cells in the cirrhotic liver sections, suggesting that hepatocytes play a significant role in collagen synthesis in cirrhosis as well. Moreover, another quantitative study (Nakamura et al. 1984) has demonstrated that L-proline is essential for induction of normal hepatocyte proliferation in primary culture through its affect on the synthesis of intracellular collagen. If a close correlation exists between the proliferation of hepatocytes and their collagenogenic activity, the continued regeneration of hepatocytes may be associated with an overproduction of collagen

and result in hepatic fibrosis or cirrhosis. Collagen synthesis by hepatocellular carcinoma cells as shown by our immunohistochemical data may also be explained on the same grounds.

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Accepted February 17, 1986