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ANIMAL AND IN VITRO MODELS IN HUMAN DISEASES

Pathogenesis of septal fibrosis of the liver. (An experimental study with a new model.) ☆

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Abstract

Septal fibrosis is an important, frequent, and non-specific type of fibrosis associated with chronic liver diseases, but its pathogenesis is still poorly understood. An interesting model of septal fibrosis occurs in rats infected with the nematode *Capillaria hepatica*. This model was used to investigate the pathogenesis, site of origin, structure, and cell-types of septal fibrosis. Forty young adult Wistar rats were inoculated with 800 embryonated eggs of *C. hepatica*. Daily liver samples were obtained from the 20th to the 39th day after inoculation to cover the critical period when septal fibrosis usually starts. Routine histology, electron microscopy, immunohistochemistry, and indirect immunofluorescence were applied to the study of liver sections. Septal blood vessels were demonstrated by India ink perfusion of the portal vein system. Prominent angiogenesis was observed to precede collagen deposition. Besides angiogenesis and mesenchymal-cell mobilization, septal fibrosis was seen to originate from portal spaces and to course through acinar zone I in between sinusoids, inducing no alterations in them, with no evident participation of stellate hepatic cells. Septal fibrosis appeared as an adaptive type of response of the liver to chronic injury, which resulted in a new structure that is normal to other species and creates accessory vessels that drain portal blood into hepatic sinusoids.
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Keywords: Hepatic fibrosis; Septal fibrosis; *Capillaria hepatica*; Angiogenesis

Introduction

Fibrosis of the liver develops through different pathways. The one involved with the production of septal fibrosis has aroused much interest due to its frequency, implication with vascular shunts, perisinusoidal location, and cells involved. Septal fibrosis is represented by thin and long fibrous septa running in between hepatic cell-plates, without causing evident alterations in neighboring sinusoids. It is seen to connect portal spaces and, rarely, portal spaces to central veins [2], sometimes originating polygonal structures mimicking that of the pig liver. Observations from two main experimental models, the pig-serum model [2,7,21] and the *Capillaria hepatica* model [4,9], have stressed that such fibrosis is not preceded by repeated episodes of hepatocellular necrosis or by overt chronic inflammation. Although septal fibrosis runs within parasinusoidal area, the participation of sinusoidal cells, especially the hepatic stellate cells (HSC), which has proved to be so prominent for many types of hepatic fibroses [6,9,18],

has not been confirmed to be so for the pig-serum model mentioned above [5,16]. Previous studies with that model have investigated the initial cellular changes of septal fibrosis formation [7,24] and disclosed that fibroblasts located in portal and peri-venular spaces play a central role in the initiation and development of the fibrous septa. It was expected that similar studies with the *C. hepatica* model, with septal fibrosis occurring in 100% of the infected rats, regardless of the inoculum size [17], would allow for a more precise timing for sample collection, representative of the real initial stages. Details on the pathology and fate of septal fibrosis have been recently described [25]. Although there occurs some small variation in time of appearance, septa are regularly present around the 23rd and 27th day after inoculation [3]. Observed changes indicated that the earliest signs of septal fibrosis appeared as a star-shaped expansion of portal spaces and contained mesenchymal cells and blood vessels, sprouting from periportal spaces. The outstanding presence of proliferating blood vessels (angiogenesis) was a constant and impressive feature and was particularly investigated.

Materials and methods

Animals

A total of 45 healthy, male and female, outbred Wistar rats, weighing 180–200 g, were used. They were maintained in good housing conditions, with free access to a commercial balanced diet and water. All the animals, except five (randomly separated to serve as intact controls), were submitted to infection with 800 mature *C. hepatica* eggs administered by a gastric tube. The eggs were isolated from the livers of experimentally infected mice through homogenization in saline, followed by repeated washing and sedimentation. The clean isolated eggs were kept in a humidified Petri dish at 25–28 °C during a period of 28 days for embryonation. Initially, two rats were sacrificed daily, from the 20th up to the 30th day following infection, for the sequential study of the lesions. The remaining rats were submitted in groups of three to a partial hepatectomy (biopsy) under anesthesia and aseptic conditions, with removal of a liver lobe on day 25, 29, 32, 36, 39, and 41 following inoculation. In addition to the liver fragments removed at necropsy or biopsy, samples of serum were collected at several intervals. Some previously biopsied animals were later sacrificed, and their portal system injected with 33% India ink in 19% gelatin for vascular observations. Two animals at each time-point received an injection of 0.3 ml of diluted India ink into the tail vein, 24 h before sacrifice for the mapping of Kupffer cells.

Normal intact control rats were used for serum samples and India ink vascular injections, both for the study of the portal circulation and for the mapping of Kupffer cells.

Histopathology

Fragments of the liver were fixed for at least 48 h in buffered (pH 7.2) 10% formalin. Paraffin-embedded sections were stained with hematoxylin and eosin, picro-sirius red for collagen, PAS-method with and without diastase digestion, Perls' method for iron, toluidine blue for intracellular metachromatic granules and orcein for elastic fibers.

Immunofluorescence procedures

Fragments of liver tissue were immediately embedded in Tissue–teck (OCT compound-Miles Inc., Diagnostic Division, Elkhart, USA) and immersed into liquid nitrogen for a few minutes and then kept frozen at −70 °C in airtight boxes, until the moment they were sectioned in a cryostat at −20 °C. The sections were submitted to the indirect immunofluorescence technique for the demonstration of collagen isotypes (I, III, and IV), laminin and fibronectin. Specific polyclonal anti-human anti-sera were obtained in rabbits (Institute Pasteur, France). They were used in dilution varying from 1:40 to 1:100. Details concerning their preparation and tests of specificity appear elsewhere [4]. Secondary fluoresceinated anti-rabbit-IgG was commercially obtained from Sigma (St. Louis, MO, USA).

Immunohistochemical procedures

All reagents were obtained from Sigma Mo USA unless otherwise stated. For the demonstration of factor VIII (mouse anti-human, DAKOPATTS) and ED3 (mouse anti-rat, BIOSOURCE INTERNATIONAL), fragments of the liver were immediately embedded in Tissue–teck, frozen in liquid nitrogen, and cryopreserved in a freezer at −70 °C until the moment of sectioning. Sections of 6 μm were obtained in a cryostat at −20 °C, placed on slides previously treated with 10% Poly-L-Lisin, fixed in dehydrated acetone, and treated with PBS containing 0.1% Saponin and 1% bovine serum albumin (BSA). For blockade of non-specific ligations, sections were treated with 10% skimmed milk in PBS for 20 min at room temperature.

For the demonstration of SM-α actin (mouse anti-SM-α actin, clone 1A4, DAKO), TGF-β (anti-human, clone TGFB17, NOVO CASTRA), TGF-β-R (mouse anti-TGFR1-β, NOVO CASTRA), desmin (anti-desmin, clone D33, DAKO), vimentin (anti-human, clone VIM 3B4, DAKO), and PDGF (polyclonal, clone AB-1,

Table 1. Data about antibodies and their sources

Antibody	Dilution	Source
Mouse anti-TGFR1-β	1:20	NOVO CASTRA
Mouse anti-smooth muscle actin (clone 1A4)	1:100	DAKO
Anti-vimentin (anti-human—clone VIM 3B4)	1:25	DAKO
Mouse anti-TGF beta (anti-human—clone TGFB17)	1:80	NOVO CASTRA
Rabbit anti-PDGF (polyclonal, clone AB-1)	1:10	CALBIOCHEM
Anti-desmin (clone D33)	1:10	DAKO
Mouse anti-macrophages (anti-rat clone ED3)	1:80	BIOSOURCE INTERNATIONAL
Anti-factor VIII (mouse anti-human)	1:100	DAKOPATTS

CALBIOCHEM), paraffin sections of formalin-fixed tissue were used. Antigen retrieval was accomplished through microwave treatment in citrate buffer at pH 6.0.

Sections were incubated with the primary antibodies (see Table 1) overnight at 4 °C in a humidified chamber. Primary antibodies were diluted in 2% BSA in PBS (pH 7.4). After washing in PBS, sections were incubated in 10% skimmed milk for 20 min for a second blockade of non-specific ligations. The slides were then incubated with the secondary antibody: a sheep-anti-Rat IgG conjugated to Peroxidase (Dako envision system-labeled polymer (Dakopatts, Denmark)) with a dilution of 1:1000 for 30 min at 37 °C in a humidified chamber. Blockade of the endogenous peroxidase was performed with 0.3% H_2O_2 for 30 min at room temperature. The color was developed with 0.06% 3,3′-diaminobenzidine tetrahydrochloride (DAB) and 0.06% H_2O_2 plus 1% dimethylsulfoxide. Sections were counterstained with 1% methyl-green for 2 min, dehydrated, and mounted with Permount. Control sections in which primary antibody was either omitted or replaced by normal rat serum were used.

Transmission electron microscopy

Small cubes of liver (about 1 mm³) were immediately fixed by immersion into 4% glutaraldehyde in 0.2 M cacodylate buffer, pH 7.4, for 1 h at 4 °C, washed in buffer and postfixed with 1% osmium tetroxide, dehydrated in graded concentrations of acetone, and embedded in Polybed 812 (Embedding Media Polysciences, Inc.). Selected ultrathin sections (50–70 nm) were mounted on uncoated copper grids, contrasted with uranyl acetate and a lead citrate. Specimens were examined in a Zeiss EM-9 electron microscope, which was operated at an acceleration voltage of 50 kV.

Results

Infected animals sacrificed 20–23 days after inoculation exhibited several focal parasitic hepatic lesions represented by collections of live and dying adult worms and their eggs, surrounded by purulent necrosis and delimited by a fibrous capsule of variable thickness, without septal fibrosis formation. From these areas, a few short fibrocellular spurs were seen radiating into the neighboring parenchyma. A few days later, the earliest signs of septum formation were noted in areas remote from the parasitic focal lesions. Not all animals developed septal fibrosis at the same time or to the same degree. However, by the 30th day, septal fibrosis became a constant and uniform feature. Septa initially sprouted from portal spaces, giving them a stellate appearance (Fig. 1A), especially evident in India ink-injected preparations (Fig. 1D). These early septa were thin, rich in vessels and mononuclear cells, and poor in extracellular matrix (Fig. 1B). With time, the septa appeared to connect neighboring portal spaces, sometimes creating a polyhedral structure, leaving a vein at its center. The presence of collagen was very scarce during the initial days, but it gradually increased with time, while the number of inflammatory cells diminished. Both collagen isotypes, I and III, were identified from the start. When examined by light or electron microscopy, both the early predominantly cellular and the late fibrous septa appeared limited by normal-looking liver cords and intact sinusoids on both sides of their extension. The general distribution of Kupffer cells in the liver, evaluated by immunohistochemistry (anti-ED3) and by in vivo staining with India ink, failed to show any evidence of concentration around the areas of septal fibrosis (Figs. 1C and 2F). In fact, no significant differences were seen in rats with septal fibrosis and normal controls with regard to the general distribution of Kupffer cells.

Cells

The combined study with routine histology, electron microscopy, and immunohistochemistry revealed that several cell-types formed a linear accumulation along the early septum, with the prominent presence of fibroblasts, loosely arranged collagen fibers (Fig. 3A)
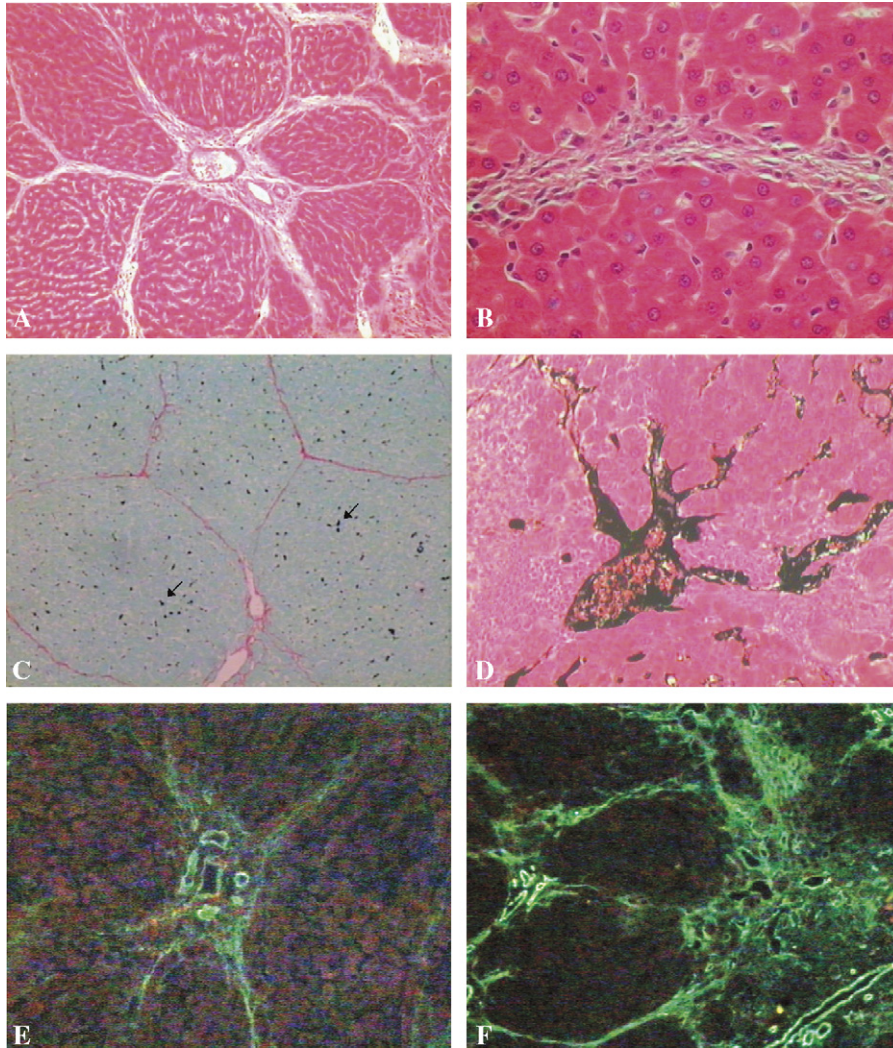
Fig. 1. (A) Thin fibrocellular septa are seen radiating from a portal space of a rat with a 32-day old *Capillaria hepatica* infection. The septa circumscribe nodular portions of the hepatic parenchyma. Hematoxylin and eosin, ×100. (B) An early septum with fusiform cells and several clefts, probably blood vessels, appears to dissect a normal-looking hepatic parenchyma. Hematoxylin and eosin, ×200. (C) Carbon-loaded Kupffer cells appear uniformly distributed throughout the liver section instead of being concentrated along the fibrous septa. Animal was intravenously injected with Indian-ink 24 h before sacrifice. Picro-sirius-red stain for collagen, 100×. (D) Portal vessels filled with Indian-ink document the sprouting of very early *C. hepatica*-induced septa. Hematoxylin and eosin, ×100. (E) A similar aspect to the previous picture is seen when immunofluorescence to collagen IV discloses the sprouting of early septa from portal area by marking basement membrane of numerous blood vessels, ×100. (F) Abundant blood vessels appear in septal fibrosis developed in the liver of a rat with a 32-day *C. hepatica* infection. Fluorescence for laminin, ×100.

and mononuclear leukocytes, and with a scattering of a few polymorphonuclear eosinophils and neutrophils (Fig. 3C). However, endothelial and mesenchymal cells were the predominating cell-types, recognized by the staining for factor VIII and vimentin markers, respectively, and by ultrastructural characteristics (Figs. 2A, B and 3C). Desmin and α-actin-positive cells appeared in a proportion of approximately 1–5 of either fibroblasts or endothelial cells (Fig. 2C). Fusiform cells with well-developed endoplasmic reticulum and the presence of myofibrils and a halo of basement membrane-like material (myofibroblasts) were only rarely found.

The same can be said for the fibroblast-like cells, containing fat droplets in the cytoplasm (SHC) (Fig. 3C). Mast-cells, identified by the presence of toluidine-positive metachromatic granules, were registered, but were not a remarkable finding. These cells were noted partially degranulated under the electron microscope (Fig. 3B). Hepatic cells located at the margins of a septum frequently underwent apoptosis and were seen within the septum as hyaline bodies. These were sometimes taken by macrophages, which appeared with diastase-resistant PAS-positive material, and a variable amount of iron.
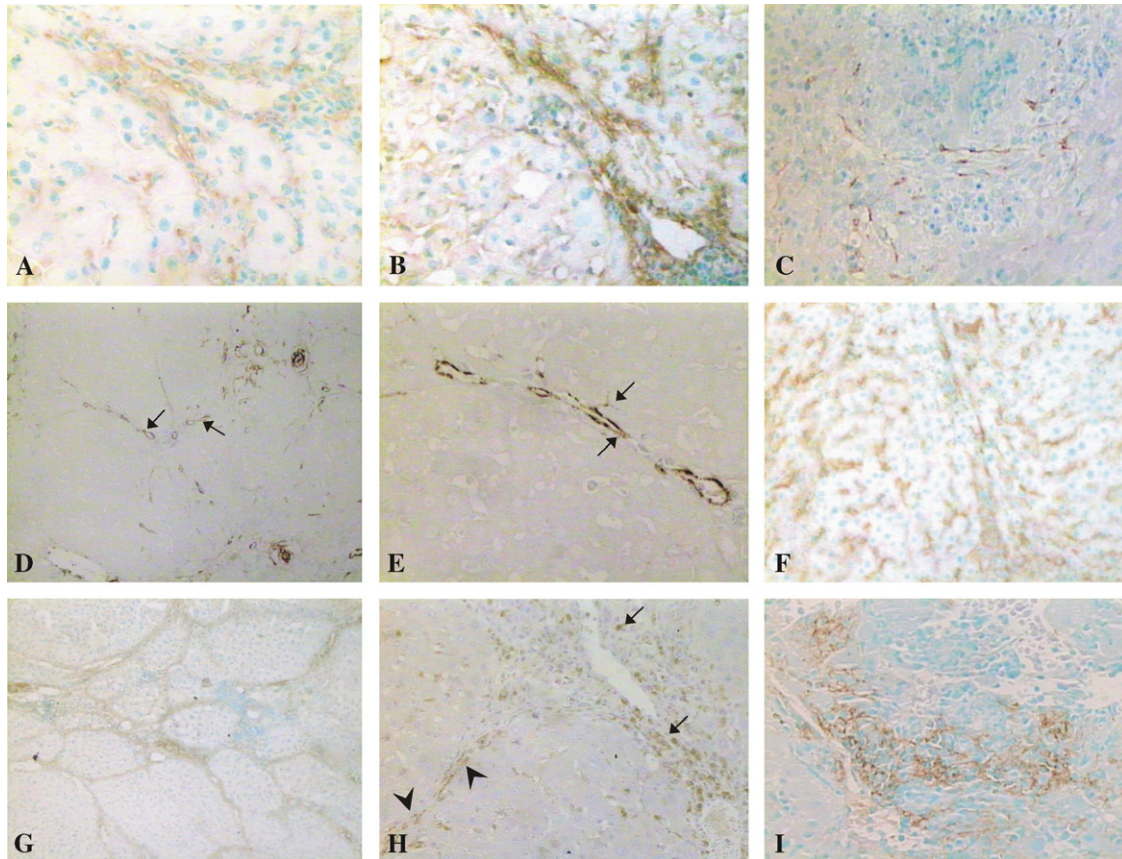
Fig. 2. Immunohistochemical demonstration of growth factors, receptors, and cell-markers in the livers of rats with early (27–40 days) *C. hepatica*-induced septal fibrosis: (A) factor VIII, (B) vimentin, (C) desmin, (D) and (E) actin. SM-α actin marks the muscular walls of blood vessels within the septum and a few stellate cells near by (arrows): (F) ED3, (G) TGF-β, (H) TGF-β-R, and (I) PDGF. × 100.

Growth factors

Treatment of the section with anti-TGF-β resulted in a selective staining of the septa (Fig. 2G), which appeared more marked in earlier infections. The marker for its receptor (TGF-β-R) was detected in at least 50% of the cells present in the septa (Fig. 2H). Around 90% of the cells infiltrating the early septa appeared positive for PDGF (Fig. 2I).

Blood vessels

Blood vessels appeared prominent, especially in very early septa. Sometimes, the vessels were seen at the advancing tip of a septum, giving the impression of leading the way to dissect the parenchyma (Fig. 1D). The increase in the amount of blood vessels was evident in preparations for the demonstration of collagen IV and laminin (Figs. 1E and F), as well as for α-actin (Figs. 2D and E), which revealed components of the blood vessel walls, such as basement membrane and smooth muscle.

Numerous capillaries, venules, and arterioles were present during the formation of the earliest septa from portal spaces. When a septum was fully formed (around the 39th day), the blood vessels were not so conspicuous. The vessels were mainly represented by arterioles and venules. Injection of India ink into the portal system revealed straight vessels running within the septa. Some appeared isolated, without collaterals for some distance, but terminating into radiating vessels, which drained directly into the sinusoids.

Discussion

Septal fibrosis is a common and non-specific change seen in many chronic liver diseases in man, appearing as a prominent finding in some of them, such as incomplete septal cirrhosis [11,22], chronic septal hepatitis [10], and schistosomiasis [1]. However, the pathogenesis of septal fibrosis of the liver is still poorly understood, including that induced by *C. hepatica* infection of rats. Since the first description of that experimental model, an
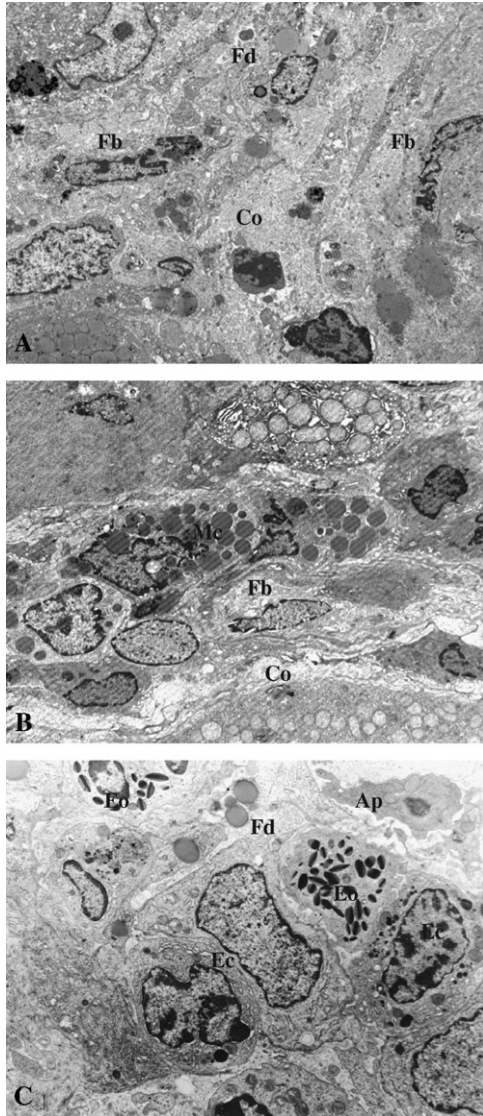
Fig. 3. A series of electromicrographs depict the diversity of cellularity found in very early (27–30 days after inoculation) *C. hepatica*-related septal fibrosis in the rat liver: (A) fibroblasts (Fb) in a background of a loose extra cellular matrix with dispersed collagen fibers (Co). (B) A mast cell surrounded by fibroblasts (Mc). (C) Presence of eosinophils (Eo), endothelial cells (Ec), a few apoptotic cells (Ap), and fat droplets (Fd). EM, magnification ×3000, 4400, and 7000 from top to bottom.

immunological basis for its pathogenesis has been suggested [8], and, recently, more convincing evidences in this regard have been brought forward [12]. Unfortunately, no antigen (s) or immunologically competent cells have yet been properly defined. The same is true for the pig-serum model, which has many features in common with the *C. hepatica* model [7]. In spite of this, other features concerning pathogenesis are worth investigating using this peculiar model, such as can be disclosed by examining the very beginning of septal fibrosis formation, by taking advantage of the fact that

septal fibrosis regularly develops within a narrow margin of time in all rats infected with *C. hepatica* [3,8]. Using this experimental model, the present investigation was able to disclose the earliest changes as located in portal spaces, away from the focal inflammatory and encapsulating parasite lesions formed shortly before within the liver. The initial septa were revealed as radiating projections sprouting from portal spaces and containing collections of small proliferating blood vessels and mesenchymal cells. The presence of this granulation tissue confirms angiogenesis as a key factor in the pathogenesis of liver fibrosis. It was seen to precede collagen deposition in the earliest septa observed in the present study. The role of angiogenesis in the pathogenesis of liver cirrhosis has long been stressed by Moschcowitz [15]. Rappaport et al. [19] stated that sprouting of vascular branches, especially of arterioles, takes the leading role in the development of mature scars in the liver, i.e. of *fibro-vascular membranes.* More recently, Rosmorduc et al. [20] provided evidence for the induction of angiogenic factors, vascular endothelium growth factor (VEGF), and fibroblast growth factor (FGF-2), the most potent angiogenic factors identified thus far, during experimental liver fibrogenesis in rats with bile-duct ligation. They found that VEGF and angiogenesis preceded the onset of cirrhotic lesions.

A similar participation of the angiogenic factors has been reported from observations of patients with primary biliary cirrhosis [14].

From the very beginning of septum formation, two elements appeared prominent: blood vessels and cells. Tuchweber et al. [26] discussed that much attention has been given to the hepatic stellate cell (HSC) as the source of the extracellular matrix proteins. However, in the bile duct ligation model, mesenchymal cells other than HSC may be involved in the early stages of fibrosis development. The periportal fibroblasts may play a dominant role in the early portal fibrosis after bile duct ligation. In the present study, periportal vascular proliferation, especially observed when actin, laminin and collagen IV were stained, strongly resembled the bile duct situation in the bile-duct ligation model. However, in the *C. hepatica* model, septum formation presents no apparent involvement of bile duct. The proliferating cells in early septa were essentially polyclonal, with predominance of fibroblast-like cells, but with the presence of many other cell-types, including polymorph nuclear eosinophils, a constant presence in early helminthic lesions. Myofibroblasts were also present, and some of them may be derived from HSCs, but no evidence of a prominent participation of such cells has been obtained from the present study. In fact, sinusoids located at the immediate vicinity of the fibro-vascular septa maintained their normal appearance, even when observed by electron microscopy, and that was true for both HSCs and Kupffer cells.

The systematized interportal connections seem to be related to evolution, since they appear as normal structures in other animals, such as pig, polar bear, camel, raccoon, etc. [13]. Vessels within the interportal connections carry portal blood to sinusoids, as observed in India ink preparations. The results apparently lead to new extra routes to preserve the physiological role of the portal circulation. Shibayama and Nakata [23] observed that rat livers with systematized pig-serum-induced septal fibrosis maintain their physiological vascular parameters within normal limits. One may conclude that the early changes observed during septal fibrosis formation are consistent with an interpretation of the significance of septal fibrosis as an adaptive response of the liver to chronic injury.

References

[1] Z.A. Andrade, Evolution and involution of hepatosplenic schistosomiasis, Mem. Inst. Oswaldo Cruz 84 (1989) 58–75.

[2] Z.A. Andrade, Contribution to the study of septal fibrosis of the liver, Int. J. Exp. Pathol. 72 (1991) 553–562.

[3] S.B. Andrade, Z.A. Andrade, Experimental hepatic fibrosis due to *Capillaria hepatica* infection (Differential features presented by rats and mice), Mem. Inst. Oswaldo Cruz 99 (2004) 399–406.

[4] Z.A. Andrade, E. Peixoto, S. Guerret, J.A. Grimaud, Hepatic connective tissue changes in hepatosplenic schistosomiasis, Hum. Pathol. 23 (1992) 566–573.

[5] G. Ballardini, S. Degli Esposti, F.B. Bianchi, L.B. de Giorgi, A. Faccani, L. Biolchini, C.A. Busachi, E. Pisi, Correlation between Ito cells and fibrogenesis in an experimental model of hepatic fibrosis: a sequential stereological study, Liver 3 (1983) 58–63.

[6] P. Bedossa, V. Paradis, Liver extracellular matrix in health and disease, J. Pathol. 200 (2003) 504–515.

[7] E. Bhunchet, K. Wake, Role of mesenchymal cell populations in porcine serum-induced rat liver fibrosis, Hepatology 16 (1992) 1452–1473.

[8] L.A. Ferreira, Z.A. Andrade, *Capillaria hepatica*: a cause of septal fibrosis of the liver, Mem. Inst. Oswaldo Cruz 88 (1993) 441–447.

[9] A. Geerts, History, heterogeneity, developmental biology, and functions of quiescent hepatic stellate cells, Semin. Liver Dis. 21 (2001) 311–335.

[10] M.A. Gerber, S. Vernace, Chronic septal hepatitis, Virchow's Arch. A Pathol. Anat. Histol. 362 (1974) 303–309.

[11] D.A. Lavison, J.G.C. Kingham, A.M. Dawson, A.G. Stanfeld, Slow cirrhosis or no cirrhosis. A lesion causing benign intrahepatic portal hypertension, J. Pathol. 137 (1982) 253–272.

[12] Q.T. Lemos, I.F. Magalhães Santos, Z.A. Andrade, Immunological basis of septal fibrosis of the liver in *Capillaria hepatica*-infected rats, Braz. J. Med. Biol. Res. 36 (2003) 1201–1207.

[13] R.N.M. MacSween, R.J. Scothorne, Developmental anatomy and normal structure, in: N.M. MacSween, P.P. Anthony, P.J. Scheuer, A.D. Burt, B.C. Portman (Eds.), Pathology of the Liver, third ed, Churchill Livingstone, Edinburgh, 2002, pp. 1–49 (Chapter 1).

[14] J. Medina, P. Sanz-Cameno, L. Garcia-Buey, S. Martin-Vilchez, M. Lopez-Cabrera, R. Moreno-Otero, Evidence of angiogenesis in primary biliary cirrhosis: an immuno-histochemical study, J. Hepatol. 42 (2005) 124–131.

[15] E. Moschcowitz, Laennec cirrhosis: its histogenesis with special references to the role of angiogenesis, Arch. Pathol. 45 (1948) 187–215.

[16] M. Nakano, Early morphological changes of porcine serum-induced hepatic fibrosis, Acta Pathol. Jpn. 36 (1986) 415–422.

[17] R.F. Oliveira, Z.A. Andrade, Worm load and septal fibrosis of the liver in *Capillaria hepatica*-infected rats, Mem. Inst. Oswaldo Cruz 96 (2001) 1001–1003.

[18] M. Pinzani, L. Gesualdo, G.M. Sabbah, H.E. Abboud, Effects of platelet derived growth factor and other polypeptide mitogens on DNA synthesis and growth of cultured rat liver fat-storing cells, J. Clin. Invest. 84 (1989) 1786–1793.

[19] A.M. Rappaport, P.J. MacPhee, M.M. Fisher, M.J. Phillips, The scarring of the liver acini (cirrhosis): three-dimensional and microcirculatory consideration, Virchow's Arch. A Pathol. Anat. Histopathol. 402 (1983) 107–137.

[20] O. Rosmorduc, D. Wendum, C. Corpechot, B. Galy, N. Sebbagh, J. Raleigh, C. Housset, R. Poupon, Hepatocellular hypoxia-induced vascular endothelial growth factor expression and angiogenesis in experimental biliary cirrhosis, Am. J. Pathol. 155 (1999) 1065–1073.

[21] E. Rubin, F. Hutterer, H. Popper, Experimental hepatic fibrosis without hepatocellular regeneration, Am. J. Pathol. 52 (1968) 111–119.

[22] R. Sciot, D. Staessen, B. Van Damme, W. Van Steenbergen, J. Fevery, J. de Groote, V.J. Desmet, Incomplete septal cirrhosis: histopathological aspects, Histopathology 13 (1988) 593–603.

[23] Y. Shibayama, K. Nakata, Significance of septal fibrosis for disturbance of hepatic circulation, Liver 12 (1992) 22–25.

[24] A. Shiga, K. Shirota, T. Ikeda, Y. Nomura, Morphological and immunohistochemical studies on porcine serum-induced rat liver fibrosis, J. Vet. Med. Sci. 59 (1997) 159–167.

[25] M.M. de Souza, M. Tolentino Jr., B.C.A. Assis, A.C.O. Gonzalez, T.M.C. Silva, Z.A. Andrade, Significance and fate of septal fibrosis of the liver, Hepatol. Res. 35 (2006) 31–36.

[26] B. Tuchweber, A. Desmouliere, M.L. Bochaton-Piallat, L. Rubbia-Brandt, G. Gabbiani, Proliferation and phenotypic modulation of portal fibroblasts in the early stages of cholestatic fibrosis in the rat, Lab. Invest. 74 (1996) 227–265.