Deletion of the Collagen-specific Molecular Chaperone Hsp47 Causes Endoplasmic Reticulum Stress-mediated Apoptosis of Hepatic Stellate Cells*

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Background: Although knockdown of heat shock protein 47 (Hsp47) attenuates liver fibrosis, the underlying molecular mechanism is unknown.
Results: Deletion of Hsp47 caused activated hepatic stellate cells (HSCs) to undergo ER stress-mediated apoptosis when autophagy was inhibited.
Conclusion: ER stress-induced apoptosis may underlie the clearance of collagen-producing HSCs.
Significance: Hsp47 could be an attractive therapeutic target for fibrosis treatment.

Chronic liver injury, often caused by alcoholism and viral hepatitis, causes liver fibrosis via the induction of collagen production. In liver fibrosis, hepatic stellate cells (HSCs) are activated and transform into myofibroblasts, which actively produce and secrete collagen into the extracellular matrix. Hsp47 (heat shock protein 47) is a collagen-specific molecular chaperone that is essential for the maturation and secretion of collagen. Here, we used the Cre-LoxP system to disrupt the *Hsp47* gene in isolated HSCs from *Hsp47* floxed mice. Immature type I procollagen accumulated and partially aggregated in Hsp47-KO HSCs. This accumulation was augmented when autophagy was inhibited, which induced expression of the endoplasmic reticulum (ER) stress-inducible proteins BiP (immunoglobulin heavy chain-binding protein) and Grp94 (94-kDa glucose-regulated protein). The inhibition of autophagy in Hsp47-KO HSCs also induced CHOP (CCAAT/enhancer-binding protein homologous protein), which is an ER stress-induced transcription factor responsible for apoptosis. These data suggest that apoptosis is induced through ER stress by procollagen accumulation in Hsp47-KO HSCs when autophagy is inhibited. Thus, Hsp47 could be a promising therapeutic target in liver fibrosis.

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Chronic liver injury causes fibrosis and cirrhosis, resulting in organ failure through excess accumulation of extracellular matrix (1). Hepatic stellate cells (HSCs)[3] store vitamin A as lipid droplets and encircle sinusoids in normal liver (2). During liver fibrosis, HSCs differentiate into myofibroblast-like cells in response to inflammatory cytokines such as TGF-β. This process is characterized by the loss of lipid droplets, accelerated production and accumulation of type I collagen, expression of smooth muscle α-actin, and an increased proliferation rate (3). Transient activation of HSCs is thought to be a protective acute response against liver injury, whereas persistent HSCs activation is responsible for fibrosis (1, 3).

Procollagen requires several molecular chaperones and enzymes for it to generate a trimer consisting of three α-chains and to fold correctly in the endoplasmic reticulum (ER). Such molecular chaperones include BiP/Grp78, Grp94 (94-kDa glucose-regulated protein), protein-disulfide isomerase, and Hsp47 (heat shock protein 47) (4–8). The enzymes prolyl 4-hydroxylase, prolyl 3-hydroxylase, and cyclophilin B are required for the stable triple-helix formation of procollagen (9–12). We previously reported that Hsp47 is a collagen-specific molecular chaperone in the ER (13–16). Disruption of the *Hsp47* gene causes embryonic lethality by 11.5 days post coitus because of the disruption of basement membranes and the misformation of collagen fibrils (17–19). When the *Hsp47* gene was disrupted in chondrocytes using the Cre-LoxP system, where *Hsp47* floxed mice were crossed with mice carrying a chondrocyte-specific *Col2α1-Cre* transgene, mice died just before or shortly after birth displaying severe chondrodysplasia and bone deformities with reduced type II collagen production (20). In Hsp47-null fibroblasts, the triple-helix formation and secretion of type

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[3] The abbreviations used are: HSC, hepatic stellate cell; ER, endoplasmic reticulum; MOI, multiplicity of infection; CQ, chloroquine; 3-MA, 3-methyladenine; MEF, mouse embryonic fibroblast; AdControl, control adenovirus; AdCre, adenovirus encoding Cre recombinase.

I collagen are severely perturbed (19, 21). Type I procollagen α-chains that are misfolded and fail to form a trimer in the ER are degraded via ER-associated degradation after retrograde transport from the ER to the cytosol, whereas misfolded α-chains that form a trimer are eliminated through autophagy, a process termed ER phagy (22, 23). In Hsp47-null fibroblasts, type I procollagen is misfolded and forms trimers, which accumulate as detergent-insoluble aggregates in the ER that are eliminated via the autophagic lysosome pathway (22).

Although expression of *Hsp47* is up-regulated by various cytosolic stresses, including heat shock, constitutive expression of *Hsp47* correlates with that of collagen in various organs and collagen-related pathophysiological conditions, including liver fibrosis, connective tissue diseases, and dermal fibrotic diseases (24, 25). A recent study showed that TGF-β, a pro-fibrotic cytokine, regulates Hsp47 production in fibroblast cell lines (26). Fibrotic progression is attenuated by down-regulation of Hsp47 expression (27–31). Because of the pivotal role of collagen-producing cells in fibrosis, clearance of activated HSCs through cell death might be expected to alleviate and recover from liver fibrosis. Therefore, the induction of the death of activated HSCs by suppression of Hsp47 expression may be an attractive therapeutic strategy for liver fibrosis. Actually, suppression of Hsp47 expression was reported to attenuate liver fibrosis via inducing the apoptosis of HSCs (28, 30); however, the reason why apoptosis is induced upon suppression of Hsp47 expression remains unknown. We previously reported that ER stress causes apoptotic cell death with the induction of CHOP expression in *Hsp47*-disrupted mice and cells (18, 20, 22). Accumulation of misfolded proteins in the ER causes ER stress and triggers activation of the unfolded protein response (32). At least three ER transmembrane sensor proteins are activated in the unfolded protein response: PERK (PKR-like endoplasmic reticulum kinase) (33), ATF6 (activating transcription factor 6) (34), and IRE1 (inositol requiring 1) (35, 36). Under ER stress, the unfolded protein response can operate as a pro-survival process through the transient termination of translation, the induction of a series of proteins that coordinately function to renature misfolded proteins, and/or the elimination of misfolded proteins. However, under conditions of persistent ER stress, proapoptotic signals are induced through the activation of several transcription factors, such as ATF4 (activating transcription factor 4) and CHOP, downstream of the PERK pathway (37).

In the present study, we investigated the mechanisms underlying the apoptosis of Hsp47-deficient HSCs and the involvement of autophagy in the clearance of misfolded type I procollagen in the absence of Hsp47. We hypothesized that misfolded type I procollagen in the ER might cause ER stress, resulting in the apoptosis of Hsp47-disrupted HSCs when autophagy is inhibited. To this end, we isolated HSCs from the livers of *Hsp47* floxed mice, disrupted the *Hsp47* gene by infecting cells with an adenovirus harboring the *Cre* gene, and performed biochemical and cell biological analyses.

EXPERIMENTAL PROCEDURES

Reagents—Collagenase type I and ascorbic acid were purchased from WAKO (Osaka, Japan). Pronase E was purchased from Roche, DNase I was purchased from Merck, and TGF-β

was purchased from PeproTech Inc. (Rocky Hill, NJ). Chloroquine (CQ), 3-methyladenine (3-MA), leupeptin, and pepstatin A were purchased from Sigma Aldrich (St. Louis, MO). Anti-Hsp47 and anti-Grp94 antibodies were purchased from ENZO Life Sciences (Plymouth Meeting, PA), an anti-BiP/Grp78 antibody was purchased from BD Transduction Laboratories, an anti-CHOP antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and an anti-GAPDH antibody was purchased from HyTest (Turku, Finland). Antibodies against mouse type I collagen and β-actin were purchased from Millipore Corporation (formerly NYSE: MIL), and those against LC3 and p62 were purchased from MBL (Nagoya, Japan).

Mice—*Hsp47* floxed mice were previously generated (20). All experiments using animals were approved by the Kyoto Sangyo University Committee for Animal Care and Welfare.

Isolation and Culture of HSCs—HSCs were isolated from $Hsp47^{flox/flox}$ mice as described by Radaeva *et al.* (38). The isolated HSCs were cultured on a plastic dish in low glucose DMEM supplemented with 10% FBS and antibiotic-antimycotic solution (Sigma-Aldrich) and maintained at 37 °C in a humidified atmosphere with 5% CO_2.

Adenovirus Infection—Adenoviruses harboring the *Cre recombinase* gene, or the *neo* gene as the control, under the control of the CAG promoter were kindly provided by Dr. Ikeda (Osaka City University, Osaka, Japan) (39). Adenoviruses were purified using the Adeno-X maxi purification kit (Takara Bio Inc., Shiga, Japan), and the titer of the purified virus was determined using the Adeno-X rapid titer kit (Takara Bio Inc., Shiga, Japan). At days 18–31 after their isolation, HSCs were infected with adenovirus at a multiplicity of infection of 17, 20, or 25 and cultured with 136 μg/ml ascorbic acid.

Immunoblot Analysis—HSCs were trypsinized and seeded onto multiwell plate on day 8 after adenovirus infection with 136 μg/ml ascorbic acid and 1 ng/ml TGF-β. At day 4 after seeding, HSCs were treated with or without 20 μM CQ or 10 mM 3-MA for 24 h. HSCs were lysed with buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5.0 mM EDTA, 1% (v/v) octylphenoxypolyethoxyethanol, and protease inhibitors (1 μg/ml of leupeptin and pepstatin A) at 4 °C for 20 min. After centrifugation, supernatants and pellets were collected and electrophoresed on a 7, 10, or 15% SDS-PAGE gel (40). Thereafter, the gels were transferred to PVDF membranes with a pore size of 0.45 or 0.22 μm. The membranes were blocked with Blocking One (Nacalai Tesque, Kyoto, Japan) and then stained with specific antibodies. The targeted proteins were detected by a chemiluminescence method using an ECL Western blotting detection reagent or an ECL prime Western blotting detection reagent (GE Healthcare Bio-Sciences) followed by visualization using the LAS-3000 system (Fujifilm, Tokyo, Japan).

RT-PCR and Quantitative Real Time PCR—Total RNA was extracted from HSCs using the RNeasy mini kit (Qiagen), and then first strand cDNA was synthesized using the SuperScript III first strand synthesis SuperMix for RT-PCR and quantitative real time PCR. For RT-PCR, the PCR primers used to amplify mouse Hsp47 were 5′-AAGATGCAGAAGAAGGCTGTCG-3′ (forward) and 5′-CTGTGACACCCCTGAATTTGGT-3′ (reverse), and those used to amplify mouse XBP-1 were 5′-TGA-GAACCAGGAGTTAAGAACACGC-3′ (forward) and 5′-TTC-
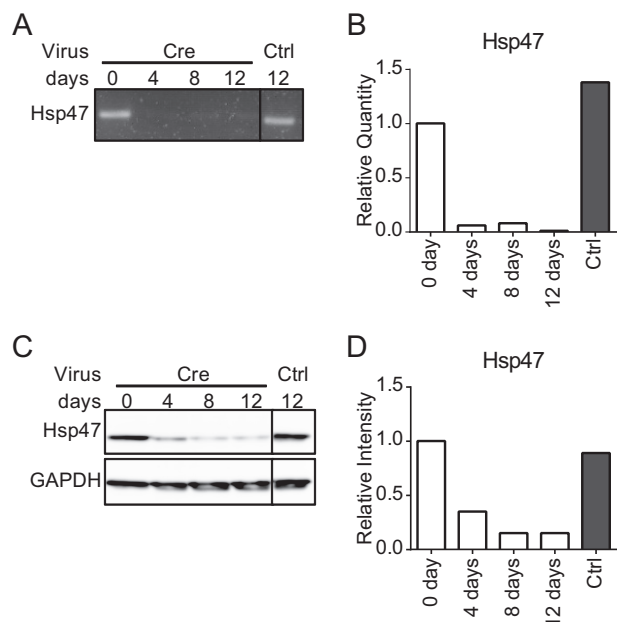
FIGURE 1. **Depletion of Hsp47 in activated HSCs.** *A–C*, the mRNA and protein levels of Hsp47 at the indicated number of days after infection with a control adenovirus (*Ctrl*) or an adenovirus encoding Cre recombinase (*Cre*) at a multiplicity of infection of 17 in activated HSCs were analyzed by semiquantitative RT-PCR (*A*), real time PCR (*B*), and Western blotting (*C*). *D*, the protein level of Hsp47 in *C* is shown relative to that of GAPDH.

TGGGTAGACCTCTGGGAGTTCC-3′ (reverse). For real time PCR, the primers and probes were designed using the Assay Design Center (Roche). The sequences of the primers and the probe used to detect mouse Hsp47 were as follows: 5′-GAAG-GCTGTCGCCATCTC-3′ (forward), and 5′-TCCTGCCAGA-TGTTTCTGC-3′ (reverse), and 5′-TGGTGGAG-3′ (probe). The Universal Probe Library Mouse GAPD Gene Assay (Roche) was used to detect GAPDH as the control. Real time PCR was performed using the Applied Biosystems StepOnePlus real time PCR System (Applied Biosystems, Foster, CA). Expression of Hsp47 was normalized against that of GAPDH.

Immunostaining—HSCs were trypsinized and seeded onto poly-L-lysine-coated cover glasses with ascorbic acid on day 8 after adenovirus infection. At day 4 after seeding, Immunofluorescence was performed as previously described (21). Briefly, HSCs were fixed with 4% paraformaldehyde for 20 min at 37 °C, treated with 0.25% collagenase type I for 3 min at 37 °C, and then treated with 0.1% Triton X-100 for 5 min at room temperature to permeabilize the cells. For staining of extracellular matrix components, HSCs were not treated with collagenase or Triton X-100. HSCs were treated with blocking buffer containing 2% goat serum and 20% glycerol for 30 min at room temperature and then incubated with specific antibodies. Thereafter, HSCs were stained with Alexa Fluor 488-conjugated anti-rabbit IgG or Alexa Fluor 546-conjugated anti-mouse IgG. Immunofluorescence was detected using a LSM-700 microscope (Zeiss, Jena, Germany). The same exposure time was used for the acquisition of all images.

Detection of Caspase-3 Activity—Caspase-3 activity was detected using the NucView 488 caspase-3 assay kit (Biotium, Hayward, CA) according to the manufacturer's instructions. Signals were observed using a BZ-710 microscope (Keyence,
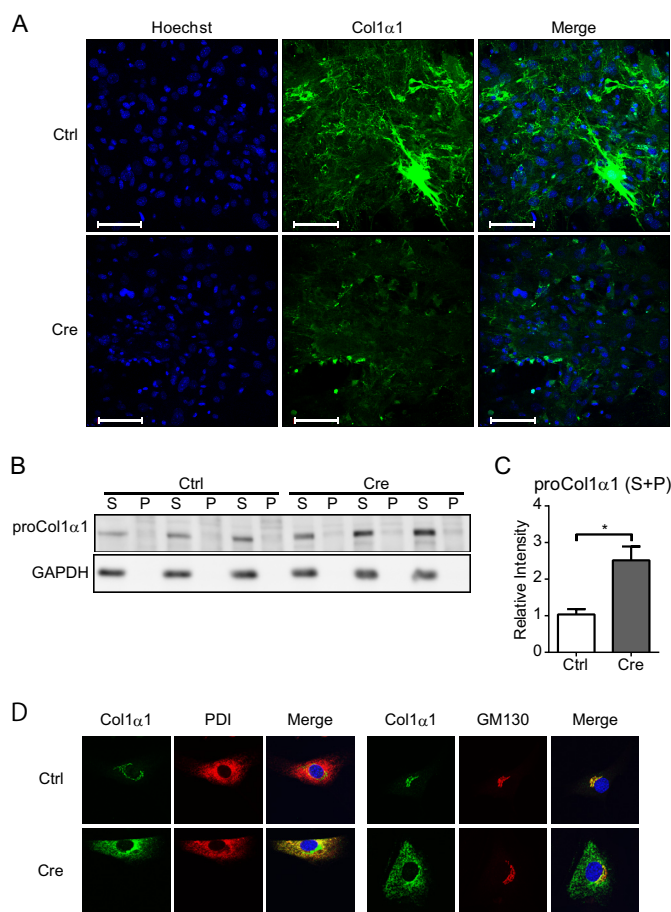
FIGURE 2. **Maturation of type I collagen in Hsp47-KO HSCs.** *A*, deposition of type I collagen in the extracellular matrix after infection of activated HSCs with AdControl or AdCre at a MOI of 17 was detected by staining with an anti-type I collagen (*green*) antibody and Hoechst 33342 (*blue*) without permeabilizing cells. *Scale bars*, 100 μm. *B*, Western blot analyses of type I procollagen α1 in activated HSCs infected with AdControl or AdCre at a MOI of 25. Cell lysates were separated by centrifugation to generate detergent-soluble (*S*) and detergent-insoluble (*P*) fractions. *C*, the protein level of type I procollagen α1 in *B* is shown relative to that of GAPDH. Experiments were performed four times independently, and values are means $\pm$ S.E. *, $p \leq 0.05$. *D*, the localization of type I collagen in activated HSCs infected with AdControl or AdCre at a MOI of 20 was determined by staining with anti-type I collagen (Col1α1; *green*), anti-protein-disulfide isomerase (*PDI*; *left*, *red*), and anti-GM130 (*right*, *red*) antibodies and Hoechst 33342 (*blue*). *Ctrl*, control.

Osaka, Japan). The same exposure time was used for the acquisition of all images.

Statistical Analysis—Quantitative data are described as means $\pm$ S.E., and the difference between the two groups was statistically analyzed using the two-tailed unpaired Student's *t* test. All quantitative data were obtained from at least three independent experiments. Densitometric analysis of immunoblots was performed using Multi Gauge version 3.0 software (Fujifilm, Tokyo, Japan), and the caspase-3 activity signal was calculated using the BZ-X analysis application (Keyence).

RESULTS

Depletion of the Hsp47 Gene in Activated HSCs—To reveal the role of Hsp47 in HSCs, we established Hsp47-KO HSCs using the Cre-loxP system. We previously established cartilage-specific Hsp47-KO mice by crossing *Hsp47^flox/flox* mice with mice expressing the *Cre recombinase* gene under the control of
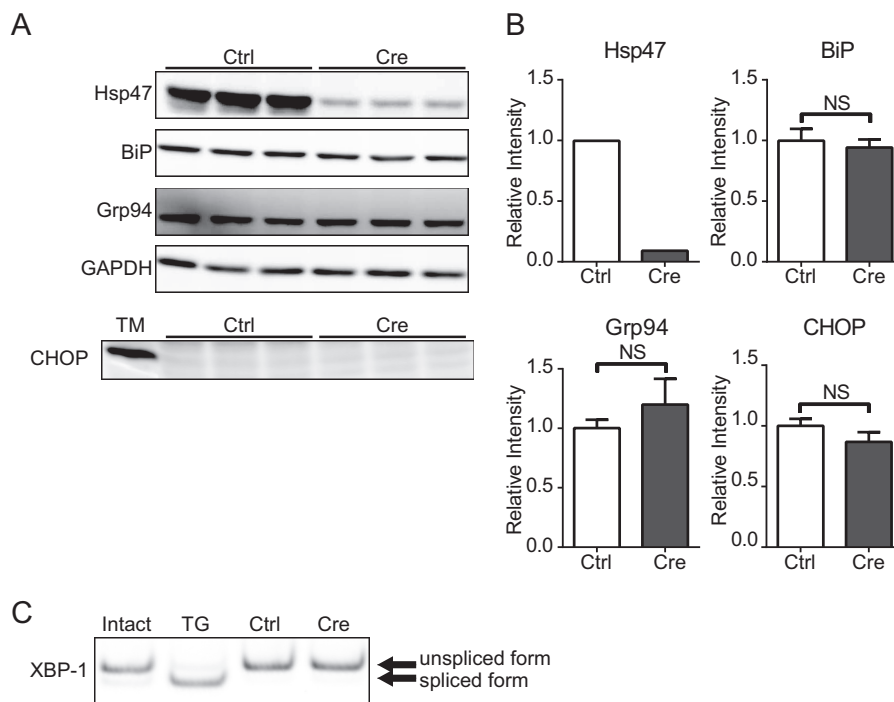
FIGURE 3. **Endoplasmic reticulum stress and apoptosis are not observed in Hsp47-KO HSCs.** *A*, Western blot analyses of activated HSCs infected with AdControl or AdCre at a multiplicity of infection of 25 were performed with the indicated antibodies. HSCs were treated with tunicamycin (*TM*) as a positive control for CHOP detection. *B*, the protein levels of Hsp47, BiP, Grp94, and CHOP in *A* are shown relative to that of GAPDH. Experiments were performed four times independently, and values are means ± S.E. **, $p \leq 0.01$. *NS*, not significant. *C*, RT-PCR for XBP-1 in activated HSCs infected with AdControl or AdCre at a MOI of 25. HSCs were treated with thapsigargin (*TG*) as a positive control for detecting of XBP-1 spliced form. *Ctrl*, control.

the type II collagen promoter (*Col2-cre*). In the current study, HSCs were isolated from *Hsp47^flox/flox* mice and activated by culture on a noncoated plastic dish. Hsp47 was depleted in the activated HSCs by infection with an adenovirus encoding Cre recombinase (AdCre). At day 4 after infection, expression of Hsp47 mRNA was decreased in HSCs infected with AdCre to less than 10% of the level in HSCs infected with the control adenovirus (AdControl), so-called control HSCs (Fig. 1, *A* and *B*). This suggests that Hsp47 was almost completely knocked out. At days 4 and 8 after infection, the protein level of Hsp47 in Hsp47-KO HSCs was decreased to ~40% and less than 20% of the level in control HSCs, respectively (Fig. 1, *C* and *D*). At day 12 after infection, the mRNA and protein levels of Hsp47 in Hsp47-KO HSCs were less than 10 and 20% of those in control HSCs, respectively. These results indicate that the *Hsp47* gene was disrupted in activated HSCs by infection with AdCre.

Accumulation of Type I Procollagen in the ER of Hsp47-KO HSCs—The effect of Hsp47 on the maturation of type I collagen in activated HSCs was examined using Hsp47-KO HSCs. The accumulation of extracellular type I collagen was analyzed by immunostaining with an anti-collagen antibody without permeabilizing cells. The level of extracellular collagen was dramatically decreased in Hsp47-KO HSCs, in comparison with control HSCs (Fig. 2*A*). The amount of type I procollagen that accumulated inside cells, as determined by immunoblot analysis, was clearly higher in Hsp47-KO HSCs than in control HSCs (Fig. 2, *B* and *C*). Accumulation of type I procollagen in the detergent-insoluble fraction was hardly observed either in control or Hsp47-KO HSCs. It might be noted that procollagen was clearly detected in the detergent-insoluble fraction in

Hsp47-KO MEFs (22). The localization of procollagen within cells was next examined by immunostaining HSCs after they had been permeabilized using Triton X-100. Type I procollagen localized primarily in the Golgi apparatus in control HSCs, as shown by its colocalization with GM130 (*cis*-Golgi marker) (41) but not with protein-disulfide isomerase (ER marker) (42). However, in Hsp47-KO HSCs, the level of type I procollagen in the ER was markedly increased, and this procollagen was also clearly observed in the Golgi apparatus (Fig. 2*D*). These results suggest that in the absence of Hsp47, type I procollagen failed to form the mature form and therefore could not be deposited in the extracellular matrix. Consequently, a portion of procollagen remained in the ER as a detergent-insoluble form in Hsp47-KO HSCs.

Induction of ER Stress and Apoptosis in AdCre-infected HSCs—The accumulation of misfolded proteins and/or detergent-insoluble protein aggregates in the ER reportedly causes ER stress and consequently apoptosis via the induction of CHOP expression, which is up-regulated by PERK in the unfolded protein response pathways (32, 37). The amount of type I procollagen in the ER was greatly increased in Hsp47-KO HSCs (Fig. 2, *B* and *C*); therefore, the induction of ER stress and/or apoptosis was examined in these cells. First, the induction of several stress proteins in the ER, including BiP and GRP94, was examined to determine whether ER stress was induced in AdCre-infected HSCs. Although Hsp47 expression was dramatically decreased in Hsp47-KO HSCs, expression of BiP or Grp94 was not clearly changed (Fig. 3, *A* and *B*). The splicing of XBP-1 by activated IRE1 is known to be induced during ER stress (32). XBP-1 splicing analyzed by RT-PCR was
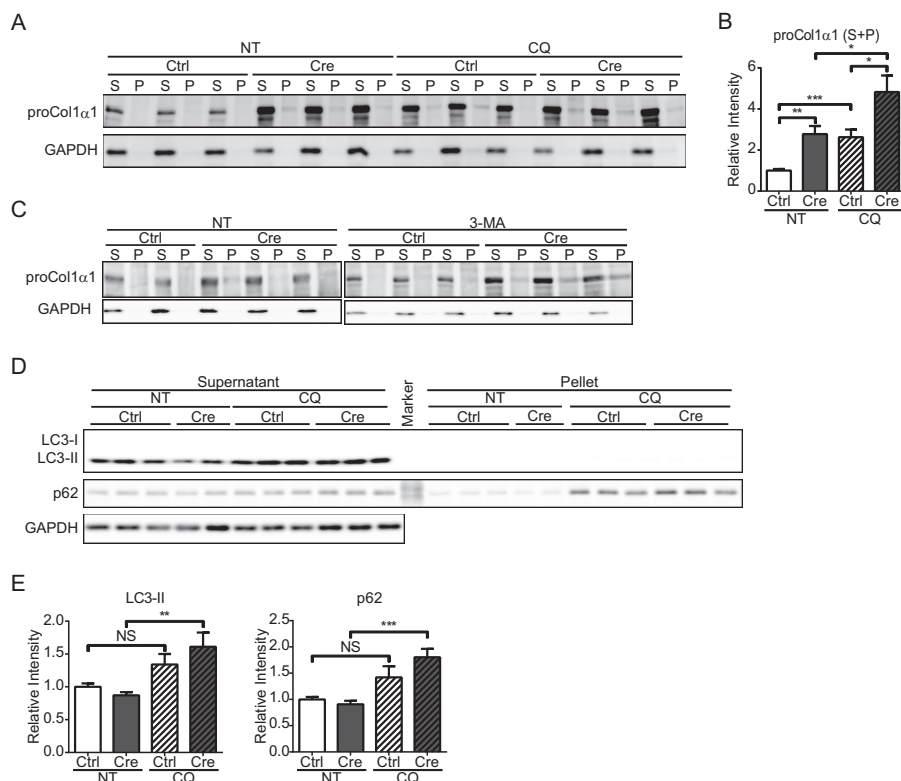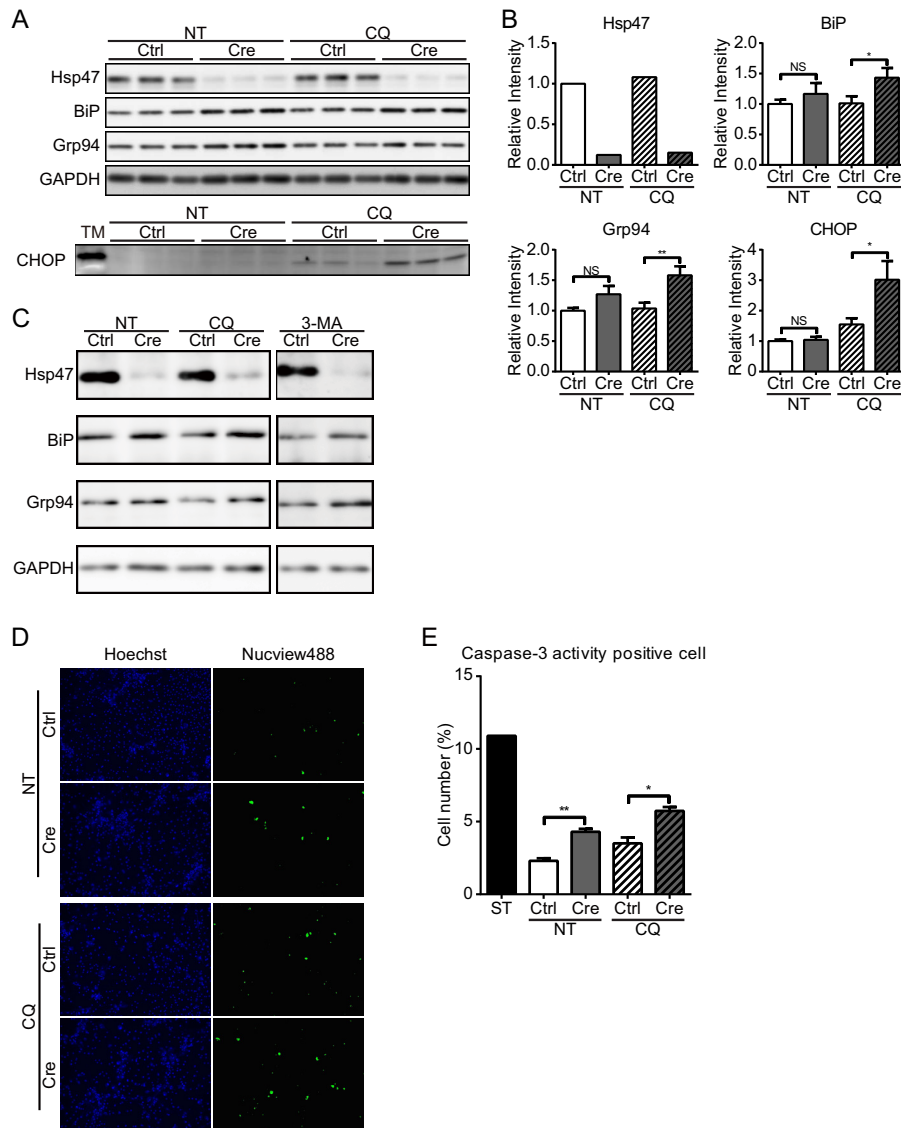
FIGURE 4. **Accumulation of type I procollagen in Hsp47-KO HSCs when autophagy is inhibited.** *A*, the protein levels of type I procollagen α1 and GAPDH in activated HSCs infected with AdControl or AdCre at a MOI of 25 and treated with or without CQ were detected by immunoblotting with anti-type I collagen (proCol1α1) and anti-GAPDH antibodies. Cell lysates were separated by centrifugation to generate detergent-soluble (*S*) and detergent-insoluble (*P*) fractions. *B*, the protein level of type I procollagen α1 in *A* is shown relative to that of GAPDH. Experiments were performed eight times independently. *C*, the protein levels of type I procollagen α1 and GAPDH in activated HSCs infected with AdControl or AdCre at a MOI of 25 and treated with or without 3-MA were detected by immunoblotting with anti-type I collagen (proCol1a1) and anti-GAPDH antibodies. Cell lysates were separated by centrifugation to generate detergent-soluble (*S*) and detergent-insoluble (*P*) fractions. The samples representing the detergent-insoluble fraction are 5-fold concentrated in comparison with the detergent-soluble fraction. *D*, Western blot analyses of LC3, p62, and GAPDH in activated HSCs infected with AdControl or AdCre at a MOI of 25 and treated with or without CQ were performed with the indicated antibodies. Cell lysates were separated by centrifugation to generate detergent-soluble (supernatant) and detergent-insoluble (pellet) fractions. *E*, the protein levels of LC3-II and p62 in supernatant fraction in *D* are shown relative to that of GAPDH. Experiments were performed nine times independently. All values are means ± S.E. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$. *NS*, not significant; *NT*, not treated; *Ctrl*, control.

not observed in Hsp47-KO HSCs (Fig. 3*C*). The induction of CHOP (Fig. 3, *A* and *B*) and caspase-3 (data not shown) expression, which are apoptosis makers, was also not detected in Hsp47-KO HSCs. These results suggest that neither ER stress nor apoptosis were induced in Hsp47-KO HSCs. We previously reported that ER stress induces apoptosis in MEFs in which Hsp47 is disrupted by homologous recombination (22). However, aggregates of misfolded procollagen in the ER of these cells are degraded by autophagy (22). Therefore, the induction of apoptosis in these cells was considered to be accentuate under the inhibition of autophagy. Thus, the effect of autophagy inhibition on the induction of ER stress and apoptosis in Hsp47-KO HSCs was next examined by treating these cells with inhibitors of autophagy.

HSCs were treated with CQ, which inhibits autophagy by preventing acidification of lysosomes (43). First, the accumulation of type I procollagen was examined by immunoblot analysis of CQ-treated cells. Type I procollagen accumulated in CQ-treated control HSCs (Fig. 4, *A* and *B*). The level of procollagen was significantly higher in Hsp47-KO HSCs than in control HSCs in the absence of CQ treatment, and the level of procollagen in Hsp47-KO HSCs was greatly increased following CQ treatment. Furthermore, the amount of procollagen was mod-

estly higher in the detergent-insoluble fraction of CQ-treated Hsp47-KO HSCs than in that of untreated Hsp47-KO HSCs (Fig. 4*A*). The increase of procollagen in Hsp47-KO HSCs was also observed when autophagy was inhibited with 3-MA, another autophagy inhibitor that inhibits type III phosphatidylinositol 3-kinases (44) (Fig. 4*C*). After treatment with CQ for 24 h on day 12 after infection with AdControl or AdCre, the levels of LC3 and p62, which are autophagy markers, were determined by immunoblot analysis. During activation of autophagy, LC3, a membrane component of the autophagosome, is converted from LC3-I to LC3-II by the conjugation of phosphatidylethanolamine (45). In control HSCs, CQ treatment did not significantly affect the levels of p62 and LC3-II (Fig. 4, *D* and *E*). By contrast, in Hsp47-KO HSCs, the levels of LC3-II and p62 were significantly increased by CQ treatment. These results clearly indicated that autophagy was up-regulated in Hsp47-KO HSCs (Fig. 4, *D* and *E*), which is consistent with our previous work (22). The levels of BiP and Grp94 were significantly increased in Hsp47-KO HSCs when autophagy was inhibited, whereas this was not observed in control HSCs (Fig. 5, *A*–*C*). CHOP was also up-regulated in Hsp47-KO HSCs, but not in control HSCs, when autophagy was inhibited (Fig. 5, *A* and *B*). The activity of caspase-3 was examined using a fluores-

FIGURE 5. **Induction of endoplasmic reticulum stress and apoptosis in Hsp47-KO HSCs when autophagy is inhibited.** *A*, the protein levels of Hsp47, BiP, Grp94, CHOP, and GAPDH in activated HSCs infected with AdControl or AdCre at a MOI of 25 and treated with or without CQ were detected by Western blot analyses using the indicated antibodies. HSCs were treated with tunicamycin (*TM*) as positive control for CHOP detection. *B*, the protein levels of Hsp47, BiP, Grp94, and CHOP in *A* are shown relative to that of GAPDH. Experiments were performed nine times independently. *C*, the protein levels of Hsp47, BiP, Grp94, and GAPDH in activated HSCs infected with AdControl or AdCre at a MOI of 25 and treated with or without CQ and 3-MA were detected by Western blot analyses using the indicated antibodies. *D*, activation of caspase-3 in activated HSCs infected with AdCre or AdControl at a MOI of 25 and treated with or without CQ was analyzed using the Nucview488 caspase-3/7 substrate (*green*) and Hoechst 33342 (*blue*). *E*, the ratio of caspase-3 activity-positive cells to Hoechst 33342-positive cells in *D* is shown. HSCs were treated with staurosporine (*ST*) as positive control for apoptosis. Experiments were performed three times independently. All values are means ± S.E. *, $p \leq 0.05$; **, $p \leq 0.01$. *NS*, not significant; *NT*, not treated; *Ctrl*, control.

cent marker-conjugated caspase-3 substrate (Fig. 5, *D* and *E*). The number of Hsp47-KO HSCs with caspase-3 activity was increased even in the absence of CQ (Fig. 5*E*), whereas the CHOP induction was not changed (Fig. 5*B*). CQ treatment increased the numbers of control and Hsp47-KO HSCs that exhibited caspase-3 activity (Fig. 5*E*). These results suggest that ER stress was induced in Hsp47-KO HSCs through the accumulation of type I procollagen in the ER when autophagy was inhibited and caused the apoptosis in activated HSCs.

DISCUSSION

Since we first identified Hsp47 as a collagen-specific molecular chaperone residing in the ER of mammalian cells (13, 15),

correlational expression analysis of Hsp47 and various types of collagens has been reported in numerous tissues (15) and collagen-related diseases including fibrosis (24, 25). This correlational expression of Hsp47 and collagens is important for the potential treatment of fibrotic disorders because down-regulation of Hsp47 expression by RNAi or antisense RNA methods markedly delays the progression of fibrosis and concomitantly reduces the level of collagen in the extracellular matrix (27–31). Recently, knockdown of Hsp47 in the HSCs of rat, which was achieved using a drug delivery system to target RNAi specifically to these cells, was shown to markedly prevent the progression of experimental liver fibrosis (28, 30). In this report, apoptotic death of HSCs in rats treated with Hsp47-targeting RNAi

was suggested to be a major reason underlying the dramatic therapeutic effects on liver fibrosis.

We previously reported that disruption of the *Hsp47* gene in mice causes improper molecular maturation of type I and/or IV procollagens and the accumulation of these molecules in the ER as detergent-insoluble aggregates that are not secreted (17–19). In Hsp47-KO mouse embryos, ER stress-mediated apoptotic cell death is observed in various tissues and MEFs (18, 20, 22). Thus, we hypothesized that the abnormal accumulation of misfolded and/or aggregated procollagens in the ER of mice treated with Hsp47-targeting RNAi might cause ER stress, leading to the expression of CHOP and other apoptosis-inducing genes, and that this ER stress might be the major reason underlying the apoptosis of HSCs.

To address this issue, we established an inducible knock-out system for the *Hsp47* gene by infecting activated HSCs from *Hsp47*$^{flox/flox}$ mouse liver with AdCre. ER stress was not observed in activated control or Hsp47-KO HSCs (Figs. 3 and 5), which is inconsistent with our previous results in Hsp47-KO MEFs (22). This apparent discrepancy may be due to the difference in the basal expression level of procollagen; it is higher in MEFs compared with that in HSCs. In addition, the autophagy was reported to be activated in HSCs (48, 49), which attenuates the induction of ER stress in HSCs, because the misfolded proteins in the ER could be eliminated by autophagy (ER-phagy). We previously reported that misfolded procollagen trimers in the ER are degraded by autophagy, whereas misfolded single procollagen α-chains are degraded by ER-associated degradation (22, 46, 47). Thus, we treated activated HSCs with inhibitors of autophagy. Upon treatment with autophagy inhibitor, the level of procollagens in the ER was increased in both control and Hsp47-KO HSCs (Fig. 4, *A–C*). It suggests that a part of procollagens was degraded through autophagic pathway. As mentioned above, it was reported recently that the up-regulation of autophagy is required for the activation of hepatic stellate cell (48, 49). It is conceivable that this higher activity of autophagy may be responsible for degrading procollagen even in control HSCs. The increase in the accumulation of procollagens in the ER of CQ-treated cells was reflected by the induction of ER stress, which was detected by the increased levels of BiP and Grp94 (Fig. 5, *A–C*). It should be noted that the increased accumulation of procollagens caused ER stress in Hsp47-KO HSCs, but not in control HSCs (Fig. 5, *A–C*), which suggests that the molecular conformation of accumulated procollagens differs according to whether Hsp47 is present or absent. This is supported by our observation that autophagy was up-regulated much more in Hsp47-KO HSCs than in control HSCs (Fig. 4, *D* and *E*). ER stress in Hsp47-KO HSCs induced apoptosis, which was detected by increases in the level of CHOP and the activity of caspase-3 (Fig. 5).

Thus, we clearly showed that procollagens that accumulate in the ER of activated HSCs because of the absence of Hsp47 can be degraded by autophagy. However, when autophagy is blocked or overwhelmed by the excess accumulation of misfolded procollagens, activated HSCs undergo apoptosis.

During regression of fibrosis, half of the activated HSCs undergo apoptosis, and the other half revert back to inactivated HSCs (50). Reinactivated HSCs are more sensitive to inflammatory cytokines and are more easily activated than quiescent HSCs. Therefore, reinactivated HSCs are regarded as a risk factor for fibrosis. Thus, immediate and sufficient elimination of activated HSCs is important for the treatment of fibrosis. We showed that apoptosis was induced in activated HSCs by a combination of Hsp47 KO and inhibition of autophagy.

The observations of this study are important with regards to the use of Hsp47 down-regulation as a therapeutic strategy for various fibroses, including liver cirrhosis. Down-regulation of Hsp47 blocks the deposition of procollagens in the extracellular matrix by activated HSCs, which decreases the level of collagens in fibrotic tissues. In addition, prevention of the secretion of procollagens from the ER causes these proteins to accumulate in the ER, which leads to apoptosis of HSCs, the major collagen-producing cells in the liver. Thus, down-regulation of Hsp47 can alleviate fibrosis in two concomitant ways: by inhibiting the secretion of collagen and by inducing apoptosis in collagen-producing cells. This sheds light on a new strategy to treat fibrosis.

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