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Relationship between Connective Tissue Cells and Fibronectin in a Sequential Model of Experimental Hepatic Fibrosis

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Summary. The cellular and non-cellular components of fibrous septa formed at early and late stages in a sequential model of experimental hepatic fibrosis have been investigated using ultrastructural and immunocytochemical techniques. In the early septa, cells with intermediate features between lobular Ito cells and active fibroblasts were formed. These cells frequently displayed subplasmalemmal microfilaments (myofibroblast-like cells). Macrophages were also present. Scanty typical fibroblasts were present in the late septa. This cellular recruitment might be related to an extracellular glycoprotein-fibronectin-which is at present under investigation as a chemotactic factor for fibroblasts. Strong positivity for fibronectin in early septa and its sharp decrease in late septa seems to support this view. Fibroblasts and/or macrophages are the likely source of fibronectin synthesis.

Key words: Hepatic fibrosis – Fibronectin – Ito cell

Introduction

Although the mechanisms underlying hepatic fibrosis have been partially clarified (Rojking and Dunn 1979), the role and relevance of the cells involved and the relationship between them and the extracellular collagen and non-collagen matrix are still not understood.

In the normal liver, typical fibroblasts are scanty and are found largely in the portal tracts. Thus attention has been focused on Ito cells (perisinusoidal cells or lipocytes), as being primarily involved in hepatic fibrogenesis (Bronfenmajer et al. 1966; McGee and Patrick 1972; Jezequel et al. 1980). On the basis of morphological criteria, Ito cells are regarded as resting fibroblasts displaying a number of fat droplets which typically show vitamin

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A autoflourescence (Kent et al. 1976). Ito cells are probably involved in the synthesis of type III collagen after accumulation in necrotic areas, as has been shown both in human material and in experimental animals (Jezequel et al. 1980). The histogenesis and role of the myofibroblasts in hepatic fibrosis is less clear, although typical myofibroblasts have been described in the fibrous septa of human alcoholic cirrhosis (Le Lous et al. 1976; Rudolph et al. 1979). As to the role of extracellular matrix components, glycosaminoglycans, in particular heparan sulphate, are known to promote the interaction between collagen type III and fibronectin (Del Rosso et al. 1982).

Little information is available on the role of fibronectin, a glycoprotein synthesized by fibroblasts, macrophages, endothelial and smooth muscle cells (Stenman and Vaheri 1978; Yamada and Olden 1978). It is characterized by a high affinity for collagen (Engvall and Ruoslahti 1977; Ruoslahti and Engvall 1980) and seems to be involved in the early phases of the scarring process (Kurkinen et al. 1980).

The aim of this work has been to investigate the sequential behaviour of fibronectin in an experimental model of hepatic fibrosis in rats induced by heterologous serum (Paronetto and Popper 1966), and to correlate it with the cells probably involved in the fibrogenetic process. Stereological data on the sequential behaviour of Ito cells in the same experimental model have already been presented (Ballardini et al. 1983).

Materials and Methods

Forty Sprague-Dawley male rats (initial weight 150 g) were treated with bi-weekly intraperitoneal injections of heterologous (swine) serum (0.15 ml) and sacrificed in groups of 10 after 5, 10, 20 and 40 injections (2.5, 5, 10 and 20 weeks, respectively). At each interval, 3 control rats treated with saline were also sacrificed. Liver samples were:

1) Snap-frozen in isopentane, precooled with a solid CO_2-acetone mixture, for immunocytochemical analysis (immunofluorescence-IFL-with rabbit anti-human fibronectin) and for the detection of vitamin A autofluorescence (Kent et al. 1976). Anti-fibronectin antibodies were prepared by immunizing rabbits with fibronectin (kindly supplied by Dr. Helmut Hörmann, Max Plank Institute für Biochimie, München, FRG) obtained by affinity chromatography of human plasma on denaturated collagen-substituted agarose. Non-immune rabbit serum was used as control. Anti-actin antibodies were prepared by immunoadsorption with rabbit skeletal muscle actin from the serum of a patient with chronic active hepatitis, positive for smooth muscle antibody at a titre higher than 1:640.

2) Fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, post-fixed in 1% osmium tetroxide in Veronal-acetate buffer, pH 7.4, dehydrated in graded ethanol and embedded in Araldite for ultrastructural analysis. Thin sections, cut with an LKB UM IV Ultrotome and stained with uranyl acetate and lead citrate, were examined in a Jeol 100 B electron microscope.

Results

Immunocytochemistry

Fibronectin. In control liver, fibronectin outlines the sinusoids where it appears to be localized between endothelial and liver cells producing a band-

like pattern. It is also localized in a network pattern in the portal areas and around blood vessels. After 10 weeks of treatment, a positive reaction is present in early fibrous septa. This reaction is much stronger than that of other positive structures such as sinusoids, residual portal tracts and blood vessels, which react as in the controls (Fig. 1 a). After 20 weeks, the late fibrous septa still show a positive reaction for fibronectin; however, this is weaker than that seen in early septa and of similar intensity to that of sinusoids, residual portal tracts and blood vessels (Fig. 1 b). Experiments with non-immune rabbit serum proved to be constantly negative.

Actin. Anti-actin antibodies react with the smooth muscle cells of blood vessels, both in controls and treated rats. In the latter, cells with a positive cytoplasmic filamentous reaction are also found in fusiform cells present in the early septa.

Autofluorescence

Vitamin A autofluorescent cells are found around sinusoids both in control and treated rats. Moreover, vitamin A autofluorescent cells, commonly found in the early septa of the treated rats, progressively disappear in the later stages.

Electron Microscopy

The sinusoidal cellular component in control rats is made up of endothelial, Kupffer and polygonal cells, which are located between the hepatocytes and endothelial cells and are frequently found in close contact with bundles of collagen fibrils. Perisinusoidal cells, or Ito cells, are characterized by a high nucleus/cytoplasm ratio. A number of profiles of slightly dilated rough endoplasmic reticulum (RER) and packed non-confluent lipid droplets are found regularly in their cytoplasm. Cells with similar features are never found in the portal areas of normal rats.

Early fibrous septa, seen in a substantial proportion of rats treated for 5 weeks, are characterized by a heterogeneous population of cells in an amorphous fibrillary matrix with few collagen bundles (Fig. 2). The main cellular component is represented by elongated cells whose overall morphology resembles that of lobular Ito cells. When viewed more closely this apparently homogeneous population can be divided into at least two subgroups. The first is composed of cells with ovoidal nuclei, whose cytoplasm displays numerous RER profiles, which are often dilated and full of floccular material, and a variable number (from 0 to 10) of non-confluent or confluent lipid droplets and glycogen particles. Bundles of thin filaments with focal densities, lying parallel to the main cellular axis are located under the cell plasma membrane. Focal densities are sometimes found in contact with the cell membrane. Macula adherens-like intercellular junctions are seen rarely (Fig. 3). From the above observations, one can conclude that these cells present morphological similarities with both lobular Ito cells and myofi-

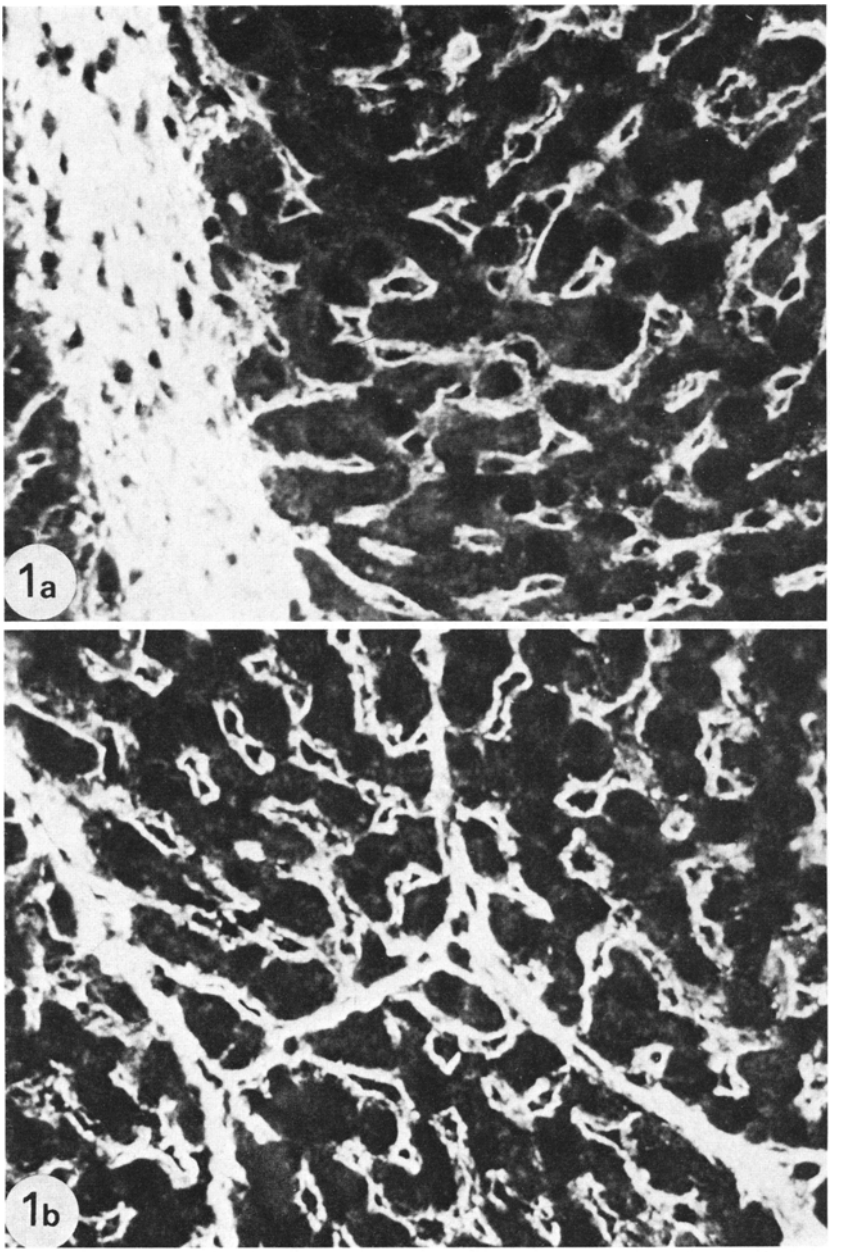

Fig. 1 a, b. Immunofluorescence. **a** A thick early septum (left) shows a positive reaction for fibronectin which is stronger than that of the lobular sinusoids. **b** Thin late septa are still positive for fibronectin. × 750 (original magnification)

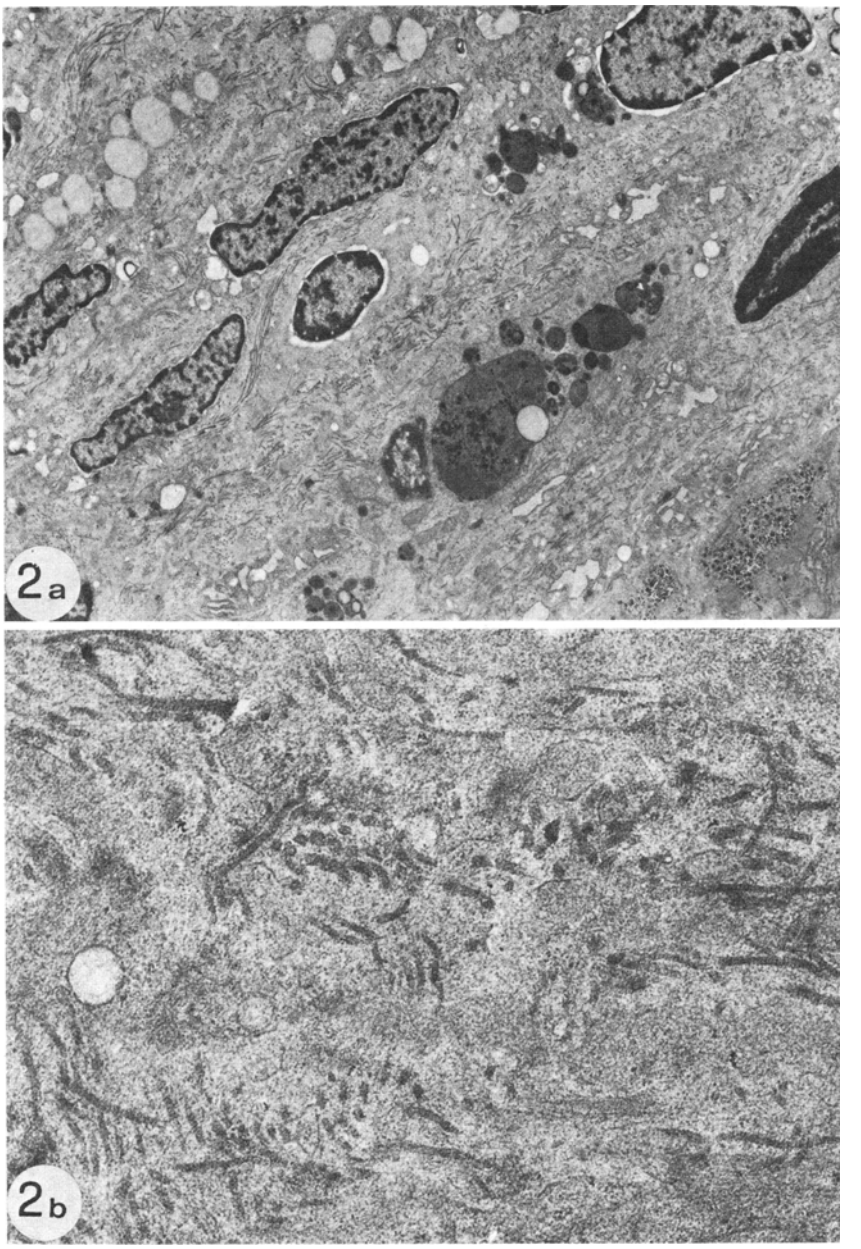

Fig. 2a, b. Early fibrous septum: **a** characterized by a heterogeneous population of cells in an amorphous fibrillary matrix with few collagen bundles. ×5,000. **b** higher magnification of collagen bundles. ×31,800

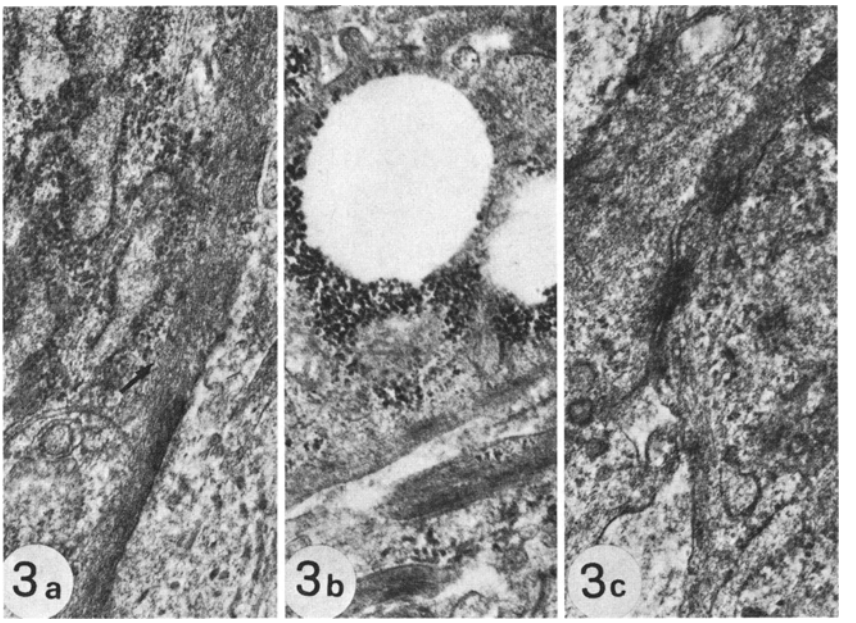

Fig. 3a–c. Early fibrous septum; cells with myofibroblastic features: **a** numerous dilated RER profiles and subplasmalemmal filament bundles (*arrow*). × 30,500; **b** non-confluent lipid droplets and glycogen particles. × 24,000; **c** macula adherens-like intercellular junction. × 36,000

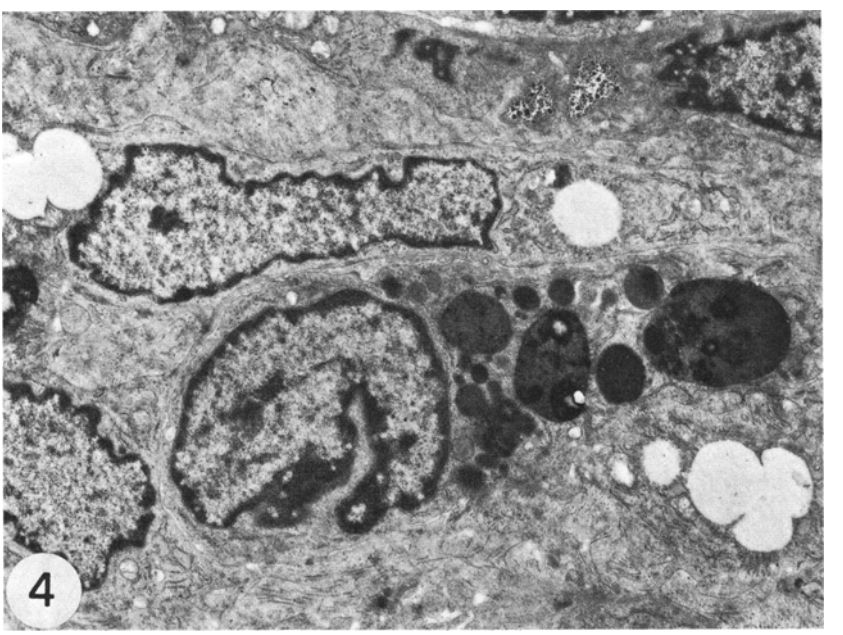

Fig. 4. Early fibrous septum. Typical macrophage with numerous phagolysosomes. × 6,800

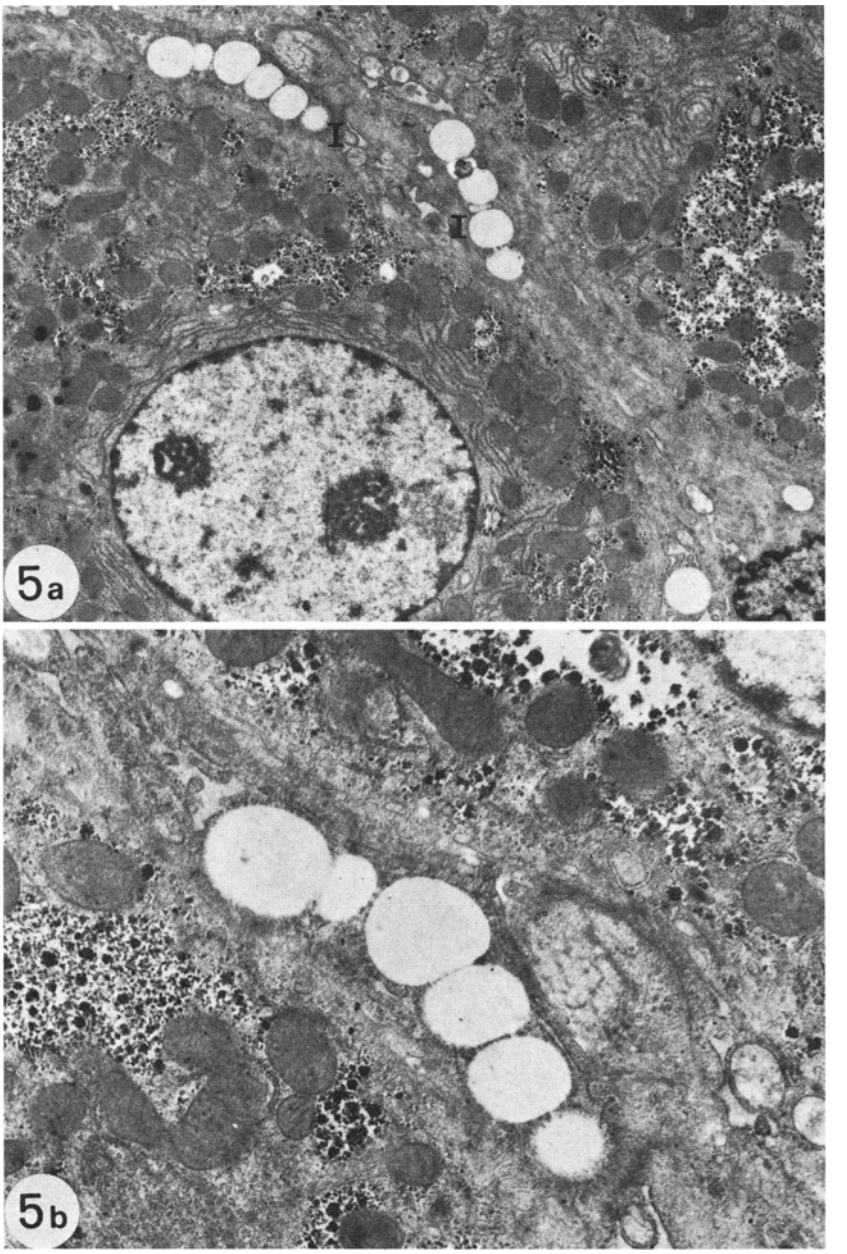

Fig. 5a, b. Early fibrous septum. **a** Low magnification of Ito cells (I) with migrating features at the periphery of lobular residues up to the septa-lobular interface. × 2,400. **b** Higher magnification of a part of the same micrograph. × 14,500

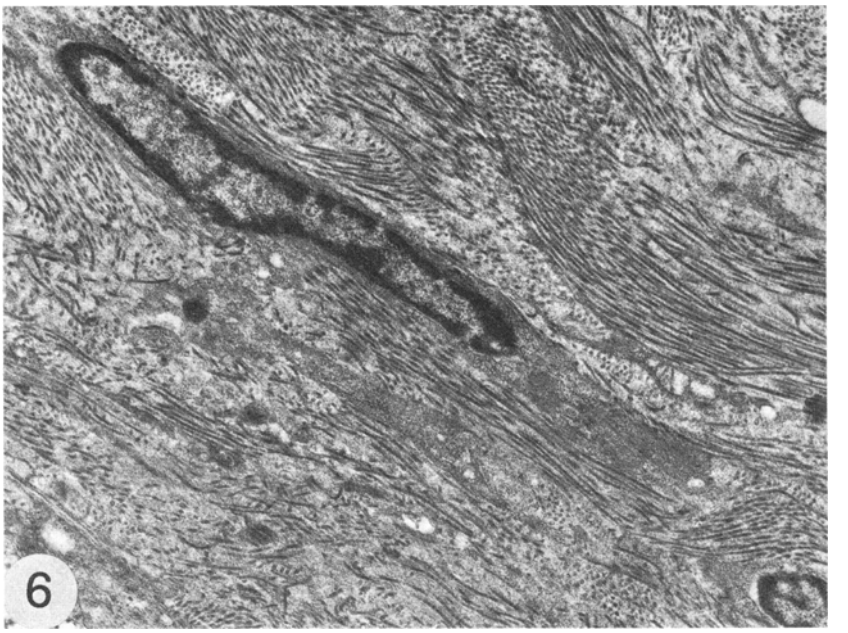

Fig. 6. Late septum. Typical fibroblast surrounded by a fibrillary matrix with numerous collagen bundles. × 11,900

broblasts. The second subgroup of cells also presents lipid droplets and dilated RER profiles, although cytoplasmic bundles of filaments are lacking. Apart from an elongated shape, these cells are quite similar to lobular Ito cells. A further relevant component of the early septum is represented by typical macrophages with the features of an active phagocytic activity (Fig. 4). Eosinophils and very occasional lymphocytes are also present.

No difference was found in the cells of the sinusoidal wall (endothelial, Kupffer and Ito cells) compared with controls. Elongated lobular cells with characteristic features of Ito cells were found frequently at the periphery of residual lobules in the region of early septa up to the septal-lobular interface (Fig. 5). These cells can be regarded as lipocytes (Fig. 5) migrating from the lobular residues to the septa.

In late septa, found regularly after 10 weeks, the fibrous component becomes prominent, while the cells decrease and are represented mainly by scanty typical fibroblasts (Fig. 6). Ito cell-like elements, generally devoid of subplasmalemmal filament bundles, are occasionally seen. Macrophages and eosinophils are rare. Migrating lobular lipocytes are found sporadically.

Discussion

The morphological findings in this experimental model of hepatic fibrosis suggests that Ito cells are deeply involved in hepatic fibrogenesis. The early

fibrous septum shows a high concentration of fusiform Ito-like cells and cells with features intermediate between Ito cells and myofibroblasts. This transitional morphology supports the hypothesis that lobular Ito cells may transform into myofibroblasts and typical fibroblasts, which are found in the later stages of the fibrotic process. Further support for the hypothesis of cellular transformation is given by the observation that Ito cells migrate from the lobule to the septum. As already mentioned, myofibroblasts have been documented in alcoholic cirrhosis (Rudolph et al. 1979), where their contractile properties have been related to the genesis of portal hypertension. Myofibroblasts with cytoplasmic lipid droplets have also been identified in atheromatous plaques (Ghadially 1982). These cells, defined as "multifunctional mesenchymal cells", seem to display several properties. They are able to synthesize actin, procollagen III and glycosaminoglycans (Ghadially 1982). The presence of numerous macrophages and eosinophils can be related to underlying immune mechanisms thought to operate in the experimental model studied.

A relevant extracellular component of the early septum is represented by fibronectin. This glycoprotein is involved in various biological processes such as intercellular and cell-substrate (type III collagen, glycosaminoglycans) adhesion, cellular motility and shape, i.e. structural tissue organization (Stenman and Vaheri 1978; Yamada and Olden 1978; Hahn et al. 1980). The fact that a strong positive reaction for fibronectin has been found in early septa supports the hypothesis that Ito cells, transitional cells and/or macrophages are able to synthesize fibronectin (Colvin et al. 1979; Alitalo et al. 1980; Rennard et al. 1981). The sequence of events leading to fibrosis cannot be fully explored in our experimental model. Regardless of the type of cell involved in its synthesis, it is nevertheless conceivable that the fibronectin present in the early septa may act as a chemotactic factor for lobular Ito cells. In this context, it is worth recalling that alveolar macrophage-produced fibronectin has been shown to exert positive chemotaxis for lung fibroblasts (Rennard et al. 1981). It might also be suggested that fibronectin may act as a differentiation matrix for Ito cells. In the later stages the positive reaction for fibronectin decreases progressively and this parallels a reduction in septal cellularity. The close correlation between the large number of cells with morphological features indicating synthetic activity (transitional and Ito-like cells) and fibronectin in early septa, together with the progressive reduction of both the septal cellularity and fibronectin in late septa, all point to fibronectin as an early marker of hepatic fibrogenesis in the experimental model studied.

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