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Raniero Mancini · Antonio Benedetti
Anne-Marie Jezequel

An interleukin-1 receptor antagonist decreases fibrosis induced by dimethylnitrosamine in rat liver

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Abstract The main pathological feature of liver fibrosis is the accumulation of extracellular matrix associated with hyperplasia and activation of perisinusoidal (Ito) cells (PSC) to myofibroblast-like cells. Interleukin-1 enhances collagen synthesis by increasing the proliferative activity of cultured PSC and has been implicated in the pathogenesis of hepatic fibrosis.
Interleukin-1 receptor antagonist (IL-1ra) can block the binding of IL-1 to its receptors and act as a natural inhibitor of IL-1. We have examined whether the administration of IL-1ra can interfere with the development of experimental cirrhosis induced by dimethylnitrosamine (DMN). Rats were divided in three groups and received respectively DMN, DMN + IL-1ra and IL-1ra. For each group the collagen content of the hepatic tissue and the volume density of the inflammatory infiltrate were measured. Immunostaining for laminin and alpha-smooth muscle actin were also performed.
In animals given DMN + IL-1ra we observed a decreased deposition of laminin and collagen, and a decreased number of laminin-positive PSC and of alpha-smooth muscle actin reactive cells, compared with animals receiving DMN alone. The present findings suggest that the early activation of PSC in vivo is at least in part mediated by IL-1 and confirm that the administration of IL-1ra may be of interest in modifying the biological effects of IL-1.

Key words Dimethylnitrosamine (*DMN*)
Interleukin-1 receptor antagonist (*IL-1ra*)
Hepatic fibrosis · Perisinusoidal cells (*PSC*)

R. Mancini (✉) · A. Benedetti
Department of Gastroenterology, University of Ancona
CP 538–60100 Ancona, Italy

A.-M. Jezequel
Institute of Experimental Pathology, University of Ancona;
CP 538–60100 Ancona, Italy

Introduction

Hepatic cirrhosis induced in the rat by dimethylnitrosamine (DMN) has been shown to partially reproduce some clinical and morphological features of human chronic liver disease (Jenkins et al. 1985; Jezequel et al. 1987; Ala-kokko et al. 1987). In this model the inflammatory infiltrate, mainly composed by CD8-positive T-lymphocytes, is associated with activation of perisinusoidal stellate cells (PSC) (Jezequel et al. 1989; Mancini et al. 1991b) and the progressive deposition of collagen and extracellular matrix (ECM) components. The early proliferation of PSC is followed by expression of alpha-smooth muscle actin (alpha-SM actin) and differentiation to myofibroblast-like cells (Jezequel et al. 1990; Mancini et al. 1992). The latter have been involved in scar formation and biomatrix deposition in the liver and other tissues (Sappino et al. 1990; Ramadori 1991). As a consequence, therapeutic antifibrotic strategies should be targeted to reduce the process of PSC activation which is considered the final common pathway of hepatic fibrogenesis (Friedman 1993). Since this process is largely mediated by fibrogenic and/or proliferative cytokines, cytokines antagonists should be able to modulate extracellular matrix protein synthesis and deposition (Henderson et al. 1992).

Interleukin-1 (IL-1), produced by activated macrophages and lymphocytes, plays basic roles in the inflammatory and immune response (Dinarello 1991). IL-1 can induce the expression of adhesion molecules and receptors on endothelial and inflammatory cells, the activation of fibroblasts and the modulation of the fibrotic response (Kovacs 1991) and finally may modulate the production of IL-6, IL-8 and TNF-alpha in autocrine and paracrine mode (Maher and Friedman 1993). In the hepatic lobule, IL-1 mRNA is expressed by portal macrophages and endothelial cells (Emilie et al. 1992). A local production of IL-1 is related to early deposition of biomatrix in experimental cirrhosis (Czaja et al. 1989). IL-1 also enhances collagen synthesis by increasing the proliferative activity of cultured PSC (Mat-

suoka et al. 1989). Different agents may interfere with IL-1 activity such as inhibitors of IL-1 converting enzyme and neutralizing antibodies to IL-1 or to IL-1 receptors (Dinarello and Wolff 1992).

In the present study we used a recombinant human IL-1 receptor antagonist (IL-1ra). IL-1ra is a 22 Kd glycoprotein secreted by monocytes/macrophages and polymorphonucleated cells which blocks IL-1 receptors thus acting as a natural inhibitor of IL-1. It seems to reduce the lethal effects of septic shock in animals and to provide protection against the effect of IL-1 in some inflammatory diseases (Dinarello 1993). Finally, it strongly decreases joint swelling and damage in animal models of rheumathoid arthritis and decreases the activity of experimental colitis (Cominelli et al. 1990), thus providing evidence for the importance of IL-1 in the pathophysiology of inflammatory diseases (Dinarello 1993).

The model of DMN-induced liver damage has already been used to test the effects of pharmacological agents on the progression of liver injury and on the development of hepatic fibrosis (Ala-kokko et al. 1989; Mancini 1991a). The purpose of the present investigation was to assess if the administration of IL-1ra is able to modulate the inflammatory response and development of fibrosis induced by DMN.

Materials and methods

Experimental design

Male Sprague-Dawley rats (175 g BW) were obtained from Charles River, Italy. They were fed GLP diet in pellets (Nossan, Italy). Animals were housed in plastic cages with a wire mesh base providing isolation from a hygienic bed and were exposed to a 12-h controlled cycle of light. Hepatic fibrosis was induced by intraperitoneal injections of 1% DMN (Sigma, St Louis) dissolved in saline solution at a dose of 10 mg/Kg/day (Jezequel et al. 1987). Human recombinant IL-1ra was kindly provided by Dr JL Vannice, Synergen INC (Boulder, Colorado) as a solution of 200 mg/ml and was given by mini osmotic pumps (Mean pump velocity = 11.5 μ l/day) inserted under the skin of the back 48 h before DMN treatment. For the purpose of this study, animals (n = 18) were divided in three groups: Group A [DMN] (n = 6) was treated with dimethylnitrosamine 3 consecutive days a week for 2 weeks; Group B [DMN + IL-1ra] (n = 6) received human recombinant IL-1ra (4 mg/Kg/day) in addition to DMN; Group C [IL-lra] received only IL-1ra and represented the control group. The treatment with IL-1ra lasted no more than 15 days to avoid possible interference due to the rat immunological response against human IL-1ra. At the moment of sacrifice each pump was checked to evaluate eventual residual IL-1ra and to control the effective delivery of the compound.

Preparation of tissue

Liver samples were routinely fixed in 4% buffered formaldehyde for 18–24 h and embedded in paraffin wax. Tissue sections (4 μ m thick) were stained with hematoxylin-eosin or with Sirius red F3BA (BDH, England) for routine examination and collagen histomorphometry respectively. For immunohistochemical studies, fragments of liver tissue were fixed overnight in 100% methanol and embedded in paraffin wax. Alcoholic fixation was chosen for the better preservation of cytoskeleton antigenicity as previously reported (Gown and Vogel 1984). Ultrastructural studies were performed after fixation of small fragments with 1% OsO_4 in 0.1 M phosphate buffer and embedding in Epon/Araldite.

Colorimetric determination of collagen

Quantitative assay of collagen was performed on formaldehyde-fixed, paraffin-embedded tissue according to Lopez de Leon and Rojkind (1985). Briefly, 15 μ m-thick sections were deparaffinized and placed in 0.2 ml of satured solution of picric acid in distilled water containing 0.1% Sirius red F3BA and 0.1% Fast green FCF (BDH, England). These dyes bind to collagen and non-collagen proteins respectively. Both dyes were eluted by 1N NaOH in 100% methanol. The absorbance obtained at 540 and 605 nm was determined in a Lamba 3 Perkin Elmer spectrophotometer and used to calculate the amount of collagen and non collagenous proteins respectively (Lopez de Leon and Rojkind 1985). Data were expressed as μ g collagen/mg total proteins.

Immunohistochemistry

Immunoperoxidase staining for alpha-SM actin and for laminin was performed on methanol-fixed and paraffin-embedded sections using respectively anti-alpha SM-1, a monoclonal IgG2a recognizing alpha-SM actin (Dako M851) (Skalli et al. 1988) and affinity-purified polyclonal rabbit IgG against laminin (Sigma L9393). The reactive sites were detected using the labelled streptavidin biotin (LSAB) technique (Dako K680). After endogenous peroxidase inhibition with 0.3% H_2O_2 in methanol for 30 min, sections were taken to water and incubated for 30 min at room temperature with anti-alpha SM-1 or anti-laminin antibodies at a dilution of 1:50 and 1:20 respectively. This was followed by LSAB staining and incubation for 10 min in TBS containing 0.06% diaminobenzidine (DAB) with H_2O_2 (0.01%) added just before use, leading to brown staining of reactive sites. The slides were briefly counterstained with hematoxylin, dehydrated and mounted in permount. Negative controls were performed using a non-immune mouse or rabbit serum instead of primary antibody.

Morphometry

Quantitative analysis was performed by two independent observers at 200 × final magnification with a standard Leitz microscope, using a 10 × 10 mm ocular grid. For each specimen at least 50 nonoverlapping fields were examined. Histomorphometric analysis of inflammatory infiltrate and fibrosis was performed in 4 μ m sections stained with Sirius red F3BA. The lobular extension of the inflammatory infiltrate and of collagen were measured by point counting. Data were expressed as Volume Density (VD = % of tissue volume). Alpha-SM actin- or laminin-positive cells were defined as perisinusoidal cells (PSC) with branching processes extending along the sinusoidal wall and occasionally around hepatocytes (therefore excluding immunoreactive elements of the portal tracts from the quantitative analysis). Only cellular elements showing the nucleus and at least one cytoplasmic process were counted. The lobular distribution of reacting cells was assessed according to the following criteria: positive cells were considered portal/periportal when location was within 150 μ m from the portal rim, as opposed to midlobular/centrilobular location (within 300 μ m from the terminal hepatic veins). Cells associated with areas of collapse and/or necrosis or associated with early fibrotic septa were defined as septal.

Statistical analysis

All results are expressed as Mean value ± SE. The differences between groups A, B and C were assessed by the Student's t-test: $p < 0.05$ was considered statistically significant.

Results

Control group

Treatment with IL-1ra alone did not affect the food intake or the growth of the rats. In this group of animals, histological examination of the liver showed a normal architecture, without evidence of spotty necrosis, inflammatory infiltration, fatty degeneration or fibrosis.

Ultrastructural examination did not reveal pathological changes in sinusoidal and perisinusoidal cell morphology. No sign of hepatocellular damage was evident.

Sirius red staining showed a normal distribution of collagen with a variable amount in the portal tracts and a thin rim around the central veins (Fig. 1). The sinusoidal wall was devoid of collagen, showing only occasional thin delicate perisinusoidal strands. Colorimetric data are reported on Table 1.

In the portal tracts, immunoreactivity for alpha-SM actin was present in the media of blood vessels, in fibroblastlike cells scattered in the connective tissue and in cells closely apposed to bile ductules. In the lobule, the total number of alpha-SM actin-positive cells was $8 \pm 1.2/mm^2$. Most (66%) were part of the wall of large and medium-sized central veins (Fig. 2), 1% were found in zone 3, within 150 μ m from the terminal hepatic vein; 14% were found scattered in acinar zone 2 as elongated, bipolar juxta sinusoidal elements.

Laminin was evident, with both intra- and extracellular localization. Immunoreactivity for extracellular laminin outlined basement membranes as a continuous layer around bile duct epithelium and blood vessels in the portal spaces (Fig. 3). The sharp reactivity for bile ducts allowed occasional observation of the smallest branches of the biliary tree. Extracellular laminin was not seen in the sinusoidal wall of acinar zones 2 and 3, but was occasionally present in acinar zone 1 close to the portal spaces. The number of laminin-positive perisinusoidal cells was $1.5 \pm 0.6/mm^2$. Most were located in acinar zone 1.

Table 1 Effect of IL-1ra on collagen accumulation during the early stages of DMN-induced hepatic fibrosis. The collagen content was calculated as μ g collagen/mg protein using the Sirius red binding procedure (see Methods). Control group was treated with IL-1ra alone. Data are expressed as M ± SEM

Group	7 Days	14 Days
Controls ($n=6$)	24.3 ± 1.2	
DMN ($n=6$)	29.1 ± 0.6	34.6 ± 0.7*
DMN + IL-1ra ($n=6$)	25.5 ± 2.6	29.5 ± 1.8**

* $p < 0.01$ vs controls
** $p < 0.05$ vs DMN

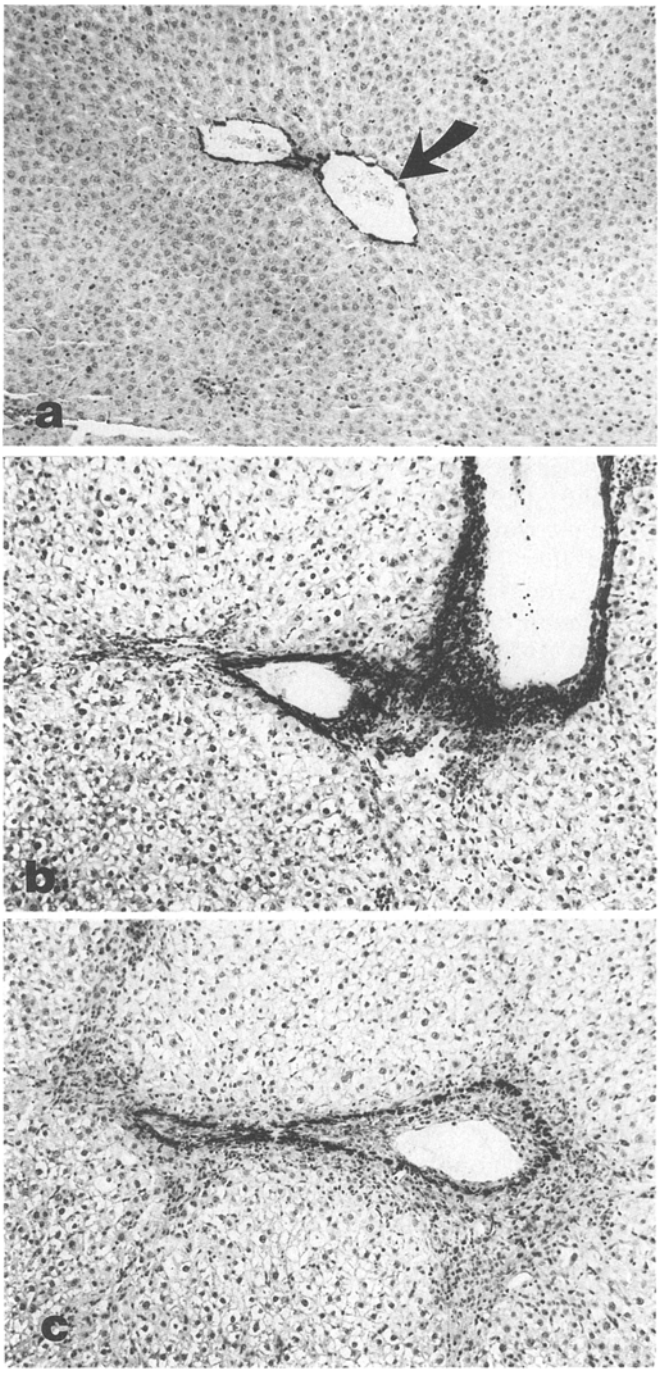

Fig. 1 Histological appearance of rat liver after treatment with IL-1ra (control group) (**a**), DMN (**b**) or DMN + IL-1ra (**c**) for 2 weeks. Sirius red staining for demonstration of collagen on tissue sections fixed in formaldehyde and embedded in paraffin. (**a**) In control animals collagen is evident as thin rim around terminal hepatic veins (*arrow*). (**b**) DMN administration for 2 weeks is accompanied by an inflammatory infiltrate with increased amount of collagen extending in acinar zone 3. (**c**) In animals simultaneously treated with DMN and IL-1ra the amount of collagen is reduced, whereas the inflammatory infiltrate appear almost unchanged

DMN-treated animals

The main histological features associated with DMN-induced liver injury have already been described in detail (Jezequel et al. 1987). The early lesion was centrilobular necrosis associated with a lymphomonocytic inflammatory infiltrate, followed by the development of fibrotic septa. After 7 days of treatment, the VD of the inflammatory infitrate was $15.3 \pm 0.8\%$ and this levelled off to $15.5 \pm 4\%$ at 14 days.

The collagen content, shown by colorimetric determination, increased by 20% and 40% with respect to controls ($p < 0.01$) (Table 1) after 7 and 14 days of treatment respectively. A significant positive correlation with the morphometric findings was evident at 7 and 14 days when the VD of collagen increased to $4.4 \pm 0.1\%$ and $11.5 \pm 0.9\%$ respectively.

An increase in alpha-SM actin-positive cells was observed after 7 days of treatment with values reaching $125 \pm 11/mm^2$ ($p < 0.001$ compared with controls). Most (70%) were located in centrilobular/midlobular areas, whereas 16% were associated with early septa. Although these cells appeared larger than the alpha-SM actin-positive cells observed in the normal liver, they maintained an elongated fibroblast-like shape and appeared particularly numerous in association with or next to necrotic areas. After 14 days of treatment, alpha-SM actin-positive cells peaked to $175 \pm 24/mm^2$ ($p < 0.01$, compared with controls); 13% were located in periportal zones and 74% in midlobular/centrilobular areas, particularly in a juxtaluminal position (Fig. 2). A statistically significant correlation was evident between the number of alpha-SM actin-positive cells and the volume density of collagen fibres ($r = 0.88$, $p < 0.05$).

Ultrastructural studies confirmed the presence of subendothelial myofibroblast -like cells exhibiting an elongated shape with a fusiform, indented nucleus showing a variable amount of rough endoplasmic reticulum, cytoplasmic dense bodies and hemidesmosomes. Typical Ito cells with cytoplasmic lipid droplets were often observed.

Most of the laminin reactivity was due to cytoplasmic staining of juxtasinusoidal cells (Fig. 3). These elements were closely apposed to or encircled one or more hepatocytes. The nuclear shape, position and slender dendritic processes suggested that these cells might represent a subpopulation of activated perisinusoidal cells. Indeed, the staining of sequential tissue sections revealed a codistribution of laminin and alpha-SM actin-positive cells in the lobule. The pattern of reactivity was a strong homogeneous or a submembranous granular cytoplasmic staining. Quantitative analysis showed that at 7 days of treatment laminin-positive cells were $84.9 \pm 1.8/mm^2$ ($p < 0.001$ compared with controls). 71% of these cells were located in centrilobular /midlobular areas, 22% in periportal zones and 7% were associated with early septa. At 14 days of treatment immunolabelled cells were $74.8 \pm 1.6/mm^2$ ($p < 0.001$ compared

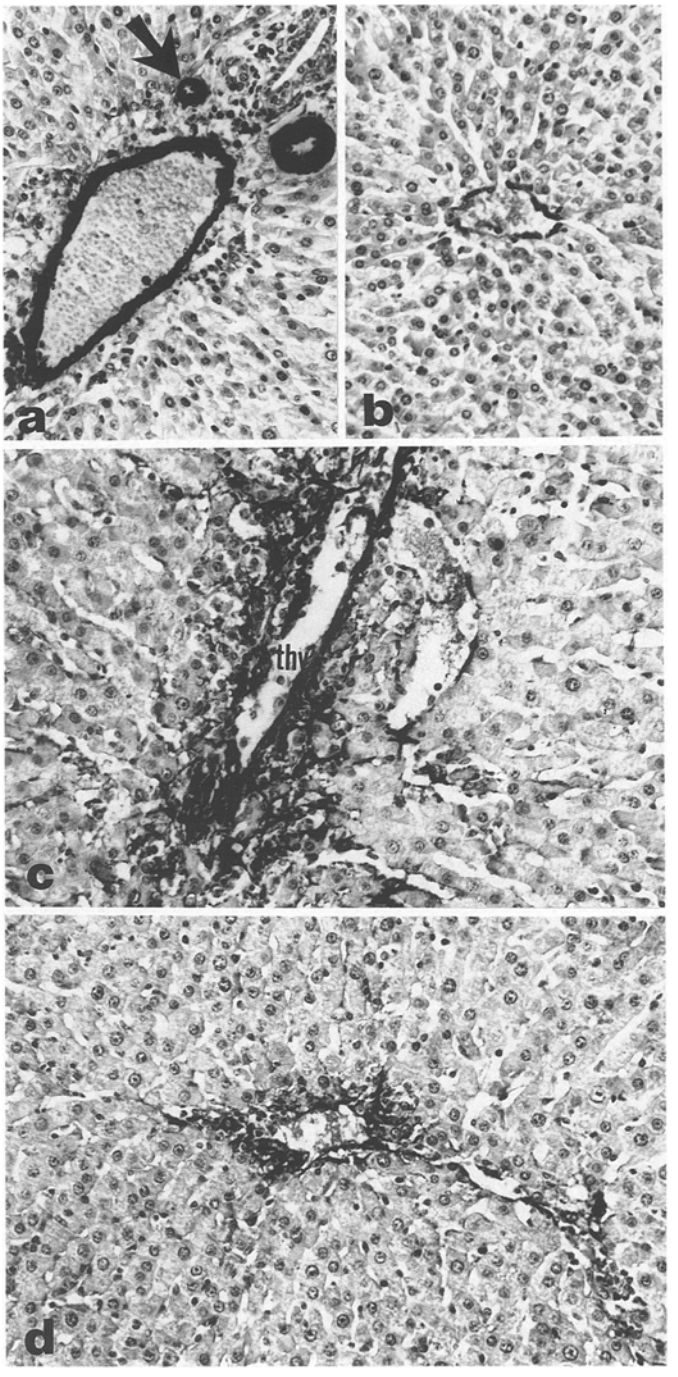

Fig. 2 Immunohistochemical demonstration of alpha-SM actin in methanol-fixed paraffin-embedded hepatic tissue of control (**a**) and (**b**), DMN (**c**) and DMN + IL-1ra (**d**)-treated rats. In portal tracts of normal liver (**a**), alpha-SM actin is strongly expressed in smooth muscle cells of the blood vessels (arrow) or in scattered elements of the portal connective tissue. Occasional immunoreactive cells are also seen in the wall of the terminal hepatic venule (**b**). In rats treated with DMN for 14 days (**c**) a hyperplasia of alpha-SM actin-positive perisinusoidal cells is evident around the terminal hepatic vein (THV) and deep in the parenchyma of acinar zone 3. In animals receiving DMN + IL-1ra (**d**) immunoreactivity for alpha SM-actin is reduced compared to DMN alone

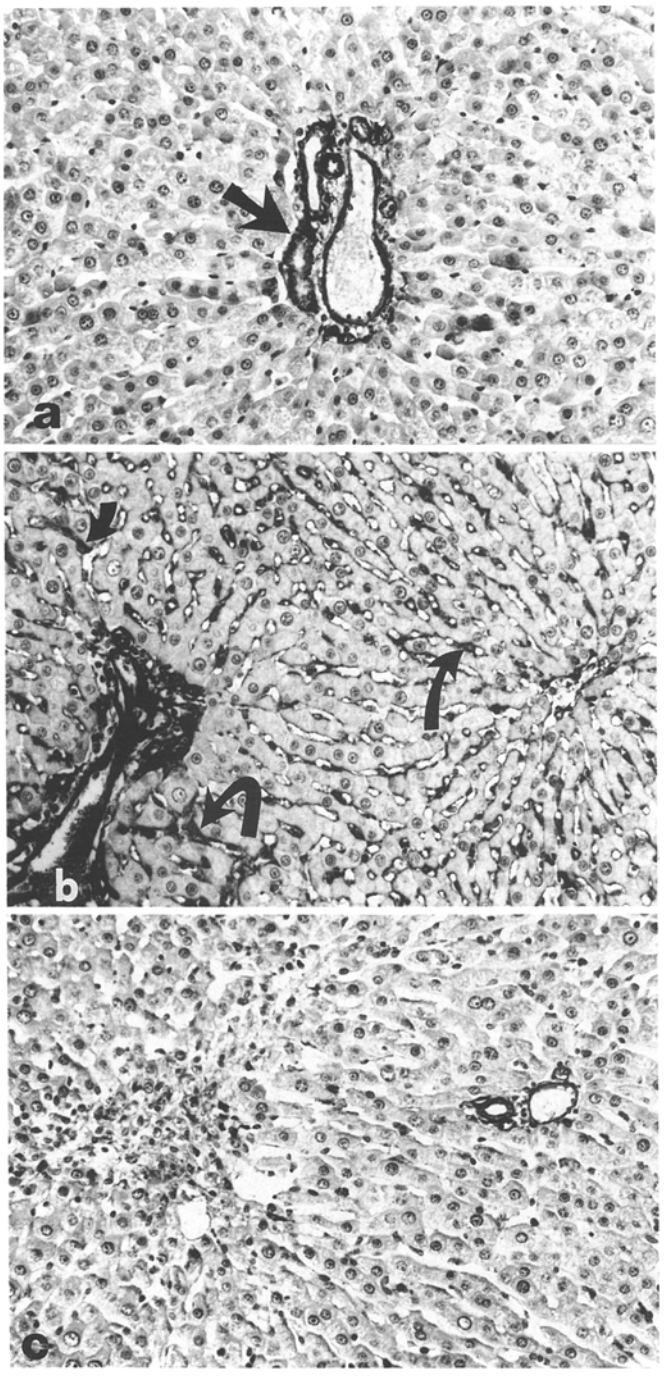

Fig. 3 Immunohistochemical detection of laminin in methanol-fixed and paraffin-embedded hepatic tissue of control (**a**), DMN (**b**) and DMN + IL-1ra (**c**)-treated rats. In portal tracts of normal liver (**a**), laminin is shown in basement membranes of bile ductules and blood vessels (*arrow*). Almost no immunoreactivity was found in the parenchyma or around the terminal hepatic veins. DMN-treated rats (**b**) showed numerous perisinusoidal cells expressing intracellular laminin (*arrows*) in acinar zone 2 and 3, together with a deposition of extracellular laminin in the sinusoidal wall (sinusoidal capillarization). In animals receiving DMN + IL-1ra (**c**) extracellular laminin appears restricted to the basement membranes of portal tracts while laminin-positive perisinusoidal cells are reduced in number compared to DMN alone

with controls) with 23% located in periportal zones and 69% in centrilobular/midlobular areas.

DMN/IL-1ra treated animals

After 7 and 14 days of combined treatment the VD of the inflammatory infiltrate was respectively $15.3 \pm 0.8\%$ ($p < 0.05$, compared with DMN) and $12.4 \pm 4.1\%$ ($p = NS$, compared with DMN). Some haemorragic and coagulative necrosis were observed in acinar zone 3 (Fig. 1).

The collagen content, as measured by Sirius red colorimetry, was increased by 5% and 21% with respect to controls after respectively 7 and 14 days of treatment, showing a reduction with respect to animals given DMN alone (−13% and −15% respectively at 7 and 14 days) (Table 1).

A comparable decrease resulted from morphometric evaluation on whole tissue sections. The VD of fibrotic areas was $3.2 \pm 0.3\%$ ($p < 0.02$, compared with DMN) at 7 days and 3.4 ± 0.7 at 14 days ($p < 0.01$, compared with DMN). In some animals the reduction in the fibrotic areas was still accompanied by pericentral necrosis but the collagen fibers assumed a characteristic distribution with an accumulation at the boundary between the inflammatory infiltrate and adjacent parenchyma (Fig. 1).

IL-1ra treatment appeared to modify the activation of PSC as shown by staining for alpha-SM actin. After 7 days of treatment, the number of reactive cells was $100 \pm 20/mm^2$ ($p = NS$ compared with DMN), with 19% located in periportal areas and 72% in midlobular/centrilobular areas (Fig. 2). The interference with PSC activation was especially evident after 14 days of treatment when alpha-SM actin positive cells were $91 \pm 17/mm^2$ ($p < 0.05$ compared with DMN) with periportal elements accounting for 10% of total reactive cells. 18% of positive cells remained associated with early septa. Laminin reactivity was different compared to that observed in DMN-treated animals, due to the disappearance of positivity in the space of Disse and reduction of the number of laminin reactive PSC (Fig. 3). Indeed, these were respectively $20.4 \pm 2.3/mm^2$ ($p < 0.001$ compared with DMN) and $29.6 \pm 11/mm^2$ ($p < 0.02$ compared with DMN) at 7 and 14 days. The staining of sequential sections showed a similar lobular distribution of laminin and alpha-SM actin reactive cells.

Discussion

The present data show that the administration of IL-1ra can modify the response to the liver injury induced by DMN. In contrast with a different experimental model (Cominelli et al. 1990), we did not observe a modification of the inflammatory response, but rather an interference with the deposition of extracellular matrix components such as laminin and collagen following initial centrilobular necrosis. Morphological evidence of a re-

duced activation of PSC (Figs. 2 and 3) observed in animals given IL-1ra together with DMN is in keeping with these data.

The distribution of intra- and extracellular laminin agrees with data of Clement et al. (1988). Moreover, the combined treatment with IL-1ra was associated with absence of extracellular laminin reactivity in the space of Disse and a significant decrease of laminin-containing PSC compared with animals given DMN alone. Sequential immunostaining for laminin and alpha-SM actin revealed that laminin-positive cells were less numerous than alpha-SM actin reactive elements, but the lobular distribution was similar. Laminin-reactive cells occasionally displayed a granular submembranous pattern possibly due to the storage of laminin inside the cisternae of endoplasmic reticulum in (Ito) PSC as previously shown by immunoelectron microscopy studies (Martinez-Hernandez 1985). Our observations are in keeping with previous reports focused on the cellular source of laminin in normal and pathological liver (Martinez-Hernandez 1984; Maher et al. 1988; Clement et al. 1988; Milani et al. 1989) and confirm that PSC play a major role in the production of laminin in the perisinusoidal space.

Rat lipocytes (Ito cells) belong to the smooth muscle cell lineage as shown by the presence of desmin (Yokoi et al. 1984) and are known to express alpha-SM actin only when activated in vivo or in vitro (Rockey et al. 1992). This activation and phenotypic modulation are associated with cell enlargement, loss of lipid droplets, and increased amounts of rough endoplasmic reticulum and thin actin-like filaments (Rockey et al. 1992). Indeed, transitional aspects have been described in a variety of pathological situations such as alcohol- (Mak et al. 1984) or CCl_4-induced liver injury (McGee et al. 1972), as well as in the present model (Jezequel et al 1990). In addition, in rats given IL-1ra together with DMN, the histological evidence of scarring was modified with a significant decrease of activated PSC cells. As shown in alcohol-fed rats, activation of lipocytes and collagen deposition appear as distinct stages of the response to injury (French et al. 1988). This is in agreement with the two- step model (initiation-perpetuation) recently proposed by Friedman (1993). Histological, morphometric and colorimetric data suggest that the administration of IL-1ra interferes with the "perpetuation stage" and the stimulation of collagen deposition, as part of decreased responsiveness to IL-1. An alternative explanation for the effects of IL-1ra may be related to a possible interference with the pathways of matrix degradation and especially with the stimulation of type IV collagenase synthesis induced by IL-1 in activated lipocytes (Emonard et al. 1990; Friedman 1993).

It is of interest that in a different experimental model using the properties of prostaglandin E1 (PGE1) to alter the response to IL-1 and IL-2, no effect of PGE1 was observed on the cell proliferation or on the inflammation following bile duct ligation, but total collagen and periductal type I collagen were significantly reduced (Beno et al. 1993). The comparable "antifibrogenic" effect suggest that both IL-1ra and PGE1 are acting on the final pathways of collagen deposition, independently of the mechanisms leading to the initial tissue injury. The use of other compounds known to interfere with activation of lipocytes such as Interferon-gamma (Rockey et al. 1992) will probably provide new information concerning the various steps of the cascade of events leading to fibrosis.

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