Coexpression of the Collagen-binding Stress Protein HSP47 Gene and the α1(I) and α1(III) Collagen Genes in Carbon Tetrachloride-induced Rat Liver Fibrosis

Hiroko Masuda,* Manabu Fukumoto,‡ Kazunori Hirayoshi,* and Kazuhiro Nagata*

**Department of Cell Biology, Chest Disease Research Institute; and ‡Department of Pathology, Faculty of Medicine, Kyoto University, Kyoto 606-01, Japan*

Abstract

HSP47 is a collagen-binding stress protein and is assumed to act as a collagen-specific molecular chaperone during the biosynthesis and secretion of procollagen in the living cell. The synthesis of HSP47 has been reported to correlate with that of collagen in several cell lines.

We examined the expression of HSP47 mRNA during the progression of carbon tetrachloride (CCl_4)-induced liver fibrosis in rats. Northern blot analysis revealed that the expression of HSP47 mRNA was markedly induced during the progression of fibrosis in parallel with α1(I) and α1(III) collagen mRNAs. By in situ hybridization, the distribution of HSP47 transcripts was similar to that of α1(I) collagen and was observed only in cells lining collagen fibrils. These collagen-positive cells were confirmed to be Ito cells by immunohistochemistry for desmin. The absence of high levels of HSP47 mRNA in the liver of rats treated with only a single administration of CCl_4 indicated that the induction of HSP47 mRNA was not due to the direct effect of CCl_4 as a stressor, but was due to the progression of liver fibrosis. The function of HSP47 in liver fibrosis will also be discussed. (*J. Clin. Invest.* 1994. 94:2481–2488.) Key words: heat shock (stress) protein • procollagen • Ito cell • liver fibrosis • in situ hybridization

Introduction

The heat shock proteins (HSPs),[1] generally called stress proteins, are a group of proteins that are induced when living cells in organisms ranging from bacteria to human are exposed to temperatures 5–10°C higher than the optimum for growth. The stress proteins are also induced in response to environmental stresses, such as exposure to heavy metals, arsenite, and oxidants. Stresses induced under pathophysiological conditions such as microbial infections, ischemia, tissue trauma, and genetic damage also induce the stress proteins. Even under normal conditions, cell cycle progression, embryonic development, cell differentiation, and hormonal stimulation in vertebrate cells and growth in microorganisms also induce the stress proteins. The major mammalian stress protein families, HSP90 and HSP70 families, as well as a small HSP, HSP28, have been well characterized (1, 2).

A 47-kD heat shock protein (HSP47) was found as a collagen-binding stress protein, the expression of which decreases after malignant transformation caused by Rous sarcoma virus or simian virus 40 infection (3–6). HSP47 is the only stress protein described to date with the ability to bind to a specific substrate. HSP47 has been found to be localized in the endoplasmic reticulum (ER) (7). Pulse-label and chase experiments combined with in vivo cross-linking and immunoprecipitation revealed that HSP47 acts like a molecular chaperone during the processing and/or secretion of procollagen in the ER (8). Collagen is a group of glycoproteins in the extracellular matrix. All the cells which express some types of collagen have been found to express HSP47, suggesting that there is functional relevancy between HSP47 and collagen synthesis. HSP47 is present in fibroblasts as well as in the connective tissues of various organs in chicken (9) and in mouse (our unpublished observations). In the chicken liver, immunohistochemical studies revealed that HSP47 is present in fibroblasts, Ito cells, Kupffer cells, smooth muscle cells, and endothelial cells (7, 9).

Fibrosis in the liver is frequently observed in chronic liver diseases such as liver cirrhosis where the accumulation and disorder of fibrous connective tissue represents a major phenomenon (10). 0.5% of total protein in normal liver consists of collagen, types I, III, IV, and V collagen (11), but CCl_4-induced rat liver fibrosis increases this to 2% of total protein (12). Interstitial collagen consisting of types I and III accounts for nearly 90–95% of all collagen found in the normal liver (11). The ratio of types I and III collagen, 60 and 40% of total liver collagen, respectively, did not change upon CCl_4-induced rat liver fibrosis (12). In CCl_4-induced liver fibrosis, types I and III collagen mRNA levels significantly increase in parallel with the increase in tissue collagen content, while type IV collagen levels increase slightly (13).

To date it is not clear whether or not HSP47 is associated with collagen biosynthesis in tissues and organs. To elucidate the biological relevance of HSP47 in relation to collagen biosynthesis, we investigated and compared the expression of HSP47 and collagen during the progression of fibrosis in the rat liver by Northern blot analysis. Furthermore, we examined the distribution of HSP47 and collagen mRNAs to identify the cells expressing both HSP47 and collagen in progressing fibrosis, using in situ hybridization and immunohistochemical techniques.

Methods

Treatment of rats. 4-wk-old male Wistar rats were fed food and water ad libitum. The rats of experimental groups were given 0.05% (wt/vol)

Address correspondence to Professor Kazuhiro Nagata, Department of Cell Biology, Chest Disease Research Institute, Kyoto University, Kyoto 606-01, Japan.

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1. *Abbreviations used in this paper:* DIG, digoxigenin; ER, endoplasmic reticulum; HSE, heat shock element; HSP, heat shock protein.

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sodium phenobarbital instead of water 1 wk before and during treatment with CCl_4 which was dissolved in an equal volume of olive oil (14). These rats were orally administered 0.2 mg of CCl_4 per kilogram of body weight every third day. The animals were killed by decapitation at each of the following time points: 2, 4, 6, 8, 10, and 12 wk. For the short-term experiments, animals were killed by decapitation at 1, 6, 12, 24, 72, 120, and 168 h, after a single administration of CCl_4 in the same manner described above.

Liver tissues were embedded in Tissue-Tec O.T.C. compound (Miles, Elkhart, IN) and in paraffin after fixing with Bouin's fixative. The remaining liver was instantly frozen with liquid nitrogen and kept at −80°C until use.

Histology and immunohistochemistry. Paraffin-embedded sections were stained with hematoxylin and eosin. Azan staining was carried out to visualize collagen fibrils in the tissue (15). For immunohistochemical staining, 6-μm-thick cryosections were fixed with acetone for 10 min at −20°C and air dried. After internal peroxidase inactivation with 0.3% H_2O_2 in methanol for 30 min, sections were incubated with 5% goat serum, and then with 10 mg/ml of a mouse monoclonal anti-desmin antibody (Boehringer Mannheim GmbH, Ingelheim, Germany) overnight at 4°C. Sections were then incubated with biotinylated goat anti-mouse IgG (Vector Labs, Inc., Burlingame, CA) for 1 h and with Vectastain ABC agent (Vector Labs, Inc.) for 50 min. Peroxidase activity was visualized with a solution containing 0.35 mg/ml of *p*-dimethyl aminobenzaldehyde and 10 mg/ml of H_2O_2 in 50 mM Tris-HCl (pH 7.6). After counterstaining with Mayer's hematoxylin, sections were mounted. As a negative control, normal mouse serum was used instead of the anti-desmin antibody.

Hydroxyproline measurement. Hydroxyproline content was measured by the method described previously (16). Briefly, small blocks of liver were freeze-dried and weighed. A block was homogenized with a Potter-Elvehjem homogenizer (Iwaki Glass Co., Ltd., Chiba, Japan). Homogenized samples (0.2 ml) were hydrolyzed in 6 N HCl at 105°C for 12 h in vacuo, dried at 65°C, and dissolved in distilled water. Samples were added to 1.5 ml of 0.066 M borate-alanine buffer (pH 8.7) and then were neutralized with 1 N NaOH. After NaCl was added to saturation, 0.6 ml of 0.2 M chloramine T and 2 mg of 3.6 M sodium thiosulfate were added. To remove proline, 3 ml of toluene was added to the tube, and the toluene layer was removed after centrifugation. 3 ml of toluene was added again to the tube, boiled for 20 min, and mixed. The toluene layer after centrifugation was mixed with 0.6 ml of Ehrlich's reagent (30 mg/ml of *p*-dimethyl aminobenzaldehyde, 68.5 μl/ml of sulfuric acid in ethanol). The color was allowed to develop for 30 min, and the absorbances were measured at 565 nm. Hydroxyl-L-proline (Nakalai Tesque, Kyoto, Japan) was used to make a standard curve. The total collagen content of each sample was also determined by hydroxyproline content/dry weight of the individual samples.

Isolation of RNA and Northern blot analysis. A small block of frozen liver was homogenized with a Polytron homogenizer (Kinematica, Switzerland) in a solution of 4 M guanidine thiocyanate according to the method of Chirgwin et al. (17). After centrifugation at 36,000 rpm (100,000 *g*) for 16 h at 15°C in a RPS55T-2 rotor (Hitachi, Tokyo, Japan), total RNA was recovered.

Total RNA was separated on 1% agarose gels containing formaldehyde (18) and transferred onto nylon membranes (Gene Screen Plus; Du Pont/New England Nuclear, Boston, MA) as recommended by the manufacturer. Blotted filters were prehybridized for 6 h at 42°C in 5× SSC (1× SSC; 0.15 M NaCl, 15 mM sodium citrate, pH 7.0), containing 50% formamide, 1× Denhardt's solution (0.02% polyvinylpyrolidone, 0.02% BSA, 0.02% Ficoll), 1% SDS, and 100 μg/ml denatured salmon sperm DNA. The filter was hybridized in hybridization solution containing ^{32}P-labeled DNA ($1.2-2\times 10^7$ cpm/ml) at 42°C for 16 h. After washing several times with 2× SSC at room temperature, 1× SSC at 65°C, and 0.5× SSC at 65°C, the filter was exposed at −80°C overnight, using RXO-H film with HR-H intensifying screens (Fuji Photo Film Co., Ltd., Tokyo, Japan). The level of mRNA transcripts was analyzed by using a Bio-imaging analyzer (Bas-2000; Fuji Photo Film Co., Ltd.).

The following ^{32}P multi-prime-labeled probes (19) were used. A 1.5-kb HindIII-EcoRI fragment of pMH47a (20) from mouse HSP47 cDNA, a 0.9-kb PstI-PstI fragment of pcmI from mouse α1(I) collagen cDNA, a 3,886–4,560-bp SacI-PstI fragment from human α1(III) collagen cDNA (a kind gift from T. Tanaka, Kyoto University), a HindIII-BamHI fragment of pH 2.3 (21) from the human HSP70 genomic DNA (a kind gift from R. Morimoto, Northwestern University, Evanston, IL), and, as a control, a 1.25-kb PstI-PstI fragment of pT2 (22) from chicken β-tubulin cDNA.

In situ hybridization. For the preparation of RNA probes, the HindIII-EcoRI 1.5-kb cDNA fragment of pMH47 from mouse HSP47 and the PstI-PstI 0.9-kb fragment of pcmI from mouse α1(I) collagen were subcloned into pGEM4Z. Antisense RNA probes of HSP47 and α1(I) collagen were labeled with digoxigenin (DIG)-labeled UTP (Boehringer Mannheim GmbH) as described by Yamamoto et al. (23).

Liver cryosections (6-μm-thick) were mounted on glass slides treated with 10 μg/ml poly-L-lysine (Sigma Immunochemicals, St. Louis, MO) and air dried at room temperature for 1 h. Thereafter the sections were fixed in freshly prepared 4% paraformaldehyde for 10 min and acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min. The slides were followed by preincubation in 50% formamide, 2× SSC for 20 min at 50°C. Hybridization was performed at 53°C for 16 h in a solution containing 50% formaldehyde, 2× SSC, 10% dextran sulfate, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1% sarkosyl, 0.02% SDS, 4× Denhardt's solution, 500 ng/ml denatured salmon sperm DNA, 500 ng/ml yeast transfer RNA, and 2.5 μg/ml of DIG-labeled riboprobe. Sections were washed six times in 2× SSC for 10 min each time, treated with 20 ng/ml of ribonuclease A (Sigma Immunochemicals) at 37°C for 30 min, and washed twice for 30 min each in 2× SSC at room temperature, in 1× SSC at 62°C, and in 0.5× SSC at 62°C. Immunological detection was performed using the Genius nonradioactive DNA labeling and detection kit (Boehringer Mannheim GmbH) according to the manufacturer's protocol. After color development, sections were counterstained with Mayer's hematoxylin and mounted. As negative controls, DIG-labeled sense RNA probes were used instead of antisense probes or an excess (×100–200) of nonlabeled antisense probes was added to the hybridization reaction.

Results

Histology of the liver of rats treated with carbon tetrachloride. In the normal liver, fine fibrous connective tissue was observed in the portal areas, around central veins, and along the sinusoids (Fig. 1 *A*). At 2 wk of CCl_4 treatment, centrilobular necrosis and fatty change were observed (Fig. 1 *B*). Hematoxylin and eosin-stained sections show inflammatory infiltrates and proliferated mesenchymal cells around the necrosis area. At 4 wk of CCl_4 treatment, necrosis of the liver significantly decreased and connective tissues began to accumulate, bridging the central to portal areas as well as the portal to portal areas (Fig. 1 *C*). Fatty degeneration and regeneration of hepatocytes were also apparent. Significant numbers of mesenchymal cells were found in and around the fibrous septa. At 10–12 wk of the treatment, prominent liver cirrhosis with pseudo-lobules surrounded by thick connective fibers was observed (Fig. 1 *D*). Fatty degeneration of hepatocytes was rare in cells adjacent to fibers. No fibrosis was observed in the control liver during these experiments (data not shown).

The expression of HSP47 and α1(I) and α1(III) collagen mRNA. The expression of HSP47 as well as α1(I) and α1(III) collagen in the liver during CCl_4 treatment was analyzed by Northern blot analysis (Fig. 2). In the control group, the expression of both HSP47 and α1(I) and α1(III) collagen mRNAs was barely detectable. However, these three mRNAs were markedly induced in the livers of rats treated with CCl_4. In our system, α1(I) collagen mRNA bands migrated as distinctly different molecular weight species. Similar results have been observed by Genovese et al. (24). The mRNA levels of HSP47

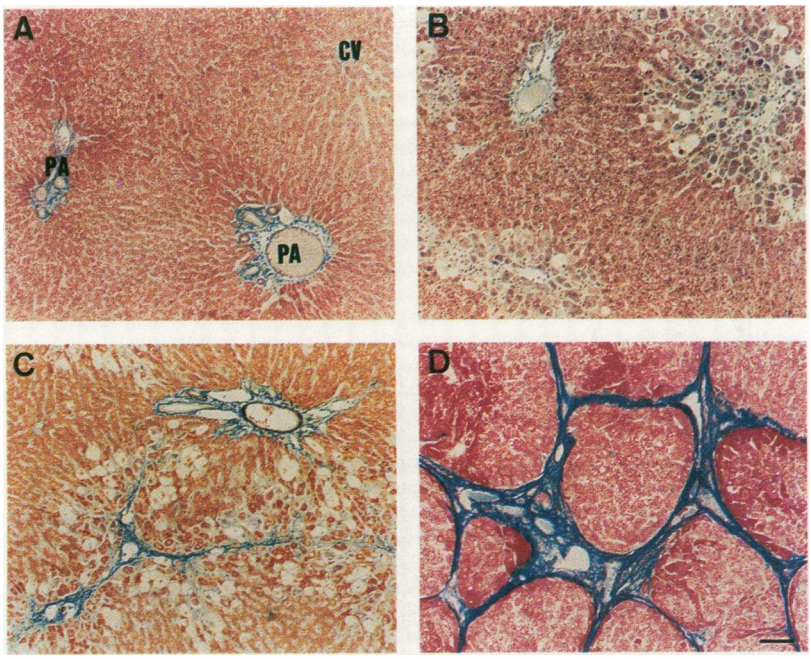

Figure 1. Azan staining of rat livers. Livers of control rats (*A*) and of rats treated with CCl_4 2 wk (*B*), 4 wk (*C*), and 12 wk (*D*) were stained with Azan to visualize collagen fibrils. *CV,* central vein; *PA,* portal area. Bar, 100 μm.

significantly increased after 2 wk of treatment and slightly decreased after 12 wk of the treatment. The mRNA levels of collagen increased in parallel with those of HSP47.

After 6 wk of treatment, the progression of fibrosis was observed to vary from rat to rat. Therefore, all the samples from the CCl_4 treatment groups were classified into the following five stages based on the degree of fibrosis. Stage I was characterized by having centrilobular necrosis, without fibrotic changes.

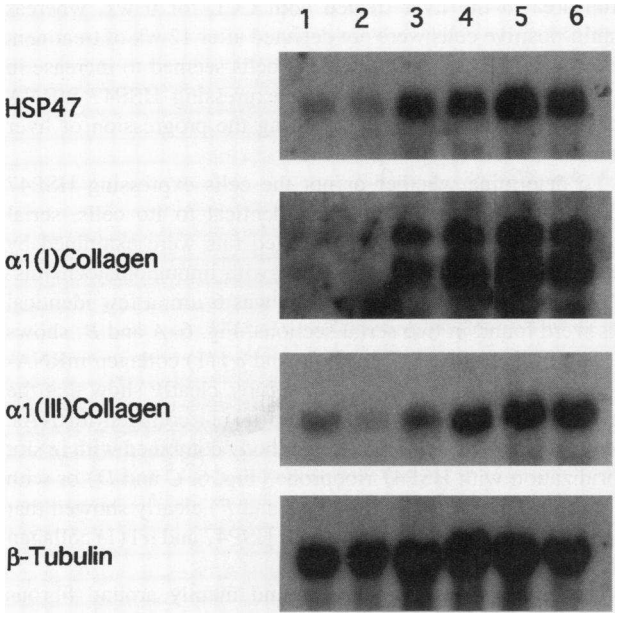

Figure 2. Northern blot analysis of HSP47 and α1(I) and α1(III) collagen mRNAs in the liver of CCl_4-treated rats. 10 μg of total RNA in each lane was electrophoretically separated, blotted onto nylon membranes, and hybridized with ^{32}P-labeled probes for HSP47, α1(I) collagen, α1(III) collagen, or β-tubulin. Lanes *1* and *2*, 2 and 12 wk of control rat liver; lanes *3–6*, 2, 4, 10, and 12 wk of the liver of CCl_4-treated rats.

Only the 2-wk group belonged to this stage. At stage II, the necrotic area decreased and fibrous connective tissue began to appear at the central area of necrosis. At stage III, progressive fibrosis became prominent, making bridges between central to portal areas and portal to portal areas. Most of the 4–6-wk samples belonged to this stage. Early cirrhosis with thin collagen fibrils as well as large hepatic nodules was defined as stage IV, and complete cirrhosis with thick fibrosis and small hepatic nodules was classified as stage V.

The levels of HSP47 and α1(I) and α1(III) collagen mRNAs at each stage of fibrosis were standardized by the level of β-tubulin mRNA. The mRNA levels of HSP47 showed a good correlation with the degree of liver fibrosis (Fig. 3 *A*). The levels of hydroxyproline were also coincident with the levels of HSP47 mRNA. Furthermore, we also calculated the correlation coefficients between HSP47, α1(I) collagen and α1(III) collagen mRNAs, and hydroxyproline content in all the samples (n = 25, Fig. 3, *B–D*). The correlation coefficients of HSP47 mRNA with α1(I) collagen mRNA, α1(III) collagen mRNA, and hydroxyproline content were 0.77, 0.80, and 0.78, respectively.

The distribution of HSP47 and α1(I) collagen mRNAs in the liver. Fig. 4 shows the results of in situ hybridization using HSP47 (*A*) and α1(I) collagen (*B*) riboprobes. In the 2- and 12-wk control livers (Fig. 4 *A*, *a* and *b*), positive stainings indicating the localization of HSP47 mRNA hyperexpressive (HSP47-positive) cells were not found. After 2 wk of CCl_4 treatment, HSP47-positive cells could be seen around the centrilobular area (Fig. 4 *A*, *c*, *arrowheads*). After 4 wk of treatment, HSP47-positive cells were observed around the portal and increased in central areas (Fig. 4 *A*, *d*, *arrowhead*). In further progressed fibrosis, many HSP47-positive cells were found in and around fibrous septa, their numbers increased after 6 wk of treatment compared with 4 wk of treatment (Fig. 4 *A*, *e*). These hyperexpressive cells were located only in fibrous connective tissues but not in liver parenchyma (Fig. 4 *A*, *f*, high magnification of *e*). In some portions of the liver after 12 wk of treatment where fibrosis was completed, HSP47-positive cells decreased

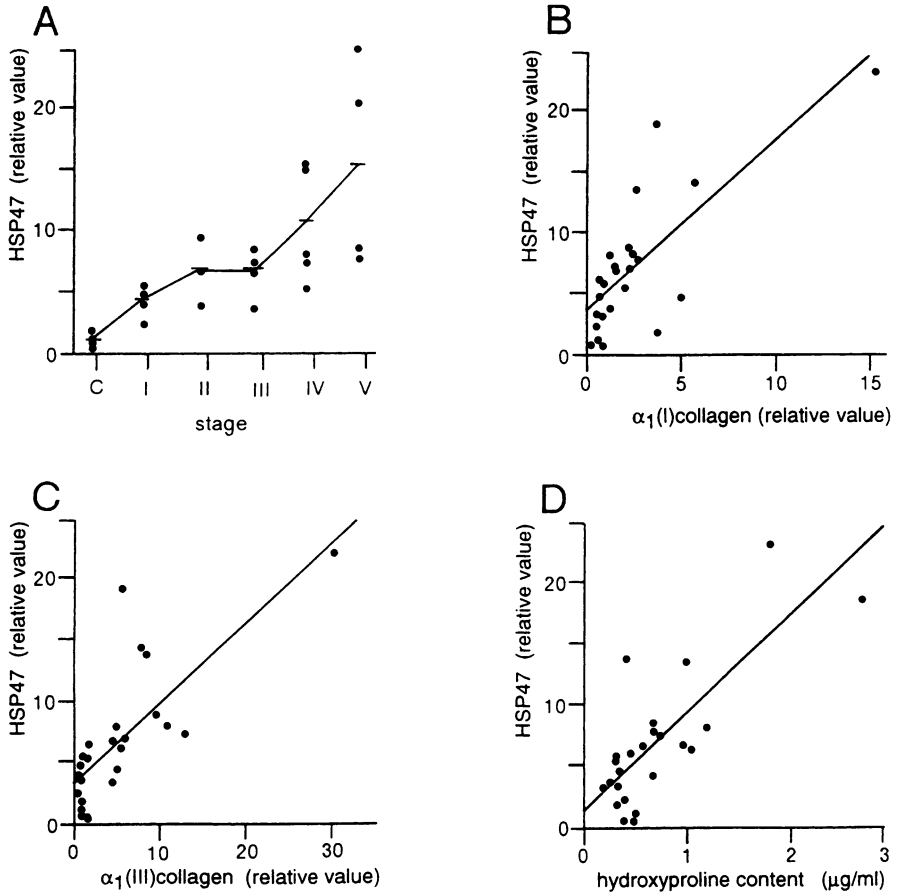

Figure 3. Correlation between HSP47 and $\alpha 1$(I) and $\alpha 1$(III) collagen mRNA levels as well as hydroxyproline content level with the progression of fibrosis. All the samples treated with CCl_4 for 2–12 wk were classified into five groups (stage I–V) according to the degree of fibrosis as described in the text. Relative amounts of HSP47 (*A*), $\alpha 1$(I) collagen, and $\alpha 1$(III) collagen were calculated by dividing the densitometric values of these mRNA bands by the β-tubulin mRNA bands for each sample. Bio-imaging analyzer, BAS-2000 (Fuji Photo Film Co., Ltd.), was used for analysis of the levels of transcript. Each value was indicated as a dot. Bars showed the means. Relative amounts of HSP47 mRNA were plotted against those of $\alpha 1$(I) and $\alpha 1$(III) collagen (*B* and *C*) and hydroxyproline contents (*D*) using all samples ranging from stages I to V (n = 25).

almost to levels seen in the group with the 2 wk of treatment (Fig. 4 *A*, *g*). Fig. 4 *A*, *h* shows hybridization with sense probes, where only background levels of signal were observed. Hybridization with HSP47 antisense probes after the addition of excess nonlabeled antisense probes showed a similar profile with that using sense probes (data not shown).

The localization of $\alpha 1$(I) collagen mRNA hyperexpressive (collagen-positive) cells was also examined by in situ hybridization (Fig. 4 *B*). In the control liver, collagen-positive cells were observed around the portal areas and central veins either at 2 wk (Fig. 4 *B*, *a*, *arrowheads*) or at 12 wk (Fig. 4 *B*, *b*, *arrowheads*). In CCl_4-treated rats, collagen-positive cells increased in and around fibrous septa after 2–6 wk of treatment, showing a similar distribution of HSP47-positive cells (Fig. 4 *B*, *c–e*, *arrowheads* of *c* and *d*). However, after 12 wk of treatment, few collagen-positive cells were found around the fibrous septa (Fig. 4 *B*, *g*, *arrowheads*). In situ hybridization using sense probe of $\alpha 1$(I) collagen (Fig. 4 *B*, *h*) or using antisense probe in the presence of excess nonlabeled probe (data not shown) showed the background level of signals.

Ito cells (also called fat-storing cells or lypocytes), which can be characterized by specific staining with anti-desmin antibodies, have been reported to produce collagen during liver fibrosis (25–27). Therefore, we examined whether or not desmin-positive Ito cells express HSP47 mRNA. First, the appearance of Ito cells during liver fibrosis was examined using a monoclonal antibody against mouse desmin by immunohistochemistry. Desmin-positive cells were not detected in the 2-wk control liver (Fig. 5 *A*) except for a few positive cells in the portal area (data not shown). After 2 wk of CCl_4 treatment, desmin-positive cells became evident around the central area. Fig. 5 *B* shows desmin-positive cells around the central and portal area in the liver treated with CCl_4 for 6 wk, whereas desmin-positive cells were not detected after 12 wk of treatment (data not shown). Desmin-positive cells seemed to increase in parallel with the number of cells expressing HSP47 mRNA and/or $\alpha 1$(I) collagen mRNA during the progression of liver fibrosis.

To determine whether or not the cells expressing HSP47 and $\alpha 1$(I) collagen mRNAs are identical to Ito cells, serial sections of 6-wk liver of CCl_4-treated rats were examined by in situ hybridization in combination with immunohistochemistry. As the thickness of the sections was 6 μm, a few identical cells were found in two serial sections. Fig. 6, *A* and *B*, shows the distribution of HSP47 mRNA- and $\alpha 1$(I) collagen mRNA-positive cells, respectively. These figures clearly show that the same cells express both HSP47 and $\alpha 1$(I) collagen mRNAs. Staining using a desmin-specific antibody combined with in situ hybridization with HSP47 riboprobe (Fig. 6, *C* and *D*) or with $\alpha 1$(I) collagen riboprobe (Fig. 6, *E* and *F*) clearly showed that desmin-positive cells expressed both HSP47 and $\alpha 1$(I) collagen mRNAs.

Desmin-positive cells were found mainly around fibrous septa but not in the septa. However, some cells in fibrous septa expressed HSP47 and $\alpha 1$(I) collagen mRNAs. These mesenchymal cells were thought to be fibroblasts which were not stained by the desmin antibody.

Is carbon tetrachloride a stressor which induces HSP47? CCl_4 is known to cause free radical injury in the liver. The toxic effect of CCl_4 is due to the conversion of CCl_4 to the highly

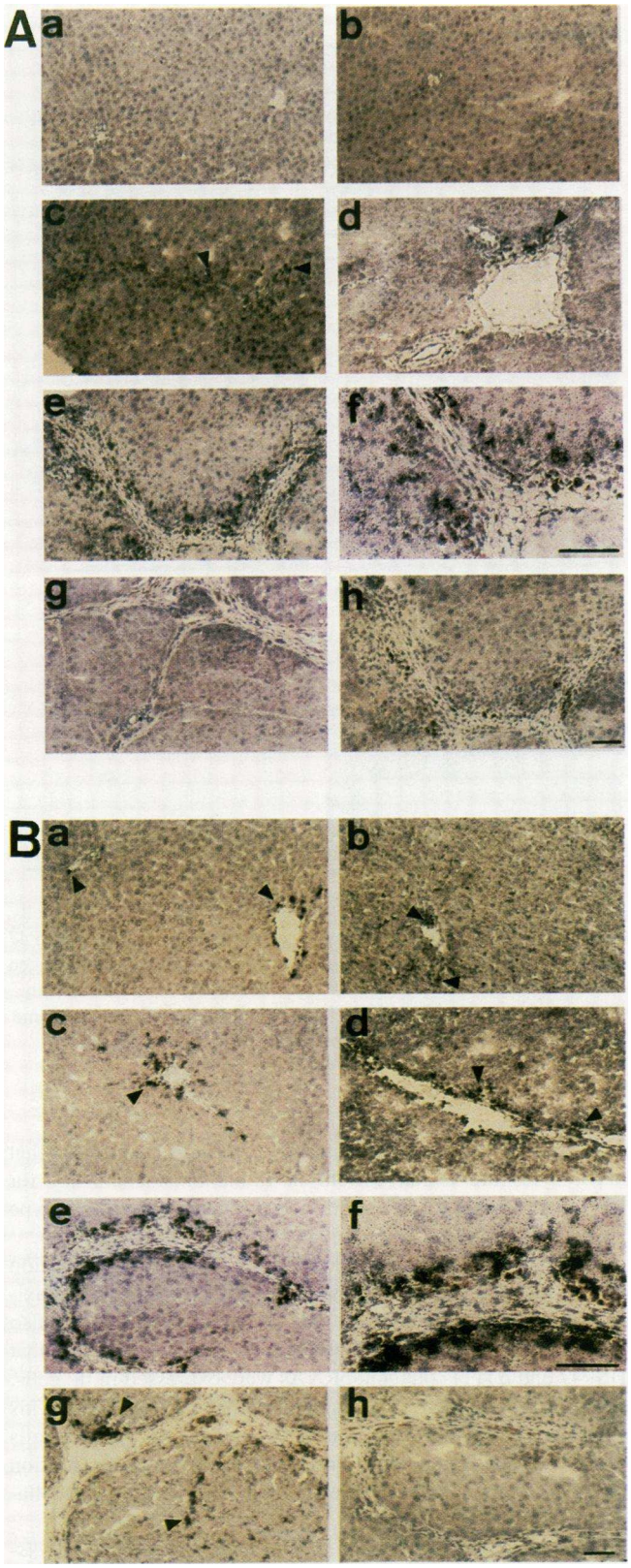

Figure 4. Time-dependent distribution of HSP47- and α1(I) collagen–positive cells examined by in situ hybridization. *A*, HSP47 riboprobes; *B*, α1(I) collagen riboprobes. Panel *a*, 2 wk; and *b*, 12 wk of control liver. Panel *c*, 2 wk; *d*, 4 wk; *e*, *f*, and *h*, 6 wk; and *g*, 12 wk of CCl_4-treated liver. Panel *f* was high magnification of *e*. Panel *h* was hybridized with sense probes as negative controls. Others were hybridized with antisense probes. Arrowheads indicate HSP47-positive cells (*A*) or collagen-positive cells (*B*). Bar, 100 μm.

reactive free radical $CCl_3\cdot$ in the smooth ER (28). As stress proteins are known to be induced by superoxide and agents which produce free radicals such as H_2O_2, it is possible that HSP47 and other stress proteins may be induced by CCl_4. Thus, the effect of a single administration of CCl_4 into rats was examined by Northern blot analysis. Fig. 7 shows mRNA levels of HSP47 and HSP70 after short periods of a single administration of CCl_4. Only low levels of constitutive HSP70 (HSC70) mRNA were observed in the controls (Fig. 7, lanes *1* and *2*). Expression of HSP70 mRNA was induced at 1 h after an administration of CCl_4 (Fig. 7, lane *3*), and the levels of HSP70 mRNA dramatically increased up to 12 h and then decreased after 24 h (Fig. 7, lanes *4–6*). On the contrary, HSP47 mRNA levels did not change until 24 h, slightly increased after 3 d of the administration, and decreased thereafter. The α1(I) collagen mRNA levels also showed modest increase after 3 d of the administration. This result is consistent with the previous observation that Ito cells are induced in the liver of rats after 3 d of a single administration of CCl_4 (29). These results suggest that HSP47 induction is associated with liver fibrosis but not caused by a direct and transient effect of the CCl_4 administration.

Discussion

In this study, we have shown that the mRNA of HSP47, a collagen-binding stress protein, was markedly induced in parallel with α1(I) and α1(III) collagen mRNAs during hepatic fibrosis in rats induced by the administration of CCl_4. In the normal rat liver, only trace amounts of collagen are synthesized, and the expression of HSP47 mRNA is undetectable. The expression levels of HSP47 mRNA were proportional to that of collagen mRNAs during the progression of fibrosis, and all of these mRNAs decreased slightly after the completion of experimentally induced cirrhosis. The expression of these three mRNAs as well as the hydroxyproline content, which reflects total collagen content, was also closely correlated with the progression of liver fibrosis.

We also demonstrated the distribution of HSP47 and α1(I) collagen mRNAs in the fibrotic liver by in situ hybridization. HSP47- and α1(I) collagen–positive cells are located around the portal area and central veins in the normal liver. During the progression of fibrosis, the cells expressing these mRNAs were observed to increase in and around the fibrous septa in the liver. However, these cells began to decrease after 12 wk when cirrhosis was almost completed. Of interest, HSP47-positive cells were observed only along the collagen fibrils and not apart from the collagen fibrils even in the fibrotic liver. This suggests the functional relevancy of HSP47 in the formation of collagen fibrils in CCl_4-treated rats.

HSP47 is an ER resident stress protein which can specifically bind to newly synthesized procollagen. HSP47 binds to types I–V collagens but does not bind to other extracellular matrix proteins such as fibronectin and laminin (30, 30a). Our previous report showed that HSP47 transiently binds to procollagen in the ER but does not dissociate from abnormal procollagens caused by heat shock or by treatment with α, α'-dipyridyl, an iron chelator which inhibits the triple helix formation of procollagens (6). Therefore, we believe that HSP47 is a collagen-specific molecular chaperone. The expression of HSP47 always correlates with that of collagens in vitro: the synthesis of both HSP47 and collagen decreases after transformation of fibroblasts and increases during the differentiation of mouse teratocarcinoma F9 cells (4, 20). Cell lines, such as mouse

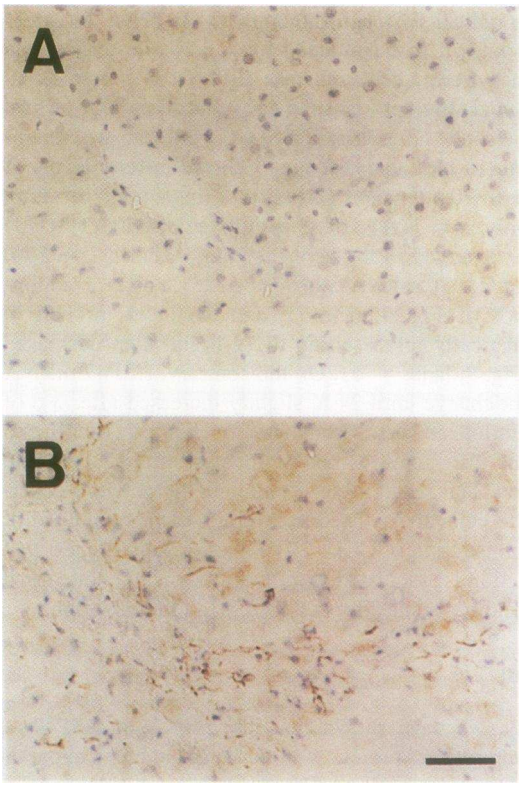

Figure 5. Immunohistochemistry with desmin antibody. *A*, 2 wk of control liver. *B*, 6 wk of CCl_4-treated liver. Bar, 50 μm.

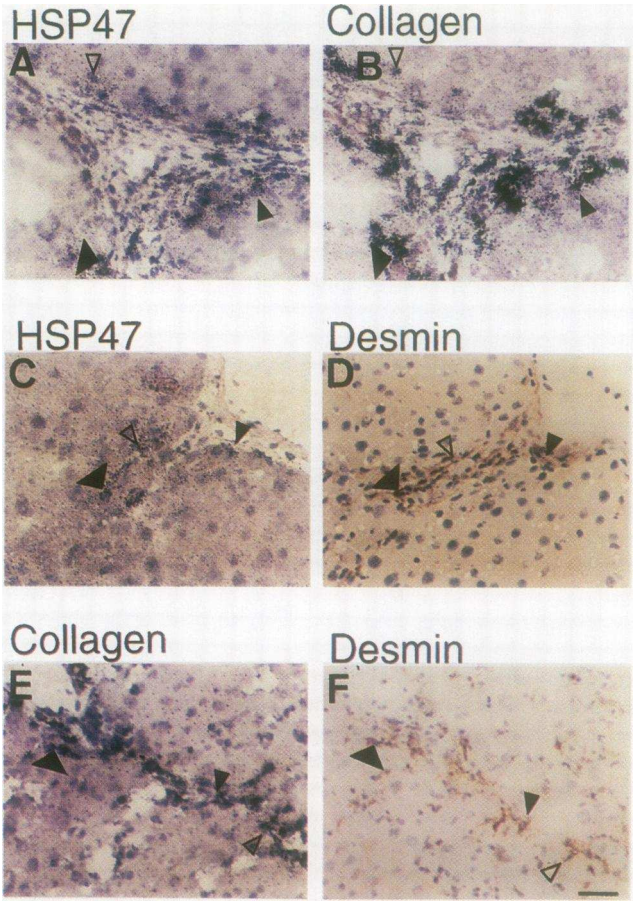

Figure 6. Identification of HSP47-positive cells, collagen-positive cells, and desmin-positive Ito cells. *A* and *C*, in situ hybridization with HSP47 antisense riboprobe. *B* and *E*, in situ hybridization with $\alpha 1$(I) collagen antisense riboprobe. *D* and *F*, immunostaining with desmin antibody. Sets of *A* and *B*, *C* and *D*, and *E* and *F* are serial sections. The same triangles indicate the same cells in serial sections. Bar, 50 μm.

myeloid leukemia M1 and rat pheochromocytoma PC12, in which collagen synthesis is not detected, do not synthesize detectable amounts of HSP47 (Satoh, M., A. Nakai, and K. Nagata, unpublished observations). Furthermore, transfection of antisense HSP47 mRNA in fibroblasts causes a decrease in the synthesis and/or secretion of type I procollagen (Satoh, M., and K. Nagata, manuscript in preparation). These observations suggest that HSP47 might play an important role for the synthesis, processing, or secretion of procollagen. It has to be elucidated whether the upregulation of HSP47 causes the progress of fibrosis.

It is a matter of interest to know which cells contribute to fibrogenesis in the liver. It has been reported that nonparenchymal cells such as Ito cells, myofibroblasts, and endothelial cells in the sinusoidal area synthesize collagens in vivo and in vitro (13, 31–33). There are contradictory reports whether hepatocytes synthesize significant amounts of collagen (34–36).

In this study, we have shown by in situ hybridization and immunostaining on serial sections of the fibrotic liver that both HSP47- and $\alpha 1$(I) collagen–positive cells were desmin-positive cells, suggesting that they are Ito cells (29, 37, 38). These cells were detected in fibrous connective tissues but not in the central or sinusoidal regions of lobuli, and the number of them increased with progressing fibrosis. Yokoi et al. (39) have reported the presence of many desmin-positive cells in the normal rat liver. However, in our experiments, desmin-positive cells were not detected in the control liver with the exception of a few positive cells in the portal area. This discrepancy may be due to differences in antibody specificity and/or activity used in each study. Hepatocytes did not hyperexpress HSP47 or $\alpha 1$(I) collagen mRNAs. From these results, we conclude that Ito cells and fibroblasts mainly express HSP47 and collagen mRNAs. This conclusion is also supported by previous observations that hepatocytes are negative both in immunohistochemistry for HSP47 in normal rat liver and in in situ hybridization for type I collagen mRNA in CCl_4-induced rat liver fibrosis (7, 33).

Northern blot analysis of livers after a single administration of CCl_4 showed that the expression of HSP47 mRNA was maximal after 3 d of the treatment. $\alpha 1$(I) collagen mRNA was also induced parallel with HSP47 mRNA. It may be suggested that HSP47 and $\alpha 1$(I) collagen mRNAs were expressed in Ito cells, which was increased by a single administration of CCl_4. This is consistent with and supported by the reports that Ito cells were increased in number at 2 or 3 d after a single administration of CCl_4 and exhibited positive staining for type I and III collagens (26, 29).

The transcription of heat shock genes is regulated by *cis*-acting heat shock elements (HSE) in the promoter region and *trans*-acting heat shock factors (40). In the current study, CCl_4 itself apparently acts as a stressor to induce HSPs in the liver since Northern blot analysis shows an induction of HSP70 mRNA upon CCl_4 treatment. The mRNA levels of HSP47 did not change until 24 h of CCl_4 treatment and slightly increased after 3 d. It may be suggested that HSP47 overexpression was not caused by CCl_4 toxicity directly but was induced in parallel

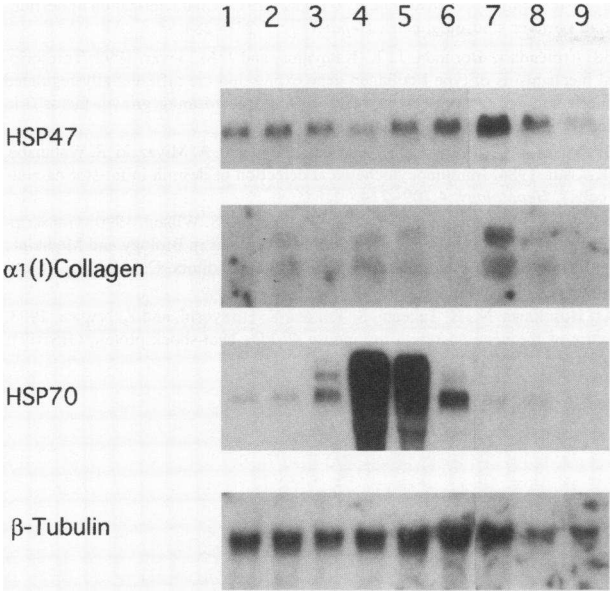

Figure 7. Northern blot analysis of HSP47, $\alpha 1$(I) collagen, and HSP70 in livers treated with a single dose of CCl_4. Lane *1*, control given water; lane *2*, control given phenobarbital water. Lanes *3, 4, 5, 6, 7, 8,* and *9:* 1, 6, 12, 24, 72, 120, and 168 h after administration of CCl_4, respectively. The three bands indicate HSP70 mRNAs, the lowest band representing constitutively expressive HSP70 and the upper two bands representing inducible forms of HSP70.

with the progression of fibrosis. HSP47 is induced by several stressors inducing heat shock through the regulation of the HSE/heat shock factor system, since there is an HSE in the HSP47 promoter (41). However, the induction of HSP47 mRNA observed during progression of liver fibrosis is probably caused by a different mechanism from heat shock stress. We are now in the process of identifying the *cis*-acting elements in the HSP47 promoter region which are responsible for the correlative induction of HSP47 with collagens as described above. Potential "collagen-responsive elements" might be involved in the induction of HSP47 during the progression of liver fibrosis.

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