Antisense oligonucleotides against collagen-binding stress protein HSP47 suppress peritoneal fibrosis in rats

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Background. Peritoneal fibrosis is a serious complication in patients on continuous ambulatory peritoneal dialysis (CAPD), but the molecular mechanism of this process remains unclear. Heat shock protein 47 (HSP47), a collagen-specific molecular chaperone, is essential for biosynthesis and secretion of collagen molecules, and is expressed in the tissue of human peritoneal fibrosis. In the present study, we examined the effect of HSP47 antisense oligonucleotides (ODNs) on the development of experimental peritoneal fibrosis induced by daily intraperitoneal injections of chlorhexidine gluconate (CG).

Methods. HSP47 antisense or sense ODNs were injected simultaneously with CG from day 14, after injections of CG alone. Peritoneal tissue was dissected out 28 days after CG injection. The expression patterns of HSP47, type I and type III collagen, α-smooth muscle actin (α-SMA), as a marker of myofibroblasts, ED-1 (as a marker of macrophages), and factor VIII were examined by immunohistochemistry.

Results. In rats treated with CG alone, the submesothelial collagenous compact zone was thickened, where the expression levels of HSP47, type I and type III collagen and α-SMA were increased. Marked macrophage infiltration was also noted and the number of vessels positively stained for factor VIII increased in the CG-treated group. Treatment with antisense ODNs, but not sense ODNs, abrogated CG-induced changes in the expression of HSP47, type I and III collagen, α-SMA, and the number of infiltrating macrophages and vessels.

Conclusion. Our results indicate the involvement of HSP47 in the progression of peritoneal fibrosis and that inhibition of HSP47 expression might merit further clinical investigation for the treatment of peritoneal fibrosis in CAPD patients.

Continuous ambulatory peritoneal dialysis (CAPD) causes morphologic [1] and functional changes in the peritoneum. Characteristic pathologic findings in the peritoneum of patients with long-term CAPD therapy include marked peritoneal fibrosis and massive accumulation of collagen [2, 3]. In particular, some patients develop encapsulating peritoneal sclerosis (EPS) associated with high mortality, which is one of the most serious complications [4]. The mechanism of peritoneal fibrosis in CAPD patients remains poorly understood and no effective therapy is available at present. Although there is no experimental model for peritoneal fibrosis similar to that of patients on CAPD, Suga et al [5] and Ishii et al [6] developed experimental models for peritoneal fibrosis in rats and EPS in mice, respectively, by peritoneal injection of chlorhexidine gluconate (CG). Many pathologic findings in the peritoneum from CAPD patients, including increased expression of type III collagen, heat shock protein 47 (HSP47), α-smooth muscle actin (α-SMA), and macrophage infiltration, are also observed in the peritoneum of animals injected with CG [7, 8].

HSP47, a collagen-specific molecular chaperone, is essential for the biosynthesis and secretion of procollagen molecules [9], and implicated in diseases associated with hepatic [10] and pulmonary fibrosis [11]. Recent studies reported the presence of high expression levels of HSP47 in various kidney diseases, such as human transplanted kidney with fibrosis [12], renal interstitial fibrosis induced by unilateral ureteral obstruction [13], experimental mesangial proliferative glomerulonephritis [14], age-related nephropathy [15], and gentamicin-induced renal injury [16]. Furthermore, we previously demonstrated the expression of HSP47 in the fibrotic peritoneal tissue from patients on CAPD, and its level correlated well with that of type III collagen [7]. Other studies showed that antisense oligonucleotides (ODNs) of HSP47 inhibited collagen synthesis in 3T3 cells [17] and in experimental proliferative glomerulonephritis induced by anti-thy-1 antibody [18]. These findings suggest the important role of HSP47 in collagen synthesis in various fibrotic disorders.

Mishima et al [8] recently demonstrated that HSP47 expression significantly correlated with accumulation of collagen type I and III in CG-induced peritoneal fibrosis.
In the present study, we investigated the importance of HSP47 in peritoneal fibrosis by examining whether ODNs against HSP47 inhibit the development of CG-induced peritoneal fibrosis. To our knowledge, our study is the first to report that inhibition of HSP47 expression results in suppression of collagen accumulation in an experimental peritoneal fibrosis model. In addition, macrophage infiltration and angiogenesis, which were other characteristic findings in peritoneal fibrosis, were also inhibited by antisense ODNs.

METHODS

HSP47 antisense ODNs

Antisense phosphorothioate ODNs against the first five codons of HSP47 that straddle the predicted translation initiation site of the mouse HSP47 mRNA were used. The antisense and sense ODNs were purchased from Beck’s (Tokyo, Japan).

Animals

Male Wistar rats weighing 250 to 300 g were used in this study. They were housed in standard rodent cages, at constant ambient temperature (22 ± 1°C) and humidity (85%), with 10 hours of lights each day. Drinking water and pelleted rodent food were provided ad libitum. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Nagasaki University School of Medicine.

Experimental protocol

In control experiments, we injected sense or antisense ODNs with 15% ethanol dissolved in 2 mL saline. Peritoneal fibrosis was induced as described previously by Suga et al [4]. Briefly, under ether anesthesia, rats received a daily intraperitoneal injection of 0.1% CG in 15% ethanol dissolved in 2 mL of saline over a period of 28 days (CG group, N = 5). In another group of rats, the same CG preparation was injected intraperitoneally at a similar dose but for only 14 days, followed by injection of antisense or sense ODNs (500 µg/kg) with CG for another 14 days (antisense ODNs–treated group, N = 5; sense ODNs–treated group, N = 5). Rats were sacrificed at 14, 17, 21, or 28 days after the first CG injection and peritoneal tissues were dissected out carefully. Control rats were also injected with 15% ethanol dissolved in 2 mL of saline for 28 days (E-saline group, N = 5). To avoid direct damage to the peritoneum caused by repeated injections, injections were made at the lower part of the peritoneum while the upper portion of the parietal peritoneum was used for histopathologic examination. The harvested tissues were fixed with 4% paraformaldehyde immediately after sampling and embedded in paraffin. For histopathologic examination, paraffin-embedded tissue sections (4 µm thick) were stained with periodic acid Schiff (PAS). We measured the thickness of the submesothelial compact zone above the muscles of the abdominal wall at 5 points in each group and the thickness was presented as mean ± SD.

Examination of ODNs transfer into peritoneal cavity

Using fluorescein isothiocyanate (FITC)–labeled antisense ODNs against HSP47, we evaluated the uptake of antisense ODNs into the peritoneal tissue. FITC-labeled antisense ODNs (Beck’s) were injected into the peritoneal cavity of rats treated with CG for 14 days and of untreated control rats. Peritoneal tissues were dissected out from both groups and embedded in O.C.T. compound (Miles, Inc., Elkhart, IN, USA). The peritoneal specimens were cut to a thickness of 4 µm. The tissue sections were washed in phosphate-buffered saline (PBS) and examined by fluorescence microscopy (Zeiss, Oberkochen, Germany).

Immunohistochemistry

Paraffin-embedded tissue sections (4 µm thick) were stained immunohistochemically using an indirect method. Deparaffinized tissue sections were incubated for 30 minutes with a blocking buffer containing 10% normal goat serum and 10% fetal calf serum (FCS) in PBS. The sections were then reacted for 1 hour with the following primary antibodies, which were diluted in the same incubation buffer: a monoclonal antibody against mouse HSP47, which also reacts with rat HSP47 diluted 1/100 (StressGen, Victoria, British Columbia, Canada); α-smooth muscle actin (α-SMA) diluted 1/500 and used as a marker for myofibroblasts (Dako, Glostrup, Denmark); ED-1 diluted 1/100 and used as a marker for macrophages/monocytes (Serotec, Oxford, UK); a polyclonal antibody against factor VIII diluted 1/200 and used as a marker for blood vessels (von Willebrand factor, A0082, Dako, Glostrup, Denmark); rat type I collagen diluted 1/75 (Chemicon International, Inc., Temecula, CA, USA); rat type III collagen diluted 1/50 (Chemicon International, Inc.); and rat type IV collagen diluted 1/75 (Chemicon International, Inc.). After reacting with the primary antibody, the sections were reacted for 30 minutes with the following secondary antibodies diluted in the same incubation buffer: horseradish peroxidase (HRP)–conjugated rabbit anti-mouse antibody diluted 1/100 (P260, Dako) and HRP-conjugated swine anti-rabbit antibody (P399, Dako) for monoclonal antibodies, and with HRP-conjugated swine anti-rabbit antibody diluted 1/50 (Z0113, Dako) for polyclonal antibodies. Positive reaction with the antibodies was characterized by color development following reaction with H2O2 and 3-3 diaminobenzidine tetrahydrochloride. Finally, the sections were counterstained with methyl green and mounted.

Peritoneal tissues were also immunohistochemically
stained for transforming growth factor-beta 1 (TGF-β1) and vascular endothelial growth factor (VEGF) using a polyclonal antibody against TGF-β1 diluted 1/100 (sc-146; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a polyclonal antibody against VEGF diluted 1/100 (sc-507; Santa Cruz Biotechnology), respectively, followed by avidin-biotin complex kit (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA, USA) according to the protocol provided by the manufacturer.

Negative control studies were performed by using irrelevant immunoglobulins (Ig) of subclasses similar to those of the primary antibodies, such as nonspecific mouse IgG1 (X931, Dako), IgG2 (X943, Dako), and rabbit IgG (X903, Dako), instead of the primary antibodies, which showed no positive cells (data not shown).

**Double immunohistochemical staining**

To identify the types of cells positive for FITC-labeled antisense ODNs of HSP47, double immunostaining for α-SMA/FITC was performed using the method described previously by Shioshita et al. [7], with some modification. After staining for α-SMA using the protocol described above, the sections were washed in PBS to stop the color reaction. They were then incubated for 90 minutes at room temperature with a blocking solution and reacted with a polyclonal antibody against FITC [peroxidase-conjugated rabbit anti-FITC (P0404, Dako)], followed by incubation with HRP-conjugated antibodies as mentioned above. A second chromogen, True Blue (71-00-64; KPL, Gaithersburg, MD, USA), was then applied, resulting in the staining of positive cells in blue. The tissue sections were not counterstained with methyl green, since such counterstaining may interfere with the color from the chromogen.

To examine whether antisense ODNs directly affected cells that expressed HSP47, double staining for FITC and HSP47 was performed as described above. Furthermore, to examine the contribution of ED-1–positive cells to TGF-β expression in the submesothelial compact zone, we performed double immunostaining for ED-1 and TGF-β in the same sections.

**In situ hybridization**

Non-radioactive in situ hybridization was performed according to a modified technique originally developed in our laboratory [19]. Sense and antisense ODNs for rat HSP47 mRNA corresponded to base number 335-373 of mouse HSP47 cDNA. The probe for rat monocyte chemoattractant protein-1 (MCP-1) mRNA corresponded to sequence numbers 83-121 of cDNA. The selected sequences are significantly different from other known sequences deposited in the latest release of gene bank data (GenBank, Release 123, 2001). Briefly, the peritoneal specimens were fixed with 4% paraformaldehyde in PBS. The sections were deproteinized using HCl and proteinase K. After prehybridization, the sections were hybridized with digoxigenin (DIG)-labeled oligonucleotide probe in prehybridization buffer. After washing with 0.075% BRIJ (430 AG-6; Sigma Chemical Co., St. Louis, MO, USA) in 2 x standard sodium citrate (SSC) and 0.5 x SSC, immunohistochemistry was performed to visualize DIG-labeled probe using mouse monoclonal anti-DIG antibody, HRP-conjugated rabbit anti-mouse antibody (P260, Dako), and HRP-conjugated swine anti-rabbit antibody (P399, Dako). Color was developed by reaction with H2O2 and diaminobenzidine. Finally, the sections were counterstained with methyl green and mounted.

To evaluate the specificity of the mRNA signal, we performed three control experiments, including pretreatment of RNase, a study with a sense probe, and a competitive study, as described previously [19]. We also checked for no cross-reaction of mouse monoclonal anti-DIG antibody, HRP-conjugated rabbit anti-mouse antibody, and HRP-conjugated swine anti-rabbit antibody (data not shown).

Furthermore, to examine the intrinsic changes induced by injection of antisense ODNs, we analyzed the expression of 28S rRNA, which is ubiquitously expressed in living cells [20], in peritoneal tissue samples obtained from rats treated with antisense ODNs, sense ODNs, or without any ODNs, by using in situ hybridization as described previously [21].

**Data processing and statistical analysis**

For semiquantitative estimation of the intensity of expression of each protein, image analysis of the stained sections was performed using Olympus Image Analyzer (Olympus, Tokyo, Japan) system as described previously [7]. In brief, the image was transformed into a matrix of 1280 x 1000 pixels, and the voltage signal at each pixel was converted to 1 to 256 intensity gray levels in proportion to the colorimetric staining. By summing the values of pixels in five sections, each measuring 0.14 mm², from different fields of the submesothelial region at ×200 magnification, we could determine the signal density for HSP47, type I and III collagen, and α-SMA. Then, we compared the density of these markers per 0.14 mm² among the four groups. The number of ED-1–positive cells and blood vessels positive for factor VIII were also counted in the same fields at ×200 magnification.

Data are expressed as mean ± SD. Differences among groups were examined for statistical significance using repeated-measures analysis of variance (ANOVA) (Bonferroni/Dunn test). A P value less than 0.05 denoted the presence of statistically significant differences.

**RESULTS**

**Transfer of ODNs into the peritoneal cavity**

To examine the toxicity of antisense ODNs, we injected antisense ODNs alone into the peritoneal cavity...
for 28 days, but no morphologic changes were found (data not shown). The toxicity of antisense and sense ODNs was further checked using an in vitro system. Rat mesothelial cells were incubated with 10% FCS with/without 75 μg/mL of antisense and sense ODNs (same concentrations of antisense ODNs were applied in subsequent in vivo experiments). The supernatants were assessed by measuring lactate dehydrogenase (LDH) levels, as described previously [22]. There was no significant difference in LDH levels in supernatants treated with antisense or sense ODNs (data not shown). In situ hybridization study for 28S rRNA also showed no difference in the expression pattern of 28S rRNA in peritoneal tissues obtained from rats treated with antisense ODNs, sense ODNs, or no ODNs. These data suggested that antisense ODNs were not toxic, as they did not induce any intrinsic changes.

To confirm the uptake of antisense ODNs into the peritoneal tissue, FITC-labeled antisense ODNs were injected into the peritoneal cavity, followed by examination of the peritoneum under fluorescence microscopy. In normal rat peritoneum, very few FITC-positive cells were observed in the peritoneum (data not shown), indicating that FITC did not bind nonspecifically to any cells in the peritoneum. As shown in Figure 1, many spindle shaped cells were positive for FITC in the submesothelial compact zone. Counting of the number of FITC-positive cells and total cells in submesothelial compact zone under immunofluorescence and light microscopy, respectively, showed that 42.3 ± 5.6% of cells in the thickened submesothelial area were positive for FITC-labeled antisense ODNs. Double staining for FITC and α-SMA was performed to determine the type of cells positive for FITC-labeled ODNs (Fig. 1). The staining revealed that 59.6 ± 1.3% of FITC-expressing cells was stained for α-SMA. Furthermore, the proportion of FITC-positive cells among α-SMA–positive cells in the submesothelial compact zone was 52.3 ± 2.1%. Double staining for FITC and HSP47 was performed to recognize FITC-labeled antisense ODNs that directly affected HSP47-expressing cells. Quantitative analysis indicated that 49.3 ± 3.2% of the HSP47-positive cells also expressed FITC in the submesothelial compact zone.

Figure 2 shows representative PAS-stained peritoneal tissue samples. In rats of the E-saline group, the PAS-stained peritoneum consisted of a monolayer of mesothelial cells that covered the surface without any thickening of the peritoneum (Fig. 2A). In rats injected with sense or antisense ODNs with 15% ethanol dissolved in 2 mL saline, the results were similar to those of the E-saline group (data not shown). At days 14 and 28, the peritoneal tissue in the CG group was markedly thickened and showed marked proliferation of collagen fibers (Fig. 2B and C). On the other hand, treatment with HSP47 antisense ODNs (Fig. 2D), but not with the sense ODNs (Fig. 2E), resulted in a decrease in CG-induced peritoneal

![Fig. 1. Uptake of fluorescein isothiocyanate (FITC)-labeled phosphorothioated oligonucleotides (ODNs) in the peritoneum and double staining for FITC (in blue)/α-smooth muscle actin (α-SMA) (in brown) in the same sections. (A) Representative fluorescence photomicrographs of ODNs peritoneal uptake 30 minutes after intraperitoneal injection of FITC-labeled ODNs. (B) Cells positive for FITC are spindle shaped (see inset). (C) Note that α-SMA–positive cells are also positive for FITC. (D) α-SMA–positive cell are not positive for FITC. (A) ×100 magnification; (B, C and D), ×200 magnification.](image1)

![Fig. 3. Immunohistochemistry for heat shock protein 47 (HSP47). In the control group, HSP47 is weakly but significantly expressed in mesothelial cells (see inset, arrowheads) (A). In the chlorhexidine gluconate (CG) group, the number of cells positive for HSP47 is increased in thickened submesothelial compact zone (see inset, spindle-shaped cells are positive for HSP47; arrowheads) (B). Antisense oligonucleotides (ODNs) decreased the expression of HSP47 (C) compared with that in sense ODNs group (D). (A to D, ×400 magnification; A and B insets, ×600 magnification.](image2)
thickening. Reduction of peritoneal thickness was seen in antisense-treated rats from day 21 and significant reduction was observed at day 28. These results were also confirmed upon measurement of the thickness of submesothelial compact zone with image analysis software (control, 6.6 ± 1.6; CG group, 265 ± 31; antisense ODNs, 160 ± 36; sense ODNs, 274 ± 35 μm; P < 0.01 between CG and control, and between CG and antisense ODNs).

Expression of HSP47

Figure 3 shows the results of immunohistochemistry for HSP47. In the control group, HSP47 was weakly but significantly expressed in mesothelial cells (Fig. 3A). In CG- and sense ODNs–treated groups, the number of HSP47-positive cells was higher in the thickened submesothelial compact zone (Fig. 3 B and D), while in antisense ODNs–treated group, the relative number of HSP47-positive cells was lower (Fig. 3C). Morphologically, most HSP47-positive cells were mesothelial cells located on the surface of the peritoneum and spindle-shaped fibroblasts located in the submesothelial compact zone (Fig. 3 A and B, arrowheads). Semiquantitative analysis showed that the expression of HSP47 was significantly higher in the CG group than in the control, while treatment with HSP47 antisense ODNs significantly reduced HSP47 expression (Fig. 4). There was no difference in HSP47 expression between CG and sense ODNs–treated group.

In situ hybridization for HSP47 mRNA

We also examined whether treatment with antisense ODNs inhibited the expression of HSP47 mRNA. In the CG group, the expression of HSP47 mRNA was evident in the thickened submesothelial compact zone (Fig. 5A), while it was markedly inhibited by treatment with antisense ODNs of HSP47 (Fig. 5B). Sense ODNs did not alter the enhanced expression of HSP47 mRNA. In the control group, only a weak expression of HSP47 mRNA was noted in some mesothelial cells (data not shown).
Expression of types I, III, and IV collagen

Figure 6 shows the results of immunohistochemistry for type III collagen. The expression of type III collagen was very weak in the control group (Fig. 6A). In the CG group, the expression of type III collagen was evident in the thickened submesothelial compact zone at days 14 and 28 (Fig. 6B and C), while this expression was clearly decreased in the antisense ODNs–treated group (Fig. 6D). No difference in type III collagen expression was observed between the CG and sense ODNs–treated groups. As for type I collagen, the expression pattern was similar to that of type III collagen (Fig. 6E and F), while the expression of type IV collagen was absent in the submesothelial compact zone in this experiment (data not shown). Semiquantitative analysis showed that the expression of type I and III collagen was higher in the CG group, but significantly lower in antisense ODNs–treated group compared with that in CG and sense ODNs–treated groups (Fig. 7A and B).

Expression of α-SMA, ED-1, and MCP-1 mRNAs

To identify the type of cells abundantly present in the submesothelial area of the peritoneum of the CG group, we performed immunohistochemistry for α-SMA, a marker for activated fibroblasts (myofibroblasts) and ED-1, a marker for macrophages. In the control group, staining for α-SMA was present only in the blood vessel wall and no positive cells for ED-1 were detected in the peritoneum (data not shown). In the CG group, α-SMA was highly expressed in the markedly thickened compact zone (Fig. 8A), while the expression was markedly decreased in the antisense ODNs–treated group (Fig. 8B). ED-1–positive cells were present in the thickened compact zone in CG group (Fig. 8C), while their number was reduced in the antisense ODNs–treated group (Fig. 8D). Table I summarizes the results of quantitative analysis of the number of α-SMA–positive and ED-1–positive...
Fig. 8. Immunohistochemistry for (A and B) α-smooth muscle actin (α-SMA), (C and D) ED-1, and (E and F) in situ hybridization for monocyte chemotactic protein-1 (MCP-1) mRNA. In chlorhexidine gluconate (CG) group, note the strong expression of α-SMA in the markedly thickened submesothelial compact zone (A), while such expression is decreased in antisense group (B). (C) In CG group, ED-1–positive cells are present in thickened submesothelial compact zone. (D) The number of ED-1–positive cells is reduced in antisense ODNs group. In CG group, the expression of MCP-1 mRNA is evident in the thickened submesothelial compact zone (E), while it is inhibited by antisense treatment (F). Magnification, ×400.

cells in the peritoneum of each group. The analysis showed increased expression of α-SMA and ED-1 in the CG group and sense ODNs–treated rats relative to the control and antisense ODNs–treated rats.

To determine the mechanism of the reduced number of infiltrating macrophages in rats treated with antisense ODNs of HSP47, we examined the expression of MCP-1 mRNA, which causes strong chemotaxis of macrophages. In the CG group, the expression of MCP-1 mRNA was evident in the thickened submesothelial compact zone (Fig. 8E), where macrophages were markedly infiltrated, while it was inhibited by antisense treatment (Fig. 8F).

Expression of factor VIII (von Willebrand factor)

Previous studies suggested that the number of blood vessels increased in peritoneal sclerosis and that neovascularization was involved in the development of peritoneal fibrosis [23]. We also examined the effect of antisense ODNs treatment on neovascularization in our model. Many vessels were observed in the CG group (Fig. 9A), while their number was decreased in antisense ODNs–treated rats (Fig. 9B). Table 1 shows the number of factor VIII–positive vessels in the peritoneum of each group. The number of vessels stained for factor VIII was significantly higher in the CG group than in the control group. Treatment with antisense, but not sense of HSP47, significantly reduced the number of vessels positive for factor VIII compared with that in the CG group.

Expression of TGF-β and VEGF

We examined the expression of TGF-β and VEGF by immunohistochemistry. The numbers of TGF-β–positive cells were increased in the thickened peritoneum in the CG group (Fig. 10A), but markedly reduced in antisense–treated group (Fig. 10B). We also examined the types of cells that expressed TGF-β in the thickened submesothelial area under a higher magnification. Expression of TGF-β was identified mainly on fibroblasts, although it was also detected on mononuclear cells, spindle-shaped fibroblasts, and some mesothelial cells. Double staining for TGF-β and ED-1 confirmed that some ED-1–positive cells were also positive for TGF-β, indicating that some macrophages express TGF-β (data not shown). Finally, the distribution of VEGF expression was similar to that of TGF-β.

DISCUSSION

In the present study, we confirmed the high expression level of HSP47 during the progression of peritoneal fibrosis induced by intraperitoneal CG injection. Administration of HSP47 antisense ODNs markedly reduced the expression of HSP47 and prevented the progression of peritoneal fibrosis in the rat model. Antisense treatment commenced 2 weeks after injection of CG, when peritoneal fibrosis had already begun, indicating the inhibitory effect of HSP47 antisense ODNs on collagen synthesis under the condition of enhanced collagen accumulation. These findings suggest that the use of HSP47 antisense ODNs could be potentially useful for suppressing the progression of peritoneal fibrosis.

Although the pathogenesis of peritoneal sclerosis in CAPD patients is still unclear, many factors are considered to be involved in the development of peritoneal fibrosis. These include endotoxin [1, 24], acetate dialysate [1, 2, 24], the use of antiseptics [24, 25], advanced glycation end products [26], and carbonyl modifications of peritoneal tissue [27]. In the present study, we used a rat experimental model of peritoneal fibrosis induced by CG. Whether this model is relevant to the peritoneal sclerosis and encapsulating peritoneal sclerosis seen in CAPD patients remains to be determined. However, it can be assumed that certain common pathways exist
during the development of peritoneal fibrosis between our model and the CAPD patients. In fact, following injection of CG as a nonspecific chemical to induce peritoneal fibrosis, many pathologic findings in the peritoneum of CAPD patients (e.g., increased expression of collagen type III, HSP47, α-SMA, and macrophage infiltration) [7], were also observed in our model. Although this model does not completely mimic human disease, further studies are necessary to confirm the inhibitory effects of HSP47 antisense ODNs on the progression of peritoneal fibrosis in another model of human peritoneal fibrosis. Furthermore, in addition to studying the morphologic changes induced by HSP47 antisense ODNs, it is also important to examine any functional changes in the peritoneum induced by such treatment.

There are two possible mechanisms for the inhibitory effects of antisense ODNs in the present study: (1) Inhibition of translation. The sites of action of antisense ODNs used in the present study are the first five codons of HSP47 mRNA, which are the translation-initiation site. The binding of antisense ODNs to this site may inhibit the translation and subsequently reduce the expression of HSP47 protein, as shown in the present study; and (2) degradation of mRNA by RNase. The binding of antisense ODNs to mRNA allows RNase H, the substrate of which are RNA/DNA duplex hybrids, to cleave the mRNA of the hybrids [28]. In fact, we showed that the expression of HSP47 mRNA in the peritoneum was markedly reduced by antisense ODNs in our model. Thus, antisense ODNs can potentially inhibit the expression of HSP47 mRNA and protein in the cells of the submesothelial compact zone. Examination of the type of cells positive for antisense ODNs showed that 59.6% of the FTIC-positive cells were α-SMA-positive myofibroblasts. Since myofibroblasts are considered to be the main site of production of collagen in peritoneal fibrosis and HSP47 is a collagen-specific chaperone, a rather good uptake efficacy of antisense ODNs by peritoneal injection inhibited the expression of collagen and prevented the progression of peritoneal fibrosis in the present study.

We have previously shown a biphasic response of macrophages in CG-induced peritoneal fibrosis [8], an initial increase in the number of macrophages followed by a fall in their number in advanced stages of fibrosis, similar to the pattern seen in human biopsy specimens of peritoneal sclerosis [7]. In this regard, Williams et al [3] demonstrated in human biopsy specimens a lack of significant infiltration of macrophages in samples collected from CAPD patients. Differences in the pathologic stage of peritoneal fibrosis may explain the discrepancies between the studies. In the present study, treatment of rats with antisense ODNs resulted in a reduction of the number of infiltrating macrophages concurrent with inhibition of progression of peritoneal fibrosis. Although the underlying mechanism of antisense ODNs–induced reduction of infiltrating macrophages in our model is not clear, our in situ hybridization study showed a decrease in MCP-1 mRNA expression with reduction of collagen in the peritoneum of rats treated with antisense ODNs against

**Table 1. Results of immunohistochemistry for α-SMA, ED-1, and factor VIII**

<table>
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<th>Control</th>
<th>CG</th>
<th>Antisense ODNs</th>
<th>Sense ODNs</th>
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<tr>
<td>Expression of α-SMA</td>
<td>214.5 ± 111.5</td>
<td>1714 ± 1023*</td>
<td>356 ± 176*</td>
<td>1208 ± 289*</td>
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<tr>
<td>ED-1-positive cells</td>
<td>2.1 ± 1.4</td>
<td>73.8 ± 21.7*</td>
<td>48.7 ± 7.6*</td>
<td>82.8 ± 22.9*</td>
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<tr>
<td>Number of vessels</td>
<td>1.9 ± 1.6</td>
<td>67.4 ± 18.8*</td>
<td>33.1 ± 8.7*</td>
<td>74.3 ± 22.6*</td>
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Abbreviations are: α-SMA, α-smooth muscle actin; CG, chlorhexidine gluconate; ODN, oligonucleotide; HSP47, heat shock protein 47.

Image analysis determined the signal density for α-SMA by summation of the values of pixels in five fields in the submesothelial region (magnification ×200). The numbers of ED-1-positive cells and factor VIII–positive vessels were determined in five fields selected at random in the submesothelial region (magnification ×200). Data are mean ± SD.

*P < 0.01 vs. control; †P < 0.01 vs. CG and HSP47 sense ODNs.
HSP47. This is consistent with the results of previous in vitro studies, which showed that collagen stimulated MCP-1 expression in hepatic stellate and endometrial cells [29, 30]. We can therefore speculate that HSP47 antisense ODNs can indirectly influence MCP-1 expression in the peritoneum by mediating collagen accumulation and subsequent reduction of the number of infiltrating macrophages. Recent studies provided evidence for the role of MCP-1 on the progression of tubulointerstitial fibrosis in the kidney through macrophage recruitment and activation [31, 32]. These studies emphasize the importance of inflammation in the progression of fibrosis. Although the relationship between inflammatory process and development of (encapsulating) peritoneal sclerosis in human remains unclear at present, further studies are necessary to clarify the relationship between inflammation and peritoneal fibrosis. If inflammation drives the fibrotic process under long-term peritoneal dialysis therapy, measurements of pro-inflammatory cytokines in the effluent could serve as a marker for the progression of human peritoneal sclerosis. Further studies are needed to determine the role of inflammatory cells, such as T cells and B cells, in this process.

Various vessel-related abnormalities have been reported in peritoneal fibrosis [33, 34]. The results of these studies suggested the involvement of vascular abnormalities during peritoneal fibrosis. In the present study, HSP47 antisense ODNs reduced the number of vessels and decreased the peritoneal thickness; however, the relationship between peritoneal fibrosis and angiogenesis remains poorly understood. In this regard, previous in vitro studies showed that culture of endothelial cells with collagen type I and III enhanced angiogenesis [35–38], indicating the importance of collagen in the initiation of angiogenesis. Since HSP47 is a collagen-specific molecular chaperone, it is speculated that antisense ODNs indirectly decreased the number of vessels through the reduction of collagen synthesis in our model. In the present study, we showed a reduction of VEGF expression in rats treated with antisense ODNs of HSP47, associated with reduction of peritoneal thickness. On the other hand, Margetts et al [39] demonstrated that treatment with angiostatin, an inhibitor of angiogenesis, resulted in a reduction of vessel number in a rat model of peritoneal fibrosis without decrement of peritoneal thickness [39]. The relationship between angiogenesis and fibrosis is still controversial. Further studies are warranted to clarify the involvement of angiogenesis in the progression of peritoneal fibrosis.

CONCLUSION

We demonstrated that treatment with HSP47 antisense ODNs results in amelioration of in vivo progression of CG-induced peritoneal fibrosis in rats. Our results suggest that HSP47 is an important molecule in the progression of peritoneal fibrosis. Further studies are necessary to confirm the effects of HSP47 in other models of human peritoneal sclerosis and clarify the suitability of HSP47 as a molecular target for the treatment of patients on CAPD with peritoneal fibrosis.

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