Pergamon

Biochemical Pharmacology, Vol. 50, No. 4, pp. 515–520, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(95)00154-9

REMISSION OF LIVER FIBROSIS BY INTERFERON-α_{2b}

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(*Received* 28 *November* 1994; *accepted* 20 *February* 1995)

Abstract—Fibrosis is a dynamic process associated with the continuous deposition and resorption of connective tissue, mainly collagen. Therapeutic strategies are emerging by which this dynamic process can be modulated. Since interferons are known to inhibit collagen production, the aim of this study was to investigate if the administration of interferon-α_{2b} (IFN-α) can restore the normal hepatic content of collagen in rats with established fibrosis. Fibrosis was induced by prolonged bile duct ligation. IFN-α (100 000 IU/rat/day; s.c.) was administered to fibrotic rats for 15 days. Bile duct ligation increased liver collagen content 6-fold. In addition, serum and liver markers of hepatic injury increased significantly; liver histology showed an increase in collagen deposition, and the normal architecture was lost, with large zones of necrosis being observed frequently. IFN-α administration reversed to normal the values of all the biochemical markers measured and restored the normal architecture of the liver. Our results demonstrated that IFN-α is useful in reversing fibrosis and liver damage induced by biliary obstruction in the rat. However, further investigations are required to evaluate the therapeutic relevance of interferons on non-viral fibrosis and cholestasis.

Key words: jaundice; bile duct ligation; fibrosis; cirrhosis; interferons; collagen; rat

Hepatic fibrosis is an important feature of chronic liver disease. The replacement of normal hepatic parenchymal tissue by connective tissue compromises the functional capacity of the liver and disrupts the normal architecture of the liver.

Interferons have been utilized widely in chronic liver diseases due to their antiviral properties [1, 2]. In addition, there is evidence for their antifibrogenic actions [3–5]. Recently, we reported that interferon-α treatment preserves hepatocyte and erythrocyte plasma membrane function and composition in CCl_4-cirrhotic rats, due probably to its antifibrogenic effect [6].

In this work, we studied the antifibrogenic effect of IFN-α† on rats with liver fibrosis and cholestasis induced by BDL. This study was designed to determine if IFN-α could restore normal hepatic content of collagen in rats with established fibrosis. In fact, we found that this compound improved liver histology and function, while collagen content was decreased.

MATERIALS AND METHODS

Materials

Recombinant leukocyte IFN-α (INTRON-A™) was obtained from Schering-Plough, Mexico, and diluted in sterile saline solution. Anthrone, picric acid, thiobarbituric acid, hydroxyproline, chloramine-T, ethylene glycol, bovine serum albumin, *p*-dimethylaminobenzaldehyde, and activated charcoal were obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A. Citric acid, sodium acetate, sodium hydroxide, glacial acetic acid, hydrochloric acid, sodium thiosulfate, sodium chloride, toluene, sulfuric acid, iodine, ethanol, xylene, potassium hydroxide, formaldehyde, and trichloroacetic acid were obtained from J. T. Baker, Phillipsburg, NJ, U.S.A.; aniline blue, orange G, and phosphotungstic acid were obtained from Merck, México.

Animal treatments and biliary obstruction

Male Wistar rats weighing around 200 g were used. They had free access to food (Standard Purina chow diet) and water. Obstructive cholestasis was induced by double ligation and sectioning of the common bile duct. Control rats were sham-operated. Randomization was performed 15 days after biliary obstruction (when fibrosis is already established [7]). Six rats (randomly chosen) were killed for biochemical and histological determinations; the remaining rats were divided randomly into two groups, one group receiving IFN-α (100 000 IU/rat/day; s.c.), while the other received vehicle only. The animals were killed under light ether anesthesia after 0, 2, or 4 weeks of biliary obstruction. Blood was collected by heart puncture, and the liver was removed rapidly.

Small liver sections fixed in Bouin were used for Mallory trichromic staining. Briefly, Bouin consisted of picric acid-saturated aqueous solution (750 mL), formaldehyde (250 mL), and glacial acetic acid (50 mL). Liver blocks were fixed for 4 hr and then stored in 70% alcohol. Liver tissues were embedded in Paraplast Plus. Paraffin sections were cut at 6 μm. The staining procedure consisted in deparaffinizing sections with xylene and alcohol, followed by transfer to aniline blue-orange G solution. Then the sections

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† Abbreviations: IFN-α, interferon-α_{2b}; BDL, bile duct ligation; BDL-rats, bile duct-ligated rats; γ-GTP, γ-glutamyl transpeptidase; MDA, malondialdehyde; and TBA, 2-thiobarbituric acid.

were washed with 95% ethanol and xylene and mounted in Permount or balsam.

Collagen quantification

Collagen concentrations were determined by measuring hydroxyproline content in fresh liver samples, after digestion with acid [8]. The procedure was as follows:

Materials. Sodium acetate/citric acid buffer, pH 6.0: one liter of the buffer contained 50 g of citric acid, 120 g of sodium acetate (trihydrate), 34 g of sodium hydroxide and 15 mL of glacial acetic acid. The buffer was kept cold under a layer of toluene to avoid bacterial growth.

Chloramine-T solution: 0.141 g of chloramine-T was mixed with 2 mL of distilled water, 3.0 mL of peroxide-free methyl cellosolve and 5 mL of the sodium acetate/citric acid buffer. This solution was prepared fresh prior to use.

Sodium thiosulfate: The solution was 2.0 M in distilled water and is stable for several weeks at room temperature.

Ehrlich's reagent: 0.5 g of *p*-dimethylaminobenzaldehyde was dissolved in 9.0 mL of absolute ethanol and 1.0 mL of 12 N HCl.

Method. Fresh liver samples (100 mg) were placed in ampules, 2 mL of 6 N HCl was added, and then the samples were sealed and hydrolyzed at 100° for 48 hr. Next the samples were evaporated at 50° for 24 hr and resuspended in 3 mL of sodium acetate/citric acid buffer, pH 6.0; 0.5 g of activated charcoal was added, the mixture was stirred vigorously, and then it was centrifuged at 5000 *g* for 10 min.

The mixture was kept for 20 min at room temperature, and the reaction was stopped by the addition of 2 M sodium thiosulfate and 1 N sodium hydroxide. The aqueous layer was transferred to test tubes. The oxidation product from hydroxyproline was converted to pyrrole by boiling the samples. The pyrrole-containing samples were incubated with Ehrlich's reagent for 30 min, and the absorbance was read at 560 nm. Recovery of known amounts of standards was carried out on similar liver samples to provide calibration samples. Liver collagen content was calculated, considering that about 50% of the weight of collagen is hydroxyproline.

Serum enzyme activities and bilirubins

Serum was obtained for the following determinations: the activities of alkaline phosphatase [9] and γ-GTP [10], and for bilirubin content (Kit Merck-México).

Determination of hepatic lipid peroxidation and glycogen content

Liver pieces were separated for glycogen quantification with anthrone reagent [11]. MDA was determined in liver homogenates using the TBA method according to Ohkawa *et al.* [12]. Protein determinations were performed according to the method described by Bradford [13].

For statistical analysis, ANOVA with the Tukey [14] test was used to compare the groups. In all cases, a difference was considered significant when $P < 0.05$.

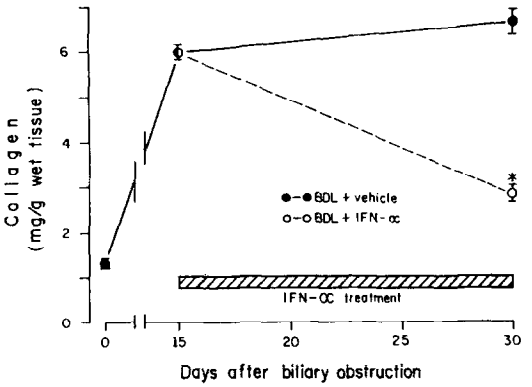

Fig. 1. Time–course of liver collagen content after bile duct ligation (BDL). Interferon-α_{2b} (IFN-α) was administered to 15 day-bile duct-ligated rats for a further 15 days. Results are the means ± SEM of 6 rats. Key: (*) significantly different from 15-day BDL-rats ($P < 0.05$).

RESULTS

Liver collagen content increased about 5-fold after 15 days of bile duct ligation. Administration of IFN-α to 15-day BDL-rats decreased collagen content nearly to control values. It is important to point out that IFN-α was administered after 15 days of biliary obstruction and for a further 15 days. Thirty days of BDL led to a 6-fold increment in liver collagen content (Fig. 1).

Figure 2 shows the degree of liver lipid peroxidation and glycogen content. Lipid peroxidation increased more than 2-fold in the BDL group during this period, while glycogen content decreased markedly after biliary obstruction. Treatment with IFN-α decreased lipid peroxidation and restored liver glycogen content to control values.

Serum markers of liver damage are shown in Table 1. Total and unconjugated bilirubins increased dramatically in the BDL group. Serum enzyme activities of alkaline phosphatase and γ-GTP also increased significantly. IFN-α treatment reversed all serum markers of liver damage that were studied to normal values.

Histological analysis of liver sections revealed that prolonged biliary obstruction is accompanied by an increase in collagen deposition around the portal triad. In the BDL group, the normal architecture was lost, and large zones of necrosis were observed frequently. IFN-α administration to BDL rats restored the normal architecture of the liver, although slight collagen accumulation and necrosis were also observed (Fig. 3).

Sham-operation and/or IFN-α administration produced no biochemical or histological alterations (data not shown).

DISCUSSION

Our results demonstrated that IFN-α can reverse fibrosis and liver damage (cholestasis) induced by biliary occlusion in the rat.

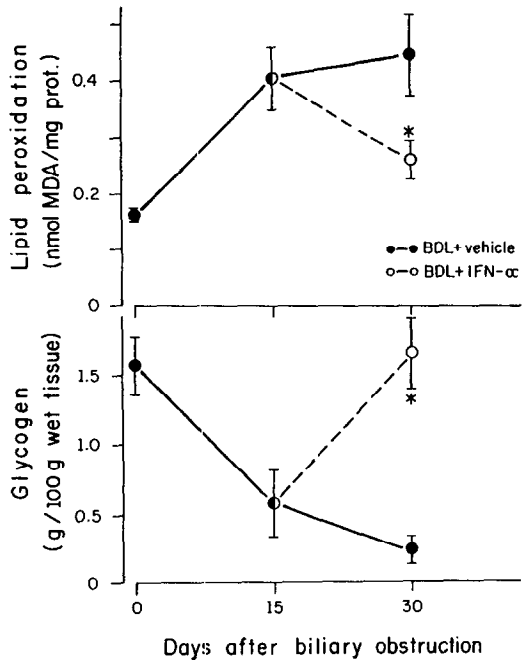

Fig. 2. Time-courses of liver lipid peroxidation (upper panel) and glycogen content (lower panel) after bile duct ligation (BDL). Interferon-α_{2b} (IFN-α) was administered to 15-day bile duct-ligated rats for a further 15 days. Results are the means ± SEM of 6 rats. Key: (*) significantly different from 15-day BDL-rats ($P < 0.05$).

In this work, IFN-α reversed collagen accumulation that occurred during days 0–15. Thus, it seems likely that IFN-α also stimulates collagen catabolism, as suggested by Duncan and Berman [15], who found that IFN-α markedly increases collagenase production. However, with the present data we cannot discriminate between an antifibrogenic and a fibrolytic effect of IFN-α. In addition, a synergistic antifibrotic action due to both effects cannot be ruled out.

Czaja *et al.* [5] reported that 100 000 IU/rat/day of IFN-α produces a better antifibrogenic effect than 50 000 IU. Thus, in the present work we decided to use 100 000 IU/rat/day.

An important consequence of liver fibrosis is the deposition of connective tissue around the hepatic sinusoids so that vascular diffusion barriers are disrupted and sinusoidal blood flow tracts are narrowed. The resultant portal hypertension and impaired clearance of endogenous and exogenous metabolites contribute to hepatic dysfunction [16]. Portal hypertension also leads to pooling of blood in the splanchnic circulation, decreasing effective arterial blood volume and activating volume receptors so that sodium and water are avidly retained, and eventually ascites ensues [17]. Hepatic fibrogenesis ultimately results in cirrhosis, a condition marked by diffuse lobular fibrosis and conversion of normal liver architecture into structurally abnormal nodules.

Since collagen is the major component of the extracellular matrix deposited in hepatic fibrosis, most anti-fibrotic therapies have been directed toward the control of collagen metabolism. A number of drugs can specifically modulate collagen biosynthesis at the transcriptional level or at various post-translational stages. These anti-fibrotic drugs include corticosteroids, azathioprine, penicillamine, colchicine, zinc, prostaglandins, cyclosporin, and interferons [18].

The mechanism of action of interferons on liver damage is far from clear, and their antiviral properties are inconsequential in BDL and in CCl_4-cirrhotic rats [6]. However, interferons may act by reducing hepatic collagen content, since fibrosis is a very important factor in chronic liver damage. In fact, interferons have been shown to inhibit fibroblast and chondrocyte collagen production *in vitro* and decrease mRNA steady-state levels of types I, II and III procollagen in these cells [3, 19–21]. *In vivo* studies have demonstrated that interferons can decrease the fibrotic reaction around implanted osmotic pumps in mice [4], as well as reduce the collagen accumulation in the lungs of bleomycin-

Table 1. Serum bilirubin levels and activities of alkaline phosphatase and γ-glutamyl transpeptidase (γ-GTP)*

Treatment	Bilirubins (μmol·L^{-1})		Serum enzyme activities (μmol·L^{-1}·min^{-1})	
	Total	Unconjugated	Alkaline phosphatase	γ-GTP
Sham	12.5 ± 3.2	2.1 ± 0.5	60.9 ± 8.2	18.2 ± 3.4
BDL (15 days)	54.0 ± 26.2†	39.2 ± 20.2†	158.4 ± 26.4†	45.3 ± 9.9†
BDL (30 days)	225 ± 20.1†	42.1 ± 4.3†	154.4 ± 18.2†	51.2 ± 11.9†
BDL + IFN-α	9.2 ± 3.9‡	5.6 ± 4.0‡	83.4 ± 1.5‡	24.1 ± 5.2‡

* Results are expressed as the means ± SEM of experiments performed in duplicate assays with samples from 6 animals.

Treatments: Sham: sham-operated rats; BDL: bile duct-ligated rats; and BDL + IFN-α: 15-day bile duct-ligated rats that were administered interferon-α_{2b} (100 000 IU, s.c.) daily for a further 15 days.

† $P < 0.05$ vs the Sham group.

‡ $P < 0.05$ vs BDL (both 15 and 30 days) groups.

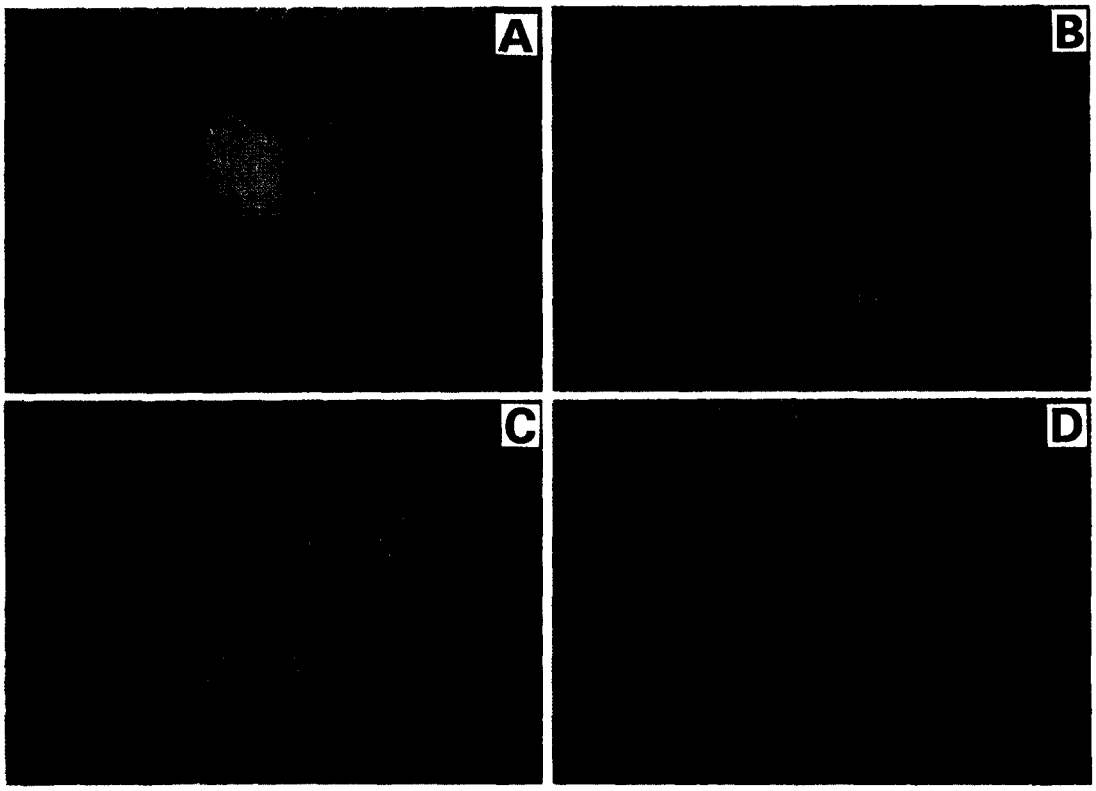

Fig. 3. Liver sections from a sham-operated rat (A); bile duct-ligated rats for 15 (B) and 30 days (C); and bile duct-ligated plus interferon-α_{2b} (D). Collagen is stained blue. Trichromic stain, 100 magnification.

treated rats [22]. Furthermore, interferon treatment inhibits collagen deposition in murine schistosomiasis [5]. However, further investigations are required to evaluate the therapeutic relevance of interferons on non-viral hepatic fibrosis and cirrhosis.

Omata *et al.* [23] have shown an important improvement of liver histology in patients with non-A, non-B hepatitis after treatment with IFN-α. However, they attributed that effect to an acute reduction of viral titers. In contrast, no histological improvement was observed in patients with B-viral chronic hepatitis by short-term (4 weeks) treatment with large doses of recombinant leukocyte IFN-α [23]. In humans, fibrosis (of any etiology) requires years to be established. Thus, it seems likely that any antifibrotic treatment will require the same time. Long-term treatments with interferon are required to evaluate its antifibrogenic effects in both viral and non-viral chronic liver diseases. In fact, Hoofnagle *et al.* [24] demonstrated, in a preliminary report, that after 1 year of IFN-α treatment of chronic non-A, non-B hepatitis, biopsy specimens showed marked improvement in hepatic histology, mainly on portal inflammation and hepatocytic necrosis; unfortunately, fibrosis was not measured.

In several clinical studies [24–27], the histopathological score for liver fibrosis changed little during treatment with interferon, although the scores for portal inflammation, piecemeal necrosis, and lobular injury decreased. Perhaps the degradation and resorption of fibrous tissue require more than 12 months, as it does in patients with cirrhosis treated with colchicine [28].

Recent investigations [29] have demonstrated the ability of several cytokines to modulate matrix protein synthesis. It has been suggested [25] that the effect of interferon on type II procollagen mRNA may be a consequence of its action on TGFβ1 expression, in addition to its antiviral properties. In BDL-rats, the antiviral effect of IFN-α is not important. Thus, in this case its effects on TGFβ1 seem likely because TGFβ1 may have an important role in fibrogenesis [25]. Manabe *et al.* [30] treated patients having non-A, non-B hepatitis with IFN-α for 24 weeks. They found that the fibrosis score was not altered significantly. In contrast, liver collagen measured colorimetrically and morphometrically was decreased significantly in patients treated with 3 and 10×10^6 IU IFN-α compared with the increase observed in placebo-treated controls.

Activation of hepatic lymphocytes is a fundamental feature of liver injury. *In vivo*, activation is characterized by the transition from a resting vitamin A-storing cell to a myofibroblast-like cell that is highly proliferative and fibrogenic [31–35]. Recently, it has been demonstrated that interferon-γ is a potent inhibitor of lymphocyte activation [35]. These properties make interferons attractive agents in the treatment of fibrotic liver diseases and other organ injury in which mesenchymal cellular proliferation and fibrogenesis are prominent.

Capra *et al.* [36] have demonstrated that IFN-α

administration for 6 months to patients with chronic viral hepatitis or active cirrhosis results in a decreased stimulus for fibrogenesis by reducing liver inflammation and necrosis, thus preventing evolution to cirrhosis.

MDA is a product of lipid peroxidation, so that an increase in liver MDA levels indicates an increase in lipid peroxidation, a very well-known mechanism of liver damage [37]. Our results show increases in MDA levels after BDL, as reported by others [38]. However, we have demonstrated that, particularly in the model of liver damage induced by BDL, lipid peroxidation does not play an important role [39]. Thus, the effect of IFN-α on MDA levels is interpreted as a consequence of its antifibrogenic and hepatoprotective actions.

Glycogen is the main source of liver energy; without it, the liver cannot carry out energy-coupled hepatic functions. Thus, restoration of glycogen content mediated by IFN-α demonstrates the beneficial effect of this compound.

Alkaline phosphatase is an ectoenzyme of the hepatocyte plasma membrane: an increase in serum alkaline phosphatase activity has been related to damage to the liver cell membrane [40]. γ-Glutamyl transpeptidase is an enzyme embedded in the hepatocyte plasma membrane, mainly in the canalicular domain; again, the liberation of this enzyme to serum indicates damage to the cell and thus injury to the liver (particularly cholestasis). It is important to point out that serum γ-GTP activity is considered to be one of the best indicators of liver damage [41, 42]. The beneficial effect of IFN-α on alkaline phosphatase and γ-GTP, as well as on the levels of bilirubins, indicates that IFN-α is a promising drug in the treatment of chronic liver diseases.

Acknowledgements—The authors want to express their gratitude to Ms. Concepción Avalos for secretarial assistance and Mr. Alfredo Padilla for preparing the figures. The authors acknowledge Patricia González, B.Sc., for excellent technical assistance. The authors also acknowledge Schering-Plough Laboratories for the gift of interferon-α_{2b} (INTRON-A™). This work was supported, in part, by grant 4265-N9406 from CONACYT, México.

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