ORIGINAL

Interferon-α enhances biological defense activities against oxidative stress in cultured rat hepatocytes and hepatic stellate cells

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Abstract : Oxidative stress has been implicated as a cause of hepatic fibrosis, and hepatic stellate cells (HSCs), which are the most important collagen-producing cell types, have been reported to be activated by lipid peroxidation products. Antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) provide a defense system that plays a critical role in protecting the cell from free radical damage, particularly lipid peroxidation. To elucidate the antioxidant activity of interferon-α (IFN-α), the effects of IFN-α on rat hepatocytes undergoing oxidative stress and HSCs in primary culture as well as isolated rat liver mitochondria were examined. IFN-α was observed to dose-dependently increase the immunoreactive protein levels of copper, zinc-and manganese-dependent SOD as well as the enzyme activities of GPx, and decrease the lipid peroxidation product levels and oxidative burst both in stressed hepatocytes and activated HSCs ; GPx activities, however, were not detected in the latter cells. IFN-α also inhibited HSC activation and lipid peroxidation in liver mitochondria. These findings suggest that IFN-α may enhance biological defense activities against oxidative stress and function as a potent fibrosuppressant by protecting hepatocytes and hepatic stellate cells from lipid peroxidation *in vivo*.
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Keywords: *interferon-α, oxidative stress, lipid peroxidation, hepatic fibrosis, hepatic stellate cell, antioxidant enzyme*

INTRODUCTION

Interferon-α (IFN-α) is a cytokine that has multiple biological functions, including antiviral and immunomodulatory activities (1), and is commonly used for the treatment of patients with chronic hepatitis C virus (HCV) infection (2-6). The results of several recent clinical reports suggest that IFN-α treatment is effective in decreasing serum alanine aminotransferase levels (2-6), reducing and eliminating serum HCV

Abbreviations : IFN-α, interferon-α ; HCV, hepatitis C virus ; HSC, hepatic stellate cell ; α-SMA, α-smooth muscle actin ; MDA, malondialdehyde ; CuZn-SOD, copper, zinc-dependent superoxide dismutase ; Mn-SOD, manganese-dependent superoxide dismutase ; GPx, glutathione peroxidase ; WE, Williams medium E ; FBS, fetal bovine serum ; FeNTA, ferric nitrilotriacetate solution ; LDH, lactate dehydrogenase ; DMEM, Dulbecco's modified Eagle's medium ; DIC, differential interference contrast ; DCFH-DA, dichlorofluorescein diacetate ; DCF, 2', 7'-dichlorofluorescein ; ELISA, enzyme-linked immunosorbent assay.

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RNA (4-6), and improving liver histology (2-4) in patients with chronic hepatitis C. These conclusions suggest that the role of IFN-α might be to elicit antifibrogenic effects in the liver (3, 4). Its mode of action (direct or indirect mechanism) on hepatic fibrosis, however, is not entirely clear at present.

Hepatic fibrosis, or the deposition of the extracellular matrix, is often associated with the hepatocellular necrosis and inflammation that accompanies repair processes (7), and is a consequence of liver damage (8, 9). In the injured liver, hepatic stellate cells (HSCs)(10, 11) in the space of Disse, which are regarded as the primary target cells for inflammatory stimuli (12), are transformed into α-smooth muscle actin (α-SMA)-positive myofibroblast-like cells, and are responsible for much of the collagen hypersecretion and nodule formation that occurs during hepatic fibrosis and cirrhosis (10, 11). It should be noted that oxidative stress (13-15), including oxygen-derived free radicals and lipid peroxidation, is also implicated as a cause of hepatic fibrosis. It has recently been demonstrated that HSCs are activated by free radicals induced by Fe^{2+}/ascorbate, as well as by malondialdehyde (MDA) (16), a product of lipid peroxidation, and that HSC activation by type I collagen is blocked by antioxidants (16).

In addition, histopathological studies of chronic HCV infection have shown fatty changes in 31% to 72% of patients (17), indicating that hepatic steatosis is a characteristic feature of chronic HCV infection. It has been suggested that hepatic steatosis may reflect a direct cytopathic effect of HCV and play a role in the progression of the disease. In support of these hypotheses, a transgenic mouse model expressing the HCV core gene, has been observed to develop progressive hepatic steatosis (18). Hepatic steatosis leads to an increase in lipid peroxidation, which, in turn, activates HSCs, increases collagen deposition (16), and, thus, induces hepatic fibrosis.

Many cells have their own enzymatic defense systems against oxidative stress, including superoxide dismutase (SOD) and glutathione peroxidase (GPx) (19, 20), which play a critical role in protecting the cell from free radical damage, particularly lipid peroxidation. Eukaryotic cells possess two main forms of SOD : a predominantly cytosolic copper, zinc-dependent SOD (CuZn-SOD) and a manganese-dependent SOD (Mn-SOD) that is found in the mitochondrial matrix (21). However, little is known about the antioxidative role of IFN-α in the liver. Therefore, the effects of IFN-α on rat hepatocytes undergoing oxidative stress and HSCs in primary culture as well as isolated rat mitochondria were examined in order to assess the antioxidant activity of IFN-α.

MATERIALS AND METHODS

Hepatocyte Isolation and Induction of Oxidative Stress.

Hepatocytes were isolated from the livers of male Wistar rats (500～600g) as described elsewhere (22). Inocula of 5×10^5 cells were introduced into 20-mm diameter plastic dishes. The cells were cultured in 1 ml of Williams medium E (WE) supplemented with 5% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 1% L-glutamine at 37°C in a 5% CO_2 atmosphere and 100% humidity. After 4 h, the cell medium was removed, and lipid peroxidation was induced in the hepatocytes by incubation in serum-free WE with 100 μmol/l ferric nitrilotriacetate solution (FeNTA) in the presence or absence of natural human IFN-α (OIF, Otsuka Pharmaceutical Co., Osaka, Japan ; 0.5, 2, 5 and 20×10^3 U/ml) for 24 h. This system represents a well-established method for the induction of lipid peroxidation both *in vivo* (23) and *in vitro* (24). The eventual damage of cultured hepatocytes in the presence of FeNTA was evaluated by measuring lactate dehydrogenase (LDH) activity in the culture medium using a Hitachi model 7350 Autoanalyzer (Hitachi, Tokyo, Japan).

Isolation and Cultivation of HSCs.

HSCs were isolated from the livers of male Wistar rats (500～600 g) as described previously (22, 25). Cells were plated at a density of 5×10^5 per well in 1 ml Dulbecco's modified Eagle's medium (DMEM) on 20-mm diameter plastic dishes. The culture medium was changed at 48-h intervals with DMEM supplemented with 10% FBS in the presence or absence of IFN-α (OIF ; 0.5, 2, 5 and 20×10^3 U/ml), and the cells were maintained for up to 10 days at 37°C in a 5% CO_2 atmosphere and 100% humidity.

For the immunohistochemical examination of α-SMA, a marker of HSC activation, HSCs that were initially cultured for 2 days were then maintained in DMEM with 10% FBS in the presence or absence of IFN-α for an additional 4 days. Cells were processed for indirect immunohistochemical techniques using monoclonal anti-α-SMA antibody (DAKO,

Glostrup, Denmark; diluted 1:50) as described elsewhere (22, 25). The reaction products were photographed using a differential interference contrast (DIC) microscope (Axioskop, Carl Zeiss, Heidenheim, Germany). For the Western blot analysis of α-SMA, α-SMA in HSCs cultured at a density of 10^6 per well was detected immunologically, as described previously (22, 25). Immunoreactive bands were visualized with an ECL Western blotting detection system (chemiluminescence) kit (Amersham, Arlington Heights, IL) according to the manufacturer's recommended protocol, and evaluated by densitometric analysis.

Fluorescence Measurement of Oxidative Burst.

Oxidative stress in hepatocytes and HSCs was examined using a CellProbe™ fluorescence assay kit (DCFH-Oxidative Burst; Coulter, Miami, FL). The conversion of nonfluorescent dichlorofluorescein diacetate (DCFH-DA) to the highly fluorescent compound, 2', 7'-dichlorofluorescein (DCF) can be used to monitor the oxidative burst (26). The fluorescence of DCF is a measure of the production of H_2O_2 and the presence of peroxidase. CellProbe™ DCFH-Oxidative Burst contains DCFH-DA as a substrate. To this end, cells were incubated in Lab-Tek Chamber 8-well slides (Nunc, Naperville, IL) with nonfluorescent DCFH-DA for 10 min, which was subsequently converted to the fluorescent DCF by subjecting it to oxidative stress. After washing, the cells were fixed with 4% paraformaldehyde for 2 h at room temperature, and the DCF fluorescence was observed by laser-scanning confocal fluorescence microscopy (TCS-4D, Leica, Heidelberg, Germany). A multiple-line argon-ion laser beam was used for single fluorescein emission after excitation at 488 nm with a filter for fluorescein.

Antioxidant Enzyme Assays.

Hepatocytes or HSCs cultured at a density of 5×10^5 per well were washed twice with ice-cold phosphate-buffered saline, lysed directly in 150 μl of 50 mmol/l Tris-HCl (pH 7.5), 5 mmol/l ethylenediaminetetraacetic acid, and 1 mmol/l dithiothreitol, and disrupted by freeze-thawing and sonication (Sonic Disseminator 50; Fisher Scientific, Pittsburgh, PA) for 15 s at 50% energy output, at 4°C. The suspension was then centrifuged at 14,000 g at 4°C for 30 min. The samples of the supernatants were analyzed for antioxidant enzyme assays. Protein levels of CuZn-SOD and Mn-SOD were detected using enzyme-linked immunosorbent assay (ELISA) system kits (Amersham, Little Chalfont, UK)(27, 28), and were expressed as nanograms of immunoreactive protein levels per milligram of protein. GPx activity was determined using a Cellular Glutathione Peroxidase Assay kit (Calbiochem, San Diego, CA) with the spectrophotometric method of Lawrence and Burk (29), and were expressed as units per milligram of protein. Enzyme assays were performed according to each manufacturer's protocol. Protein concentrations were determined by the Lowry method (30) using bovine serum albumin as a standard.

Lipid Peroxidation.

For the determination of MDA levels in hepatocytes and HSCs, the cells were washed with phosphate-buffered saline followed by harvesting with a rubber policeman, and were used in the analysis involving the thiobarbiturate method as described previously (22).

Preparation of Liver Mitochondria.

Liver mitochondria were prepared from 250-300 g male Wistar rats (31). Lipid peroxidation in isolated rat liver mitochondria (0.7 mg/ml) after freezing overnight and thawing the next day was measured by monitoring oxygen consumption at 25°C with a Clark-type oxygen electrode on the assumption that the saturated oxygen concentration at 25°C is 258 μmol/l, in the presence of 100 mmol/l of iron (II) sulfate heptahydrate ($FeSO_4$) and 1 mmol/l adenine diphosphate with or without IFN-α (0.5, 2, 5, 10, and 20×10^3 U/ml) in a total volume of 2.53 ml of 10 mmol/l Tris-HCl (pH 7.5) and 175 mmol/l KCl. The inhibition rate (%) of IFN-α against lipid peroxidation was calculated based on the amount of consumed oxygen as described by Pan and Hori (32).

Statistical Analysis.

Data are presented as means ± SD unless otherwise indicated. The means were compared between two groups using Wilcoxon's signed-rank test and the Mann-Whitney U test. All p values are two-tailed. A p value of less than 0.05 was considered to be statistically significant.

RESULTS

Effects of IFN-α on Lipid Peroxidation and Antioxidant Enzyme Levels in Cultured Rat Hepatocytes Undergoing Oxidative Stress.

Increased levels of extracellular LDH (489 ± 81 U/ml) and intracellular MDA (3.12 ± 0.56 nmol/well) were observed in hepatocytes incubated with

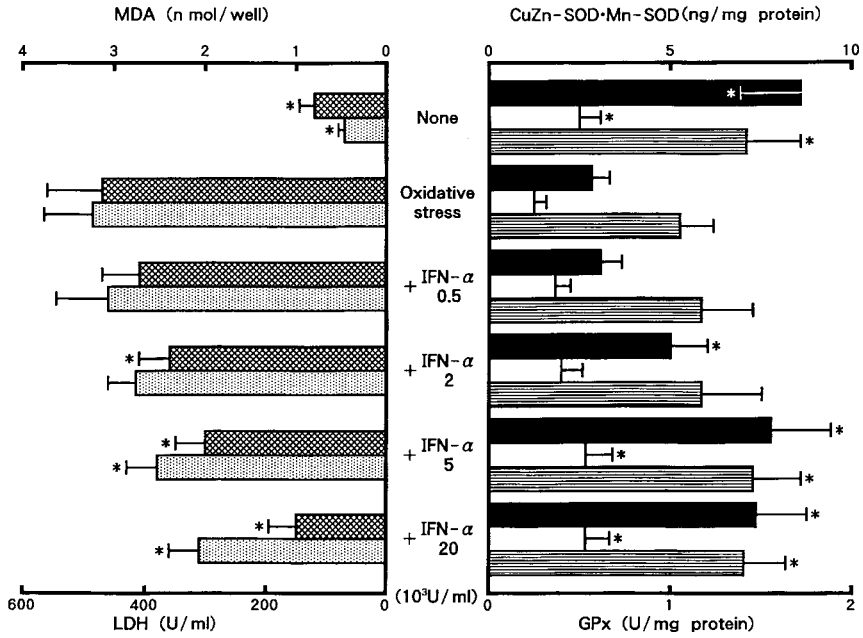

Fig. 1. Effects of IFN-α on intracellular MDA (▩) and extracellular LDH (▤) levels (left panel) and antioxidant enzyme [CuZn-SOD (■), Mn-SOD (□), and GPx (▤)]levels (right panel) in cultured rat hepatocytes undergoing oxidative stress. Cells were cultured in WE supplemented with 5% FBS for 4 h and then incubated with 100 μmol/l FeNTA (Oxidative stress) in the presence or absence of IFN-α (0.5～20×10³ U/ml) for 24 h. Values are means ± SD for 5 dishes. *P<0.05 compared to cultures incubated with FeNTA in the absence of IFN-α.

100 μmol/l FeNTA for 24 h compared to controls (65 ± 12 U/ml, 0.68 ± 0.18 nmol/well, respectively) (left panel in Fig. 1). However, the FeNTA enhancement of lipid peroxidation in the cultures was significantly inhibited by IFN-α in a dose-dependent manner (0.5-20×10³ U/ml) (Fig. 1). Furthermore, ELISA analyses of enzyme activities in the cells revealed that oxidative stress significantly attenuated protein levels of CuZn-SOD (2.8 ± 0.5 ng/mg protein) and Mn-SOD (1.2 ± 0.3 ng/mg protein) and GPx activities (5.2 ± 0.9 U/mg protein) compared to the controls (8.5 ± 1.7 ng/mg protein, 2.4 ± 0.6 ng/mg protein, 7.1 ± 1.5 U/mg protein, respectively), while IFN-α induced their activities in a dose-dependent manner (0.5-20×10³ U/ml) (right panel in Fig. 1).

When the effect of IFN-α on hepatocyte oxidative bursts was examined using the fluorescence probe DCF in conjunction with laser-scanning confocal microscopic techniques, the DCF fluorescence generated via oxidative stress in hepatocytes for 24 h of IFN-α-supplemented culture was found to be dose-dependently attenuated (Fig. 2). These findings suggest that IFN-α functions as an antioxidant in hepatocytes.

Effects of IFN-α on Oxidative Stress and Antioxidant Enzyme Levels in Isolated Rat HSCs.

When isolated HSCs were cultured on uncoated plastic dishes in DMEM supplemented with 10% FBS, they underwent transformation within 5 days to myofibroblast-like cells with enlarged cell bodies contain-

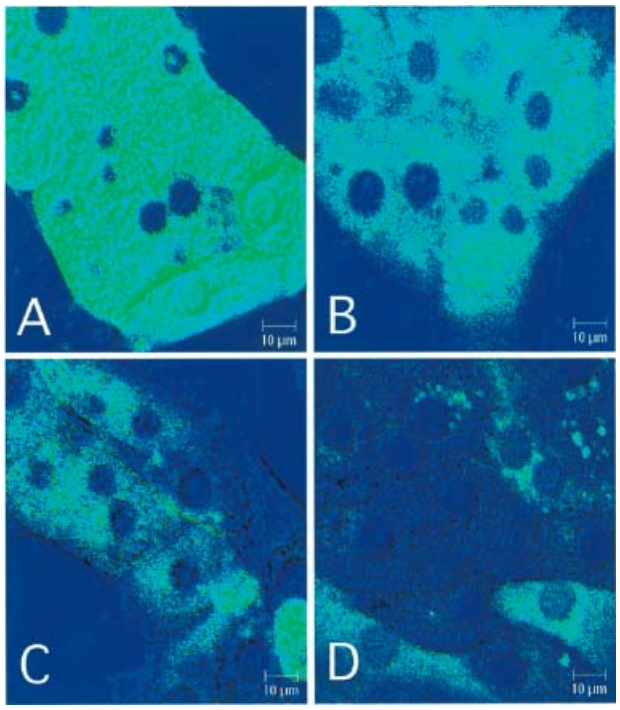

Fig. 2. Effects of IFN-α on oxidative bursts in hepatocytes using the fluorescence probe DCF and laser scanning confocal microscopic techniques. Cells were cultured in WE supplemented with 5% FBS for 4 h and then incubated with 100 μmol/l FeNTA (Oxidative stress) in the presence or absence of IFN-α for 24 h. (A) Oxidative stress ; (B) 2×10³ U/ml IFN-α ; (C)5×10³ U/ml IFN-α ; (D)20×10³ U/ml IFN-α. Nonfluorescent DCFH-DA supplementation for 10 min resulted in its conversion to fluorescent DCF through the action of H_2O_2 and peroxidase. The micrographs show typical results of three independent experiments. Original magnification ×200.

ing fewer lipid particles and exhibiting immunoreactive α-SMA. Six days after cell plating, most of the cells spread well and showed α-SMA-positive microfilaments (Fig. 3A). Supplementation of the medium with IFN-α (20×10^3 U/ml), however, inhibited both cell spreading and staining with the antibody to α-SMA (Fig. 3B) and induced the formation of numerous lipid droplets in the cytoplasmic space compared with the control cell culture (data not shown). Western blotting experiments also resulted in a reduction in α-SMA expression that was dependent on IFN-α concentration (Fig. 4).

In addition, the production of MDA in cultured HSCs was dose-dependently inhibited by IFN-α (left panel in Fig. 5). We assayed the effects of IFN-α on antioxidant enzyme levels in cultured HSCs. IFN-α was found to increase CuZn-SOD and Mn-SOD protein levels in a dose-dependent manner (right panel in Fig. 5), although GPx activities were not detectable, but under the lower detection limits for our GPx assay system that corresponds to approximately 5.6 mU/ml.

Moreover, a dose-dependent attenuation of DCF fluorescence was observed (Fig. 6). The DCF fluorescence, which was generated via oxidative stress, increased in HSC bodies in the IFN-α-unsupplemented culture (data not shown). These findings suggest that IFN-α plays an inhibitory role in processes that are mediated by oxidative bursts in HSCs.

Effects of IFN-α on Lipid Peroxidation in Isolated Rat Liver Mitochondria.

The addition of adenine diphosphate and Fe^{2+} to a suspension of rat liver mitochondria induced a

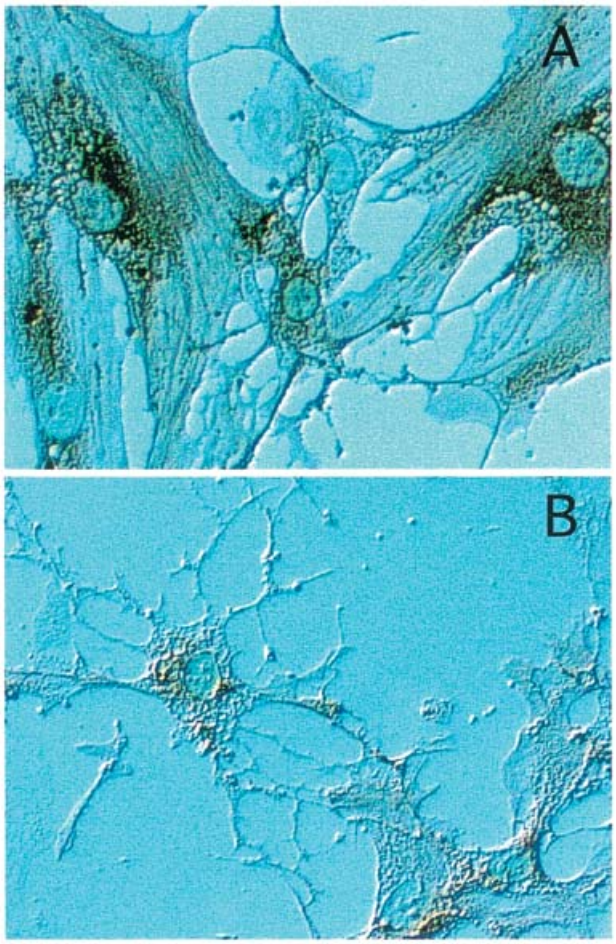

Fig. 3. Reaction of cultured rat HSCs with an antibody to α-SMA. Cells were cultured in DMEM supplemented with 10% FBS for 2 days and then incubated in the absence (A) or presence of IFN-α at a dose of 20×10^3 U/ml (B) for 4 days, immunologically stained for α-SMA, and then photographed using a differential interference contrast microscope. The micrographs represent the typical results of three independent experiments. Original magnification ×300.

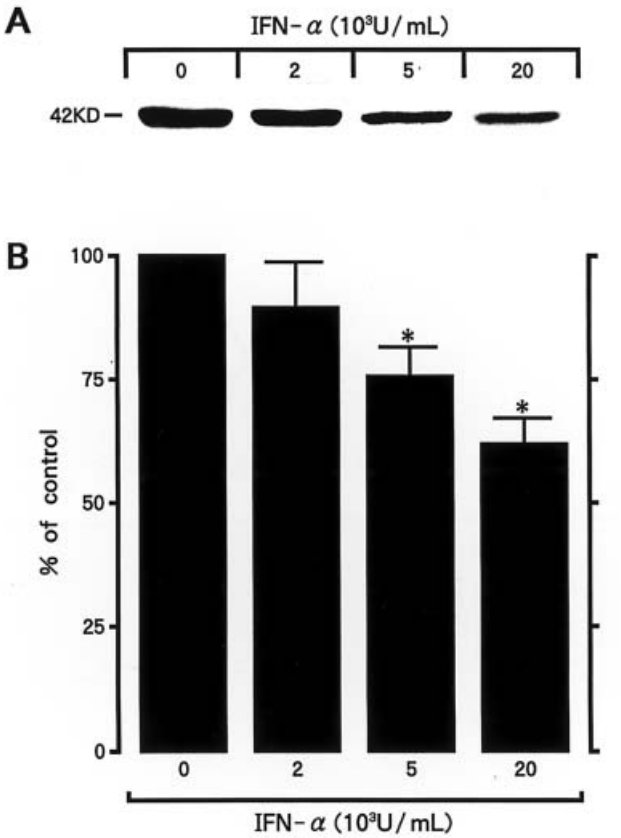

Fig. 4. (A) Effect of IFN-α on α-SMA expression in cultured rat HSCs. Cells cultured in DMEM supplemented with 10% FBS for 2 days were incubated in the presence or absence of IFN-α (0.5～20×10^3 U/ml) for 8 days. Cell lysates were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nylon membranes, and α-SMA was detected immunologically. The graphs represent the typical results of four independent experiments. (B) Densitometric analysis of α-SMA expression in cultured rat HSCs induced in the presence of IFN-α (0.5～20×10^3 U/ml) shown in (A). The results of the densitometric analysis are expressed as mean percentages (± SD) of the control values (n=5). *P<0.05 compared with controls.

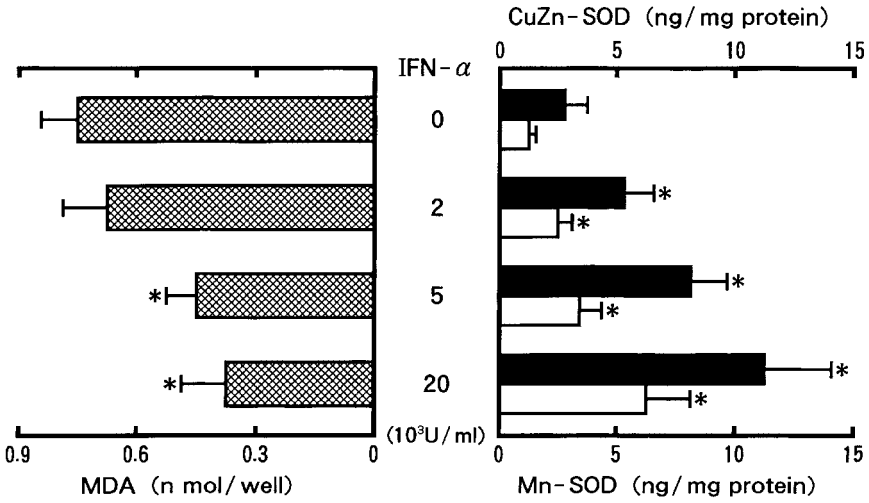

Fig. 5. Effects of IFN-α on MDA (▨) levels (left panel) and antioxidant enzyme [CuZn-SOD (■) and Mn-SOD (□)] levels (right panel) in cultured rat HSCs. Cells were cultured in DMEM supplemented with 10% FBS for 2 days and then in the presence or absence of IFN-α (0.5～20×10^3 U/ml) for an additional 8 days. Values are means ± SD for 5 dishes. *P<0.05 compared with controls.

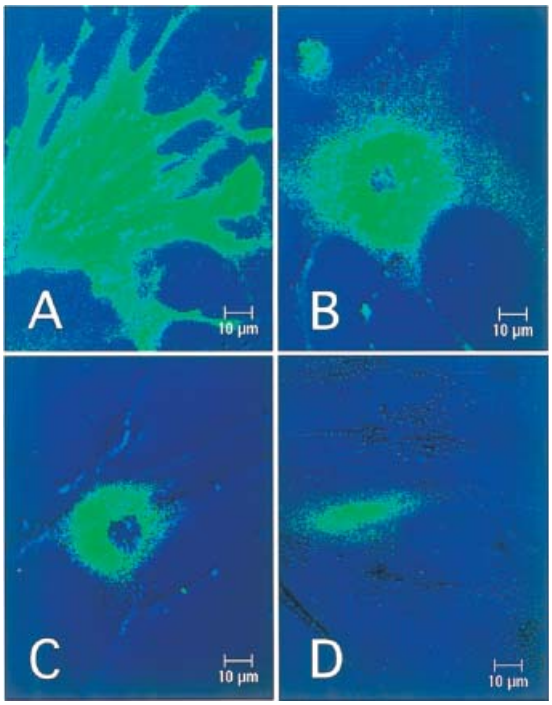

Fig. 6. Effects of IFN-α on oxidative bursts in HSCs using the fluorescence probe DCF and a laser-scanning confocal microscopic technique. Cells, cultured in DMEM supplemented with 10% FBS for 4 days, were incubated in the presence or absence of IFN-α for 24 h. (A) Control; (B) 2×10^3 U/ml IFN-α; (C) 5×10^3 U/ml IFN-α; (D) 20×10^3 U/ml IFN-α. Nonfluorescent DCFH-DA supplementation for 10 min resulted in the conversion of DCFH-DA into fluorescent DCF through the action of H_2O_2 and peroxidase. The micrographs represent the typical results of three independent experiments. Original magnification ×300.

lower oxygen consumption for about 2 min, after which consumption became more rapid until nearly all the oxygen in the incubation medium had been consumed (data not shown). The amount of MDA formed from the oxidized fatty acid chains of mitochondrial phospholipids was well correlated with the extent of oxygen consumption at this stage (data not shown). IFN-α ($0.5\text{-}20\times10^3$ U/ml) caused a dose-dependent inhibition of oxygen uptake in the second stage after the lag time (data not shown), indicating that IFN-α inhibits lipid peroxidation in rat liver mitochondria. From the dose-response plots of the antiperoxidative effect of IFN-α, the concentration of IFN-α required for the 50% inhibition of lipid peroxidation was determined to be 12×10^3 U/ml (Fig. 7).

DISCUSSION

There is evidence that free radicals cause tissue injury by initiating lipid peroxidation and inducing irreversible modifications of cell membrane structure and function (33, 34), and that the products of lipid peroxidation modulate collagen gene expression in the liver (13). This evidence suggests that lipid peroxidation may be a link between liver injury and hepatic fibrosis (35). Although it is difficult to discriminate between the profibrogenic effect of the necrotic event and the true fibrogenic effect induced by free radical species, an independent stimulation of extracellular matrix deposition, including collagens, appears to occur at a prenecrotic stage during oxidative stress-associated liver injury (35). Recently, it has been reported that paracrine stimuli derived from hepatocytes undergoing oxidative stress induce HSC proliferation and collagen synthesis (36). Further, it has been shown that HSCs are activated by the generation of free radicals and by MDA(16) and 4-hydroxynonenal (13), aldehydic products of lipid peroxidation, and antioxidants have been observed to inhibit HSC activation by type I collagen (16).

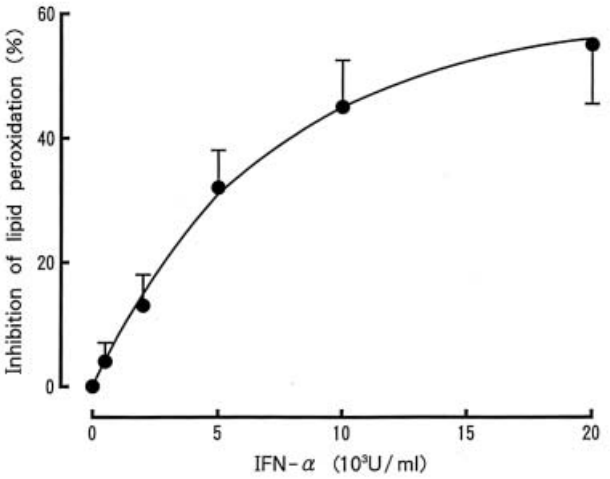

Fig. 7. Inhibition of IFN-α on lipid peroxidation in freeze-thawed rat liver mitochondria induced by Fe^{2+} and adenosine diphosphate. Values are means ± SD for five independent experiments. The calculation method was described in the Materials and Methods.

We have shown here that IFN-α inhibits the prooxidant (FeNTA)-enhanced lipid peroxidation and oxidative burst in cultured rat hepatocytes, and suppresses the α-SMA expression and oxidative burst as well as MDA production during the activation of isolated rat HSCs cultured on uncoated plastic dishes. In addition, IFN-α was observed to inhibit lipid peroxidation in rat liver mitochondrial membranes. These results are in agreement with a report that IFN-α inhibits collagen synthesis, α-SMA expression, and proliferation in cultured human HSCs (37). Therefore, IFN-α may exert its direct and fibrosuppressive effects in the liver, at least in part, through its antioxidant activity.

Despite the multiplicity of antioxidant defense mechanisms, the generation of lipid peroxidation during the course of hepatofibrogenesis may exceed the capacity of antioxidant defense mechanisms and contribute to the development of liver injury. In this study, the two variables of hepatic levels of antioxidant defense enzymes, CuZn-SOD, Mn-SOD, and GPx, and a product of oxidative stress, MDA, were reciprocal; hepatocytes undergoing oxidative stress and HSCs undergoing activation had significantly lower antioxidant enzyme levels and higher lipid peroxidation product levels. Very recently, Whalen *et al*. have also reported that the activation of rat HSCs leads to a loss of glutathione S-transferases, which detoxify lipid peroxidation products (38). These findings suggest that enzymatic defense systems may be impaired in hepatic fibrosis. In the present study, however, IFN-α was found to dose-dependently increase the immunoreactive protein levels of CuZn-SOD and Mn-SOD as well as the enzyme activities of GPx, and to decrease the MDA levels in oxidatively stressed hepatocytes and activated HSCs, although GPx activities were not detected in the latter cells with or without IFN-α supplementation. Lalazar *et al*. have reported the early gene expression of GPx during HSC activation in a rat model of hepatic fibrosis (39). It should be pointed out that immunoreactive protein and enzyme activity changes are not necessarily related to changes in mRNA levels in many instances (40), and that differences might exist in the cellular defense mechanisms against oxidative stress under different conditions and cell types (40, 41). At any rate, it appears that IFN-α can enhance biological defense activities against oxidative stress in the liver.

After administration of a single therapeutic dose of IFNs, the peak peripheral plasma levels have been reported to anywhere from 40 to 150 U/ml (42, 43). However, there is evidence for drug accumulation by a factor of 2 to 5 after repeated administration (44). Our results showed that the lowest effective IFN-α concentration in cultured hepatocytes and HSCs is 2×10^3 U/ml, although the concentration required for 50% inhibition of lipid peroxidation in isolated liver mitochondria was 12×10^3 U/ml. These results suggest that this may be the lowest concentration at which IFN-α is capable of exerting antioxidative effects *in vivo*.

When hepatocytes are continuously damaged and replicated, the frequencies of genetic alterations also probably increase along with hepatic fibrosis, leading to the development of HCC and cirrhosis. It is generally accepted that multiple genetic alterations, which are induced by mutations, are important in carcinogenesis. Several reports have suggested that the IFN-α treatment of patients with chronic HCV infection may lower their risk of developing hepatocellular carcinoma (45, 46), and that IFN-α expresses potent antitumor effects both by exerting direct antiproliferative effects on target tumor cells and by activating host cytotoxic effector cells to more efficiently lyse target tumor cells (1). Thus, our findings, which show that IFN-α plays a role as a direct antioxidant and defensive enzyme-stimulant in damaged hepatocytes and activated HSCs, suggests that IFN-α may have beneficial effects, not only on hepatic fibrosis, but also on hepatocellular carcinoma development in patients with chronic hepatitis C.

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