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Effect of free iron on collagen synthesis, cell proliferation and MMP-2 expression in rat hepatic stellate cells

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Abstract

Various studies on hepatic fibrosis occurring in iron overload suggest that excess of tissue iron may be involved in the stimulation of collagen synthesis. Anyway, up to date, direct evidence on the role of iron in hepatic fibrosis is lacking. Moreover, it is not clear whether iron acts as direct initiator of fibrogenesis or as mediator of hepatocellular necrosis. In the present study, we investigated the effect of nontoxic doses of iron on collagen metabolism and proliferation, key features of liver fibrosis, by means of cultures of hepatic stellate cells, the liver cells responsible for collagen production. Iron treatment increased collagen synthesis without affecting noncollagen proteins. The maximum effect was observed at 5 μM iron (+132%). At this dose, no cell damage or proliferation was detected. Conversely, higher doses of iron (10 and 25 μM) induced cell proliferation and a lower increase in collagen synthesis, suggesting the prevalence of proliferative effect on the synthetic one. These effects occurred without the intervention of serum factors and were not mediated by lipid peroxidation. Our results strongly support the hypothesis that iron "*per sé*" may act as a profibrogenic agent. Finally, we provide evidence that iron plays a role also in matrix degradation, by stimulating some metalloprotease activities. Iron treatment increased metalloprotease-2 activity in hepatic stellate cells, while no changes were observed for interstitial collagenase activity suggesting that, in these conditions, a pathological accumulation of hepatic extracellular matrix may occur.

Keywords: Collagen metabolism; Hepatic stellate cells; Iron chelates; Iron overload; Liver fibrosis; Metalloproteases

1. Introduction

The pathogenesis of hepatic fibrosis in patients with iron-storage diseases (e.g. genetic hemochromatosis; GH) is not well understood. Iron overload in humans leads to liver injury, fibrosis, and eventually cirrhosis [1]. It is conceivable that excess iron might result in parenchymal cell injury and necrosis. However, many patients with GH demonstrate extensive fibrosis and signs of portal hypertension without any or minimal hepatic biochemical abnormalities to suggest previous cell necrosis [2,3]. These observations emphasize the possible role of iron as a profibrogenic agent itself.

In last years, investigators have tried to give experimental support to these clinical observations and understand the cellular pathways mediating the fibrogenic effect of iron [4–6]. A major difficulty in approaching this issue experimentally has been the lack of an animal model producing the full pathological picture of the cirrhotic liver seen in GH. In fact, in these models, despite a significant increase in tissue iron, only a mild fibrosis occurs.

In an attempt to elucidate the role of iron in the onset of liver fibrosis, we studied *in vitro* the effect of iron on collagen metabolism by means of hepatic stellate cells (HSC), the liver cell type mainly responsible for enhanced collagen production during chronic iron overload.

HSC (also designated as fat-storing cells, lipocytes, or Ito cells) are identified by the presence of intracytoplasmatic lipid vacuoles containing Vitamin A [7]. During the onset of fibrosis, HSC proliferate and transform to "activated" cells, and express the activation marker α-smooth muscle actin (α-SMA) [8].

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Abbreviations: DFO, deferoxamine; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; FBS, fetal bovine serum; GH, genetic hemochromatosis; HSC, hepatic stellate cell; LDH, lactate dehydrogenase; MDA, malondialdehyde; MMP, metalloprotease; α-SMA, α-smooth muscle actin; TCA, trichloroacetic acid.

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In previous studies, we demonstrated that crocidolite (a kind of asbestos with high iron content) increases collagen production in lung fibroblast cultures [9]. This effect is specific for collagen and is highly enhanced when iron is mobilized from crocidolite by citrate. The absence of cell damage, proliferation or lipid peroxidation leads to the supposition that the iron "*per sé*" acts as a profibrogenic stimulus. We hypothesized that a similar finding may occur also in HSC.

Alterations in extracellular matrix (ECM) may result not only from changes in deposition but also in degradation. Matrix degradation is catalyzed by the activity of matrix metalloproteinases (MMP), which consist of collagenases, gelatinases, stromelysins, and membrane type MMP [10]. Many studies demonstrate that in the fibrotic liver there is an increased expression of interstitial collagenase and MMP-2 [11,12]. Interstitial collagenase digests native fibrillar collagens I, II, and III. Gelatinase A (MMP-2) digests denatured collagens I and III and native collagen IV.

Currently available information regarding synthesis and release of MMP in the liver is scant and in part contradictory. HSC isolated from rat and human liver release MMP-2 activity into the culture media [13,14]. Both an increase and a decrease of collagenase activity have been variably reported in experimental models of liver fibrosis in rodents [15,16].

No data concerning the effect of iron on MMP activity in HSC cultures are available.

Therefore, we investigated whether iron is able to modulate MMP-2 and interstitial collagenase activities in HSC cultures.

2. Materials and methods

2.1. Materials

Collagenase A and DNAse were purchased from Roche s.p.a., pronase E from Bracco s.p.a. and deferoxamine (DFO) from Ciba Geigy. All other chemicals were of reagent grade and purchased from Sigma.

2.2. Cell isolation and culture

HSC were isolated from Sprague–Dawley rats (Nossan) as previously described [17]. HSC were prepared by means of sequential pronase/collagenase digestion and purified by density-gradient centrifugation, using Nycodenz 18% (Nycomed). HSC harvested from the top of gradient were seeded (10^6 cells/mL) in 25 cm^2 flasks in Dulbecco's modified eagle's medium (DMEM) plus 20% fetal bovine serum (FBS) and antibiotics. The viability and purity of preparation was tested by trypan blue esclusion and autofluorescence of Vitamin A, respectively. Cells were incubated in 95% air–5% CO_2 at 37° and allowed to adhere. Five days after plating, HSC started to proliferate and medium was replaced with DMEM plus 10% FBS. Once cultures reached confluency, they were trypsinized and subcultured.

2.3. Determination of α-SMA expression

Activation was determined in cells cultured for 1, 3, 5, and 7 days in chamber slides. Cells were fixed in cold acetone at −20° for 5 min and α-SMA expression was detected by means of α-SMA Immunohistology Kit (IMMH-2, Sigma).

2.4. Culture treatment

Cells were seeded at a density of 2.5×10^4 mL^{-1} of DMEM supplemented with 10% FBS in 12- or 24-well culture plates and allowed to grow to confluence. Twenty-four hours before treatment, medium was changed with serum-free DMEM. HSC were treated with a solution of $FeCl_3$:citrate (molar ratio of 1:5) for 48 hr in presence or absence of iron chelator DFO (iron:DFO, molar ratio of 1:1). When iron chelator was present in control cultures, the cells received 0.25 μM DFO (corresponding to iron concentration contained in DMEM). These solutions were freshly prepared and immediately used for cell treatment.

2.5. Cytotoxicity assays

Preliminary experiments were carried out to assess the range of iron concentrations nontoxic for HSC, by incubating the cells with iron for 48 hr. Iron toxicity was assessed by measuring the release of lactate dehydrogenase (LDH) activity in culture media using the LDH-P kit (Cat. No. 81282, Sclavo Diagnostics). Cell viability was assessed by trypan blue exclusion test.

2.6. DNA estimation and cellular proliferation assay

DNA content was evaluated in HSC treated with iron for 48 hr, according to Taylor *et al*. [18]. DNA synthesis was evaluated in 24-well plates by measuring [^{3}H]thymidine incorporation in HSC, according to Boscoboinik *et al*. [19]. Subconfluent cells were incubated with iron-citrate (5–50 μM) for 48 hr in serum-free DMEM. Twenty-four hours later, [^{3}H]thymidine (20 μCi/well) was added. After additional 24 hr exposure, cell layers were washed, fixed for 20 min with ice-cold 5% trichloroacetic acid (TCA), and solubilized in 0.4 mL of 0.1 M NaOH/2% Na_2CO_3. Samples were mixed with 8 mL of Ultima Gold (Packard) and counted for radioactivity in a Packard 2100 TriCarb liquid scintillation analyzer.

2.7. Collagen synthesis assay

To evaluate the effect of iron on collagen metabolism, HSC were seeded on 12-well plates and grown to visual confluency. Medium was changed to serum-free DMEM for 24 hr to allow the cells to become relatively quiescent.

In addition to DMEM, 11.5 μg/mL L-proline, 50 μg/mL ascorbic acid, 50 μg/mL β-aminopropionitrile were added. After incubation, cells were treated with a solution of iron-citrate (5, 10, and 25 μM) with or without DFO for 48 hr.

Collagen synthesis was assessed as previously described [20]. Briefly, 16 hr before the end of experiment, 10 μCi/mL of [^{3}H]proline (Amersham International; specific activity 23 Ci/mmol) were added to each well. Media were harvested for determination of [^{3}H]proline incorporation into collagen and noncollagen proteins following the collagenase digestion method, using highly purified bacterial collagenase (Boehringer Mannheim Cod. 602426). Incorporation of radioactivity into collagen and noncollagen proteins was determined following precipitation with TCA. Collagen-incorporated radioactivity was recovered in the TCA-soluble fraction while noncollagen radioactivity from the TCA precipitate. Data were expressed as tritiated proline incorporation (dpm) per microgram DNA. Percentage collagen synthesis was estimated using the formula of Diegelmann and Peterkofsky [21]:

$$\frac{(\text{dpm in the supernate}) \times 100\%}{(\text{dpm in the pellet} \times 5.4) + (\text{dpm in the supernate})}$$

(the factor 5.4 corrects for the smaller amount of proline in noncollagen proteins).

2.8. Malondialdehyde (MDA) evaluation

Lipid peroxidation was evaluated as malondialdehyde (MDA) production [22] at 10, 30, 60, and 120 min after iron treatment. Samples were precipitated with 5% TCA and centrifuged at 600 *g* for 10 min. Supernatants were treated 1:1 with thiobarbituric acid and boiled for 10 min. Data were expressed as moles of MDA per milligram of protein.

2.9. Metalloprotease zymography

MMP activity was evaluated in the media of HSC cultures treated with iron-citrate by means of zymography as previously described by Kleiner and Stetler-Stevenson [23]. Media were electrophoresed in 10% SDS-polyacrylamide gels copolymerized with 1 mg/mL gelatin or casein. For gelatinase assay, 8 μL of medium were applied to SDS-PAGE gels. Caseinolytic activity was detected in media concentrated about 25 times. The gels were washed 30 min with 2.5% Triton X-100 and subsequently incubated overnight at 37° in 50 mM Tris–HCl, pH 7.4, containing 10 mM $CaCl_2$. Gels were stained with Coomassie Brilliant Blue R-250. The bands of lysis were analyzed by densitometry scanning of the gels. All samples were normalized on the basis of cell number of each well.

2.10. Statistical analysis

Data are the mean ± SD from four replicate cultures. Statistical evaluation was performed using the Student's *t* test for single group comparison and one-way ANOVA (*F* test) for multiple group comparison. $P < 0.05$ was considered significant.

3. Results

3.1. Determination of α-SMA expression

HSC were used for the following experiments on 7° day of culture, when spontaneously became activated and expressed α-SMA. Immediately following isolation (not shown) and after 1 day in culture (Fig. 1A), HSC did not express α-SMA. After 3–5 days in culture, α-SMA could be detected in conjunction with features of activation (Fig. 1B and C), including cell spreading and nuclear enlargement. With time in culture thereafter, a progressively larger proportion of cells expressed α-SMA. At 7 days, all cells displayed this protein and labeling was fibrillar (Fig. 1D).

3.2. Cytotoxicity associated with iron-citrate treatment

The range of concentration nontoxic for the cells was assessed as cell viability and LDH release. Trypan blue evaluation revealed no significant difference between controls and 5–25 μM iron-treated cells (Table 1). At 50 μM iron, a decrease in cell viability was observed (87% viable cells compared to control).

Table 1 also shows the effects of increasing concentrations of iron-citrate on LDH release by HSC. Controls showed a basal release of about 10% of total LDH activity. At doses between 5 and 25 μM, there were nonsignificant effects, but at 50 μM a slight but significant increase in enzyme release was found (about 38%), suggesting cell

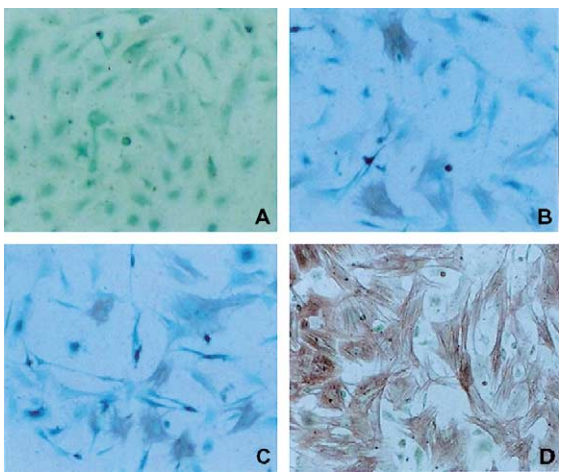

Fig. 1. Expression of α-SMA in HSC cultures. Cells were isolated and processed as described in Section 2. Activation was evaluated by means of an Immunohistology Kit for the α-SMA after 1, 3, 5, and 7 days in culture. (A) After 1 day, cells appear quiescent and negative for the marker; (B) at 3 days in culture, occasional HSC are positive; (C) after 5 days most of the cells display α-SMA; (D) by 7 days in culture, all cells are activated. α-SMA labeling appears fibrillar (original magnification 120×).

Table 1
Toxicity of iron-citrate in rat HSC

Iron treatment	Viability (%)	LDH (% release)
Control	98.7 ± 0.5	10.7 ± 2.5
5 μM	98.1 ± 0.8	11.5 ± 2.8
10 μM	97.9 ± 1.2	11.1 ± 3.0
25 μM	97.1 ± 1.3	12.3 ± 2.6
50 μM	85.5 ± 0.4*	19.6 ± 3.0*

Cells were treated for 48 hr with iron-citrate. Cell viability was assessed by trypan blue exclusion test. Percentage of total LDH released was determined as described in Section 2. Data are means ± SD from four experiments.
* $P < 0.01$ vs. control values.

death. On the basis of these findings, the range of treatment was limited to doses between 5 and 25 μM iron.

3.3. Effect of iron-citrate on HSC proliferation

The eventual proliferative effect of iron on HSC was evaluated as DNA content and [^{3}H]thymidine incorporation. A significant increase in DNA content could be observed after iron treatment 10 and 25 μM (Fig. 2). On the contrary, 50 μM iron determined a significant decrease in DNA compared to controls. At this dose, the toxic effect is prevalent on proliferation.

Fig. 3 shows the effect of iron-citrate on HSC proliferation at doses between 5 and 25 μM, assessed as [^{3}H]thymidine incorporation. These results are comparable with those reported for DNA. There was nonsignificant difference in replication between controls and cells exposed to 5 μM iron. At 10 and 25 μM, thymidine incorporation increased 41 and 44%, respectively.

3.4. Effect of iron-citrate on collagen synthesis

The effect of various doses of iron-citrate (5–25 μM) on collagen synthesis is reported in Fig. 4. A marked increase in collagen synthesis was obtained after treatment with 5 μM iron (+132%). A lower, but significant, increase was observed at the dose 10 μM, while at 25 μM there was no difference compared to controls. In any case, when DFO was present, collagen synthesis went down to control

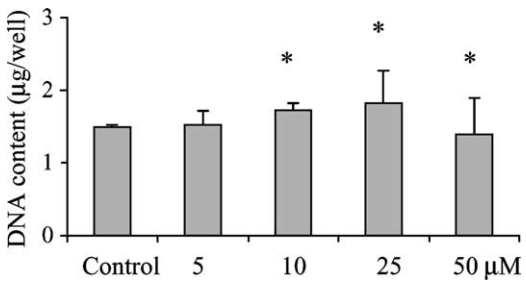

Fig. 2. Effect of iron-citrate on DNA content in HSC cultures. Cells were treated for 48 hr with iron-citrate in 12-well plates. Values are expressed as microgram per well. Data are means ± SD from four replicate cultures. (*) $P < 0.05$ vs. control.

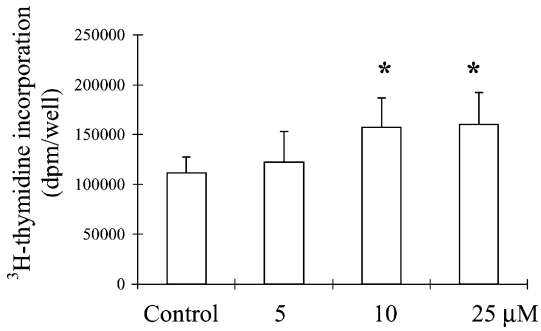

Fig. 3. Effect of iron-citrate on proliferation in HSC. Cells were treated for 48 hr with iron-citrate. Tritiated thymidine was added to the incubation medium 24 hr before the end of the treatment. Results are expressed as [^{3}H]thymidine incorporation (dpm) per well. Data are means ± SD from four replicate cultures. (*) $P < 0.05$ vs. control.

values. These results, expressed per microgram of DNA, indicated an increased production per cell.

When collagen synthesis was expressed as percentage of total protein production (Fig. 5), a significant increase (+34%) was detected only at the dose 5 μM, while slight but no significant difference was at 10 (+16%) and 25 (+14%) μM.

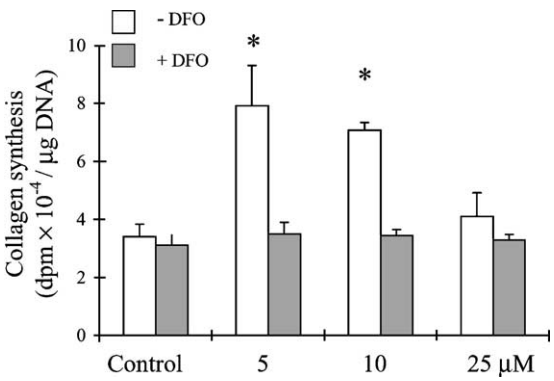

Fig. 4. Effect of iron-citrate on collagen synthesis in HSC cultures. Cells were treated for 48 hr with iron-citrate in presence or absence of the chelator DFO. Data are expressed as [^{3}H]proline incorporation (dpm) per microgram of DNA. Each value represents mean ± SD from four experiments. (*) $P < 0.05$ vs. control.

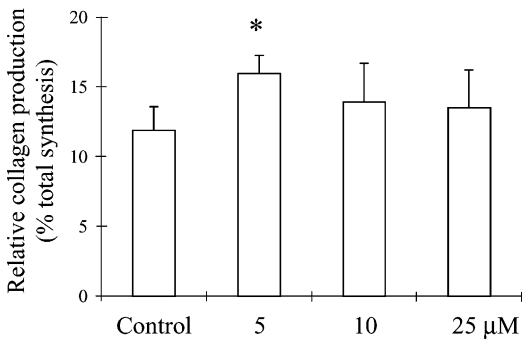

Fig. 5. Percentage collagen synthesis in iron-treated HSC. Relative collagen production was determined by calculating collagen production as a percentage of total protein production (mean ± SD), using the formula of Diegelmann and Peterkofsky [21] as reported in Section 2. (*) $P < 0.05$ vs. control.

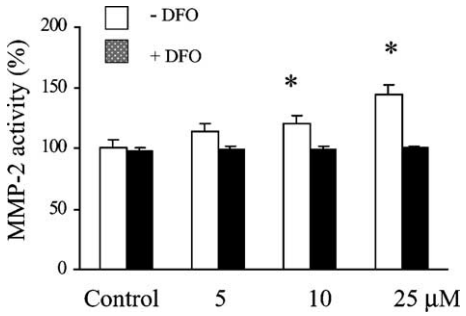

Fig. 6. MMP-2 activity released in the media of iron-treated HSC. Cells were treated for 48 hr with iron-citrate in presence or absence of the chelator DFO. The activity of MMP-2 was evaluated by means of zymography in SDS-polyacrylamide gels containing 1 mg/mL gelatin. The density of lysis bands was calculated by three densitometric scanning of the gels (mean ± SD). Data are reported as percentage assuming the control as 100%. (*) $P < 0.05$ vs. control.

Thus, the optimal dose to appreciate the fibrogenic effect of iron is 5 μM, since in this case neither toxic nor proliferative effects were observed.

3.5. Effect of iron on MDA production

No detectable changes in MDA were found in HSC cultures treated with iron-citrate (not shown). These results suggest that, at least under our experimental conditions, iron-induced collagen increase is not associated with lipid peroxidation.

3.6. MMP detection after iron treatment

The activity of MMP was evaluated in culture media of HSC treated for 48 hr with iron-citrate 5–25 μM. The data were expressed as percentage of caseinolytic (interstitial collagenase) or gelatinolytic (MMP-2) activity in comparison with controls. As shown in Fig. 6, there was no difference between iron-treated cells and controls at 5 μM iron, while a progressive increase in MMP-2 activity was detected at higher doses, in particular at 25 μM (+44%). In the presence of DFO, MMP-2 activity went down to control values. No difference in caseinolytic activity was detected between iron-treated cells and controls (data not shown).

4. Discussion

The association between iron overload and the onset of hepatic fibrosis and cirrhosis is known [3,24,25]. Anyway, the pathogenesis of liver fibrosis in patients with iron-storage diseases (e.g. GH, thalassemic syndromes, transfusional iron overload, alcoholic cirrhosis, porphyria cutanea tarda) is not well understood.

Conceptually, we can consider three possible pathogenic mechanisms underlying the fibrogenic potential of iron: (1) iron as an inducer of fibrogenesis in the absence of necrosis inflammation (“direct initiator” of fibrogenesis); (2) iron as mediator of hepatocellular necrosis, known as sideronecrosis, and local inflammation (“indirect initiator” of fibrogenesis); (3) iron as an inducer of fibrogenesis in conjunction with other hepatotoxins (“co-initiator,” and in many instances, “propagator” of fibrogenesis) [1].

In the present study, we demonstrate that noncytotoxic doses (5–25 μM) of iron can stimulate collagen synthesis in activated HSC, without affecting noncollagen proteins. The greatest increase may be observed with the dose 5 μM (+132%), while a lower response is obtained with higher doses (10–25 μM). This latter effect may be due to the prevalence of proliferative stimulus on the synthetic one. In fact, higher doses of iron induce an enhanced HSC proliferation, an other feature of fibrotic process.

At the dose 5 μM iron, the percentage increase in collagen synthesis is not too high (+34% of total protein production). Anyway, this is in agreement with clinical observations reported in humans. In fact, in the absence of other inciting insults, in hemochromatosis the hepatic disease is “mild” and usually progresses slowly over a long time period to cirrhosis.

Generally, hepatic fibrosis is viewed as a secondary effect following cell injury and death and iron may be an important mediator of hepatocellular necrosis [1]. In animal models of iron toxicity, despite significant increase in liver iron content, necrotic events are limited and fibrotic responses are minimal [24,26].

In our experimental conditions, the collagen increase induced by iron-citrate treatment in the range of concentration 5–25 μM is not associated to cell damage, confirming the findings reported *in vivo* for humans or animal models of iron overload.

Iron may activate peroxidative process and some authors provided information describing a link between lipid peroxidation and fibrogenesis [25,27,28]. However, under our experimental conditions, the effect of iron on collagen synthesis does not appear to be correlated with membrane lipid peroxidation, since the treatment of HSC with iron does not produce detectable changes in MDA.

Our data agree with that observed *in vivo* [29] and *in vitro* [30] by other authors. These reported that, during the fibrogenesis associated to iron overload, HSC are not directly subjected to oxidative stress, but they are likely activated by paracrine signals arising by neighboring cells.

Moreover, this study (carried out under serum-free conditions) provides evidence that iron can enhance collagen synthesis without the intervention of cytokines or other serum factors, although their importance *in vivo* involvement cannot be excluded.

Our results, taken together, strongly support the idea that iron may act as “direct initiator” of liver fibrogenesis, in the absence of inflammatory necrosis and without the mediation of peroxidative process.

The exact mechanism of increased collagen synthesis in response to iron overload is still unknown. Iron is essential

for normal collagen synthesis since it is required as cofactor for prolyl-hydroxylase [31], and increased activity of this enzyme has been reported in various models of iron overload [2,32].

Iron could in some way increase the synthesis of subunits of prolyl-hydroxylase and/or enhance conversion to active tetramer. Alternatively, iron might increase gene transcription or enhance mRNA expression for the formation of the procollagen chains [2]. Further studies are necessary to clarify these questions.

In the second part of our study, we investigated whether iron influences the remodeling by interfering also in liver matrix degradation. Various studies demonstrated that HSC and other nonparenchymal hepatic cells secrete many MMP, as well as factors involved in the activation of their proenzymes and in the specific inhibition of active enzymes [11,12,33].

In this work, we evaluated the effect of iron on the activity of interstitial collagenase and MMP-2, the metalloproteases mainly expressed during hepatic fibrosis. The data obtained demonstrate that iron treatment increases MMP-2 activity, while no change was found in interstitial collagenase activity.

The differential expression of various MMP, which have different substrate spectra, may be an important factor responsible for the preferential accumulation of certain ECM components. Interstitial collagenase shows specificity for native interstitial type I and III collagens produced in the liver [10]. Absent or reduced activity of this collagenase may, therefore, shift the balance between fibrogenesis and fibrolysis, which is delicately maintained in normal tissue, toward accumulation of native interstitial collagens. The presence of MMP-2 activity is not in contradiction to the above interpretation, because this enzyme does not degrade native but only denatured interstitial collagens. However, it is likely that *in vivo* the enhanced expression of MMP-2 may contribute to the alteration of normal liver matrix, thus affecting liver cell function and favoring the progression of the fibrotic process [12].

In conclusion, the data reported in this paper demonstrate that iron may act as "direct initiator" of fibrogenesis stimulating collagen synthesis without the intervention of serum factors, cell damage, proliferation or peroxidative events. In the range of doses tested, iron treatment increases MMP-2 activity, without affecting interstitial collagenase.

Our results, on the whole, lead to suppose that, at least in our experimental conditions, the net effect of iron on matrix remodeling is in favor of collagen accumulation, typical of fibrotic process.

Acknowledgments

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