Kidney International, Vol. 57 (2000), pp. 619–631

Expression of heat shock proteins 47 and 70 in the peritoneum of patients on continuous ambulatory peritoneal dialysis

KEI SHIOSHITA, MASANOBU MIYAZAKI, YOSHIYUKI OZONO, KATSUSHIGE ABE, KOUICHI TAURA, TAKASHI HARADA, TAKEHIKO KOJI, TAKASHI TAGUCHI, and SHIGERU KOHNO

The Second Department of Internal Medicine, Division of Renal Care Unit, Department of Histology and Cell Biology, and Second Department of Pathology, Nagasaki University School of Medicine; and Nagasaki Municipal Chronic Disease Center, Nagasaki, Japan

Expression of heat shock proteins 47 and 70 in the peritoneum of patients on continuous ambulatory peritoneal dialysis.

Background. Peritoneal sclerosis, characterized by collagen accumulation, is a serious complication in continuous ambulatory peritoneal dialysis (CAPD) therapy. Heat shock protein 47 (HSP47) is a collagen-specific molecular chaperon and is closely associated with collagen synthesis.

Methods. We determined the expression of HSP47 and HSP70 (nonspecific for collagen synthesis) by immunohistochemistry in peritoneal tissues of patients on CAPD. The tissue for collagen III, α-smooth muscle actin (α-SMA), and CD68 (a marker for macrophages) were also stained. Thirty-two peritoneal samples were divided into three groups (group A1, 11 patients who had no ultrafiltration loss; group A2, 9 patients who had ultrafiltration loss; and group B, 12 specimens who had end-stage renal disease prior to induction of CAPD.

Results. In group B, staining for HSP47, HSP70, and collagen III in peritoneal tissues was faint, and only a few cells were positive for α-SMA and CD68. In contrast, HSP47, HSP70, and collagen III were expressed in areas of thickened connective tissues in fibrotic peritoneal specimens of CAPD patients. The expression level of HSP47, HSP70, collagen III, and α-SMA and the number of CD68-positive cells in group A2 were significantly higher than those in groups A1 and B. HSP47/HSP70-positive cells were mesothelial cells, adipocytes, and α-SMA–positive myofibroblasts. Furthermore, the expression level of HSP47 was significantly higher in peritoneal specimens from patients with refractory peritonitis than without it and was significantly higher in patients with more than 60 months of CAPD therapy than that in patients with less than 60 months of CAPD.

Conclusion. Our results indicate that CAPD therapy may induce HSPs in the peritoneal tissue, and that peritonitis in CAPD patients may be associated with the progression of peritoneal sclerosis at least through HSP47 expression and chronic macrophage infiltration. Our data also suggest that the progression of peritoneal sclerosis in such patients is associated with deterioration of peritoneal ultrafiltration function.

Key words: peritoneal ultrafiltration, sclerosis, end-stage renal failure, collagen synthesis, fibroblasts.

Received for publication February 22, 1999
and in revised form August 6, 1999
Accepted for publication September 9, 1999

© 2000 by the International Society of Nephrology

Continuous ambulatory peritoneal dialysis (CAPD) has been used in the treatment of end-stage renal failure during the last two to three decades. Peritoneal sclerosis was first described in 1980 [1] and is one of the most important complications of CAPD therapy. Especially, some patients develop encapsulating peritoneal sclerosis (EPS), a condition associated with high mortality [2, 3]. Although it is still uncertain whether EPS is an advanced stage of peritoneal sclerosis, the characteristic pathological feature of EPS is marked peritoneal sclerosis [2]. Peritoneal sclerosis usually occurs in patients who have been on peritoneal dialysis for several years [2], but it occasionally develops in patients who have been on CAPD for as little as six months [4]. One of the characteristic pathological features of peritoneal sclerosis is a massive accumulation of collagen; however, the mechanism of development and the progression of peritoneal sclerosis remain to be elucidated. Clinically, loss of ultrafiltration is one of the most important problems in patients with CAPD. The relationship between morphological changes in the peritoneum of CAPD patients and ultrafiltration loss is also unclear.

Heat shock proteins (HSPs) are synthesized under various forms of stress and are important in various physiological and pathological processes [5]. HSPs are molecular chaperones responsible for protein processing and protection against cellular injury by preventing inappropriate peptide interaction. Recent studies have demonstrated that one HSP, a 47 kd HSP (HSP47), is involved in collagen production. HSP47 is a collagen-binding stress protein [6] and acts as a collagen-specific molecular chaperone during the synthesis and/or secretion of procollagen [7]. Using Northern blotting, Masuda et al showed that the level of HSP47 mRNA correlated with that of

collagen type I and type III mRNAs during the progression of rat experimental liver fibrosis [8]. Moreover, using *in situ* hybridization, they also demonstrated that the distribution of HSP47 mRNA was similar to that of collagen type I and type III mRNAs in the model of liver fibrosis [8]. Similarly, in several renal diseases, including renal interstitial fibrosis induced by unilateral ureteral obstruction [9], experimental mesangial proliferative glomerulonephritis [10], age-related nephropathy [11], gentamicin nephrotoxicity [12], and human transplanted kidney with fibrosis (abstract; Abe et al, *J Am Soc Nephrol* 8:718A, 1997), the expression of HSP47 also correlates with the degree of collagen expression. Furthermore, Sunamoto et al showed that antisense oligonucleotides for HSP47 inhibited collagen synthesis in experimental mesangial proliferative glomerulonephritis induced by anti–Thy-1 antibody [13]. These findings suggest that HSP47 probably plays an important role in collagen synthesis in various disorders.

Like several other HSPs, HSP70 is induced by heat shock, hyperthermia, ischemia, toxic chemicals, and other environmental stresses, as well as during normal cell growth [6]. It acts as a molecular chaperone that mediates the correct assembly and localization of proteins [14]. HSP70 has a different inducible regulatory mechanism from HSP47 [15] and is not associated with collagen synthesis. To date, there are no data on the expression of HSP47 and HSP70 in the peritoneal cavity.

In this study, using immunohistochemistry, we examined the expression of HSP47 and HSP70 in human peritoneal specimens from patients on CAPD. To our knowledge, this is the first report that shows that peritoneal fibroblasts express HSP47 and that the expression level of HSP47 appears to correlate with the degree of peritoneal sclerosis.

METHODS

Patients

Thirty-two individuals were examined in this study (Tables 1 and 2). Twenty peritoneal biopsies were obtained from patients in whom CAPD had been discontinued. The clinical data of these patients are summarized in Table 1. CAPD patients were divided into two groups according the presence or absence of ultrafiltration loss: group A1 ($N = 11$), representing those without ultrafiltration loss, and group A2 ($N = 9$), representing those with ultrafiltration loss. CAPD was discontinued in patients of group A1 because of renal transplantation, abdominal operation, or the refractory or recurrent infectious peritonitis. "Recurrent" and "refractory" peritonitis were defined according to the recent report of Pirano [16]. Recurrent peritonitis was defined as a second episode of peritonitis caused by an organism identical to that causing the first episode within two weeks of discontinuation of antibiotics. Refractory peritonitis was defined as an episode associated with no clinical improvement five days after the commencement of appropriate antibiotic therapy. CAPD was also discontinued in patients of group A2 because of ultrafiltration failure. One patient (number 14) of group A2 developed EPS with ileus. Serum levels of C-reactive protein were negative at the time of peritoneal biopsy in patients with recurrent or refractory peritonitis episodes. The mean duration of CAPD therapy in group A1 (53.5 ± 40.8 months, $\pm$ SD) was not different from that of group A2 (78.8 ± 35.0 months). The mean age of patients of groups A1 (50.8 ± 15.2) was not different from that of group A2 (50.9 ± 11.9 years). The mean number of episodes of peritonitis in group A1 during CAPD therapy (3.4 ± 3.4) was not different from that of group A2 (3.2 ± 0.8). Table 2 shows the glucose concentration in dialysate used in our patients. Peritoneal equilibration test (PET) was performed in nine patients. Four of nine patients of group A2 showed type I membrane failure, and 5 of 11 patients in group A1 showed below average in PET.

Twelve peritoneal specimens obtained from the site of insertion of the catheter for CAPD therapy prior to CAPD were used as control (group B; Table 3). The mean age of patients of group B was 46.0 ± 13.7 years, which was not significantly different from that of group A. None of the patients in group B had previously been on peritoneal dialysis or hemodialysis. The serum albumin level was 3.50 ± 0.38 mg/dL, 3.17 ± 0.77 mg/dL and 3.74 ± 0.25 mg/dL in groups A1, A2, and B, respectively. The level of serum albumin in group A2 was significantly lower than in group B ($P < 0.05$).

The study protocol was approved by the Human Ethics Review Committee of Nagasaki University School of Medicine, and a signed consent form was obtained from each subject.

Tissue preparation

Biopsy specimens were carefully obtained from the parietal peritoneum at the border of the anterior abdominal wall incision in elective or catheter-related operation, as described by Suassuna et al [17]. Harvested tissues were fixed with 10% formalin and were embedded in paraffin.

Immunohistochemistry

Paraffin-embedded tissue sections (4 μm thick) were stained immunohistochemically using the avidin-biotin complex kit (Vectastain Elite ABC KIT; Vector Laboratories, Burlingame, CA, USA). Deparaffinized tissue sections were reacted with the following primary antibodies: a monoclonal antibody against mouse HSP47 (StressGen, Victoria, Canada), which reacts with human HSP47 [18]; monoclonal antibody against human α-smooth muscle actin (α-SMA; Dako, Glostrup, Denmark), used as a marker

Table 1. Clinical data of group A patients treated with continuous ambulatory peritoneal dialysis

No.	Age/sex	Duration of CAPD *months*	Causes of catheter removal	No. of episodes of peritonitis	UF volume *mL/day*	BUN *mg/dL*	Cr *mg/dL*	TP *mg/dL*	Alb *mg/dL*
Group A1									
1	30/F	16	renal transplantation	0	1,000	47.5	9.2	6.1	3.9
2	36/M	31	renal transplantation	0	1,400	27	2.7	5.2	3.4
3	54/M	18	abdominal operation	1	1,000	64	7.9	5.8	3.5
4	70/F	67	recurrent peritonitis	8	1,200	71	12.2	6.9	3.6
5	60/M	64	cerebral infarction	3	700	36	6.8	6.1	3.4
6	60/F	20	tunnel infection	0	1,000	43	14.2	5.1	2.6
7	77/M	160	refractory peritonitis	2	1,000	24	7.2	5.5	3.1
8	39/M	53	tunnel infection	5	1,000	91	11.2	5.6	3.7
9	40/M	66	recurrent peritonitis	8	800	57	10.9	6.6	3.8
10	55/F	62	tunnel infection	2	900	33	5.2	5.9	3.7
11	38/M	31	tunnel infection	5	1,000	69	12.5	5.8	3.8
Group A2									
12	41/F	49	UF loss	2	470	81	9.9	6.1	3.1
13	42/F	88	UF loss	3	300	71	11.6	5.9	3.1
14	44/F	88	UF loss	3	0	70	4.5	4.6	2.8
15	75/M	59	UF loss[a]	2	400	68	9.3	6.1	3.1
16	50/M	60	UF loss[a]	4	0	40	9.1	5.4	2.9
17	40/M	38	UF loss	4	450	89	11.9	8.1	5.1
18	47/F	132	UF loss	4	300	47	6.5	5.9	2.9
19	55/M	60	UF loss[a]	4	300	107	11.1	5.6	2.3
20	64/M	135	UF loss	3	400	42	10.1	6.6	3.2

Age is in years. The cause of CRF was chronic glomerulonephritis in all patients. Abbreviations are: UF loss, ultrafiltration loss; TP, total protein; Alb, serum albumin.
[a] These group A2 patients had refractory or recurrent peritonitis episodes

Table 2. Glucose concentration of dialysate and results of the peritoneal equilibration test (PET) in group A patients

	CAPD menu *glucose concentration (%), volume (L), × times*	PET category D/D0	PET category D/P_{Cr}
Group A1			
1	1.5, 2 ×4	LA	LA
2	1.5, 2 ×3/2.5, 2 ×1	L	L
3	1.5, 2 ×3/2.5, 2 ×1	L	L
4	1.5, 2 ×2/2.5, 2 ×2	LA	LA
5	1.5, 2 ×2/2.5, 2 ×2	LA	LA
6	1.5, 2 ×3/2.5, 2 ×1	NA	NA
7	1.5, 2 ×3/2.5, 2 ×1	NA	NA
8	2.5, 2 ×4/1.5, 2 ×1	NA	NA
9	2.5, 1.5 ×4	NA	NA
10	1.5, 2 ×2/2.5, 2×2	NA	NA
11	2.5, 2 ×4	NA	NA
Group A2			
12	1.25, 2 ×2/4.25, 2 ×2	HA	H
13	2.5, 2 ×4	HA	HA
14	4.0, 2 ×4	NA	NA
15	2.5, 2 ×4	HA	HA
16	2.5, 2 ×4	HA	HA
17	2.5, 2 ×4/1.35, 2 ×1	NA	NA
18	2.5, 2 ×4	NA	NA
19	2.5, 2 ×4/1.5, 2 ×1	NA	NA
20	2.5, 2 ×2/4.25, 2 ×2	NA	NA

Abbreviations are: D/D0, 4 h/0 h dialysate glucose concentration ratio; D/PCr, dialysate/plasma creatinine ratio; L, low; LA, low average; HA, high average; H, high; NA, not available.

for myofibroblasts [19]; monoclonal antibody against CD68 (Dako), used as a marker for macrophages/monocytes; and monoclonal antibody against human type III collagen (Fuji Chemical Co., Tokyo, Japan). Because several HSPs are expressed in a variety of tissues under various forms of stress, we also stained the tissues for HSP70 using a monoclonal antibody against HSP70 (StressGen). Negative control studies were performed by using irrelevant monoclonal antibodies of the same subclass, nonspecific mouse IgG1 (Dako) and IgG2 (Dako) instead of primary antibodies, which showed no positive cells (data not shown).

To identify myofibroblasts among the population of cells positive for HSP47 in peritoneal specimens, double immunolabeling for HSP47 and α-SMA was performed with some modification using the methods described by Furusu et al [20]. Briefly, deparaffinized peritoneal sections were incubated with blocking serum and anti–α-SMA antibody. Then the sections were washed with phosphate-buffered saline and incubated with the biotinylated antimouse antibody. After washing, sections were incubated with alkaline phosphatase-conjugated avidin (Vector), following a reaction with BCIP/NBT (Dako). Cells positive for α-SMA were stained in blue color. After staining for α-SMA, tissue sections were stained for HSP47 using the avidin-biotin complex kit, as described earlier in this article. HSP47 protein was stained brown.

To estimate the signal intensity for each protein semi-

Table 3. Clinical data of group B patients treated with continuous ambulatory peritoneal dialysis

Case no.	Age *years*	Sex	Cause of CRF	BUN	Cr	TP	Alb
				mg/dL			
1	24	F	CGN	57	8.2	6.0	3.9
2	24	M	CGN	70	8.7	5.4	3.5
3	41	M	CGN	71	8.1	6.2	3.8
4	60	M	CGN	67	10.1	5.6	3.8
5	64	M	NS	71	7.1	5.9	3.8
6	34	M	CGN	76	9.9	6.4	4.2
7	41	M	CGN	87	8.5	5.5	3.5
8	58	F	CGN	74	9.5	6.0	3.7
9	58	M	CGN	70	8.9	5.6	3.2
10	44	M	CGN	118	17.4	6.2	3.8
11	55	M	CGN	87	8.1	6.7	3.9
12	49	F	CGN	92	7.2	6.5	3.8
Mean ± SD	46.0 ± 13.7			78.3 ± 15.9	9.31 ± 2.7	6.0 ± 0.4	3.7 ± 0.2

Abbreviations are: CGN, chronic glomerulonephritis; NS, nephrotic syndrome.

quantitatively, image analysis of stained sections was performed using an Olympus Image Analyzer system. The image was transformed into a matrix of 1280×1000 pixels, and the voltage signal at each pixel was converted to 1 of 256 intensity gray levels in proportion to the colorimetric staining. By summation of the values of pixels in five fields contained submesothelial region at $\times$ 200 magnification, we could determine the signal density for HSP47, HSP70, α-SMA, and type III collagen. Fat tissue, vessel, and muscle were excluded in the field. Then the density of these markers per 0.14 mm^2 were compared among the three groups. The number of CD68-positive cells was also counted in the same field at ×200 magnification.

Statistical analysis

Data were expressed as mean ± SD. Differences among groups were examined for statistical significance using one-way analysis of variance with Scheffe's *F*-test. Correlation coefficients were tested for significance using the Spearman rank correlation test. A *P* value of less than 0.05 denoted the presence of a statistically significant difference.

RESULTS

Hematoxylin-eosin staining

Histologic examination of hematoxylin-eosin–stained sections of group B showed a monolayer of mesothelial cells covering the surface of the peritoneum (Fig. 1A, B), as well as the presence of small blood vessels scattered among collagen fibers. In group A1 patients, the peritoneal tissue was partly thickened by proliferation of collagen fibers (Fig. 1C). In group A2, mesothelial cells were mostly detached, and peritoneal tissue was markedly thickened by proliferation of collagen fibers with occasional hyalinous changes (Fig. 1D, E). No polymorphonuclear cells were observed even in specimens from patients with tunnel infection and recurrent or refractory peritonitis episodes.

Immunohistochemistry

Peritoneal sections obtained prior to CAPD. A weak immunoreactivity for HSP47 was observed in the submesothelial region (Fig. 2A, B). Almost all mesothelial cells were negative for HSP47, although some occasionally expressed HSP47 (Fig. 2C). HSP70 and type III collagen were hardly detected in peritoneal specimens obtained prior to CAPD therapy (Fig. 2D, E). No cells positive for CD68 were detected (Fig. 2F), and staining for α-SMA was present only in blood vessel walls (Fig. 2G).

Peritoneal tissue from CAPD patients. In peritoneal tissues of patients of group A1, HSP47 and type III collagen were expressed in the thickened connective tissue of the submesothelial region (Fig. 3A, B, E). The remaining mesothelial cells strongly expressed HSP47 (Fig. 3C). HSP70 was also expressed in the thickened submesothelial region (Fig. 3D) and mesothelial cells, and CD68-positive cells were occasionally detected (Fig. 3F). Many cells were positive for α-SMA in the submesothelial area (Fig. 3G). In group A2, peritoneal tissues showed intense immunoreactivity for both HSP47 and HSP70 (Fig. 4A–C). Intense staining for type III collagen was also observed in the area (Fig. 4D). Moreover, several cells positive for CD68 were present in markedly thickened areas with abundant proliferation of collagen fibers (Fig. 4E). α-SMA was expressed very strongly in the markedly thickened submesothelial area (Fig. 4F).

In order to identify the type of cells that expressed HSP47, we performed immunohistochemistry for HSP47 and α-SMA, a marker for activated fibroblasts (myofibroblasts) using serial sections (Fig. 5). Moreover, for precise localization of HSP47 and α-SMA, we performed double staining for these markers (Fig. 6). From the

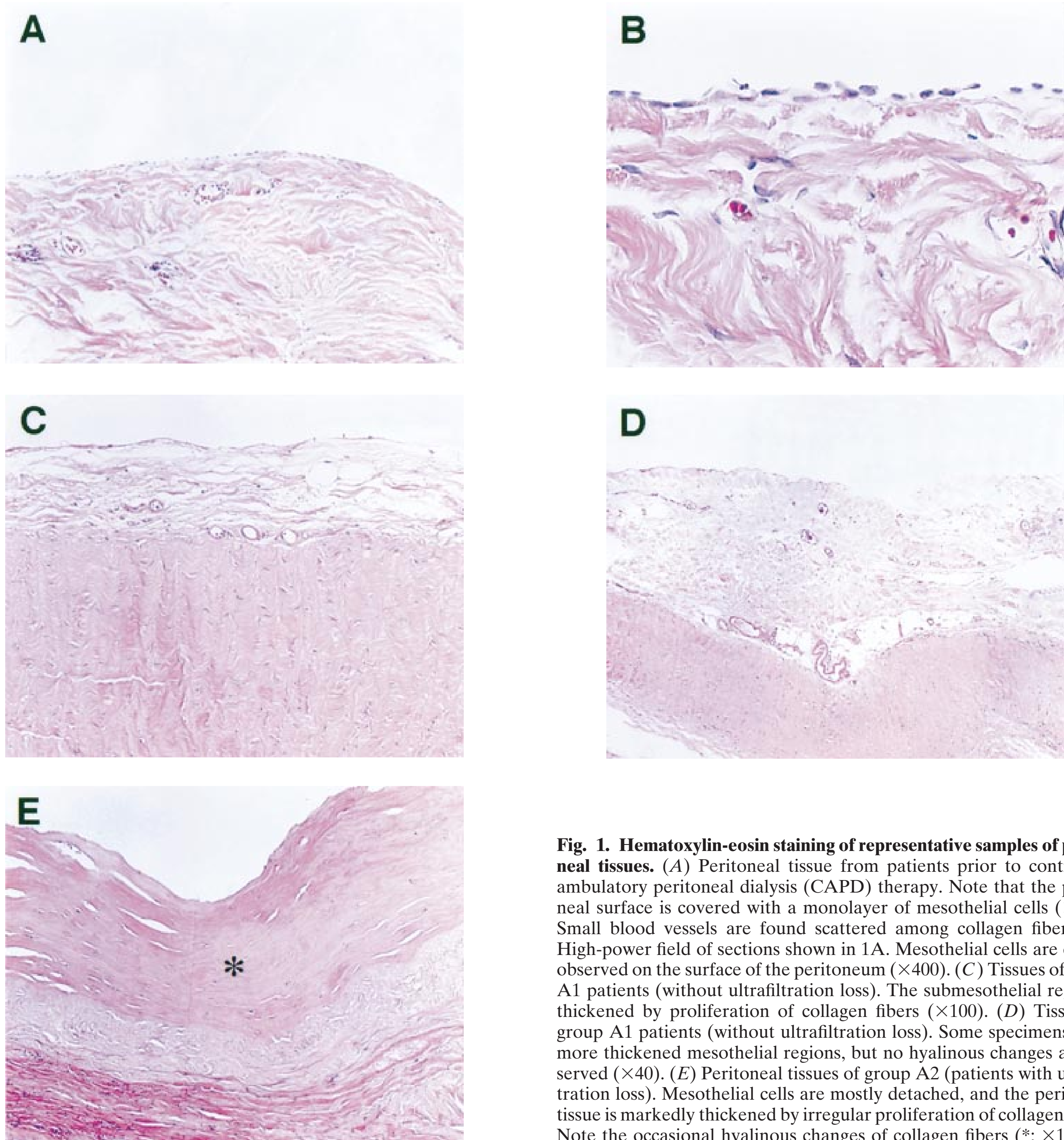

Fig. 1. Hematoxylin-eosin staining of representative samples of peritoneal tissues. (*A*) Peritoneal tissue from patients prior to continuous ambulatory peritoneal dialysis (CAPD) therapy. Note that the peritoneal surface is covered with a monolayer of mesothelial cells (×100). Small blood vessels are found scattered among collagen fibers. (*B*) High-power field of sections shown in 1A. Mesothelial cells are clearly observed on the surface of the peritoneum (×400). (*C*) Tissues of group A1 patients (without ultrafiltration loss). The submesothelial region is thickened by proliferation of collagen fibers (×100). (*D*) Tissues of group A1 patients (without ultrafiltration loss). Some specimens show more thickened mesothelial regions, but no hyalinous changes are observed (×40). (*E*) Peritoneal tissues of group A2 (patients with ultrafiltration loss). Mesothelial cells are mostly detached, and the peritoneal tissue is markedly thickened by irregular proliferation of collagen fibers. Note the occasional hyalinous changes of collagen fibers (*; ×100).

results of staining in serial sections (Fig. 5) and double staining (Fig. 6), the majority of cells that expressed α-SMA were also positive for HSP47. However, the distribution of HSP70-positive cells was not always identical to those positive for α-SMA. A comparison between localization of HSP47 and cells positive for CD68, a marker for macrophages, in serial sections showed that HSP47 was expressed in the area containing many infiltrating macrophages (Fig. 7). A comparison of expression of HSP47 and type III collagen in adjacent sections showed that some cells were stained for HSP47 and collagen III, whereas others were stained for only one of these proteins (data not shown). Figure 8 shows that HSP47 and collagen III were also expressed in adipocytes present in the fat tissue. Moreover, adipocytes were stained for HSP70 (data not shown).

Table 4 compares the level of expression of HSP47, HSP70, collagen III, α-SMA, and CD68 among the three groups. Because fat tissue, vessel, and muscle were excluded in the selected areas, almost all signal intensity of HSP47, HSP70, collagen III, α-SMA shown here represented the expression in collagen fibers. The expres-

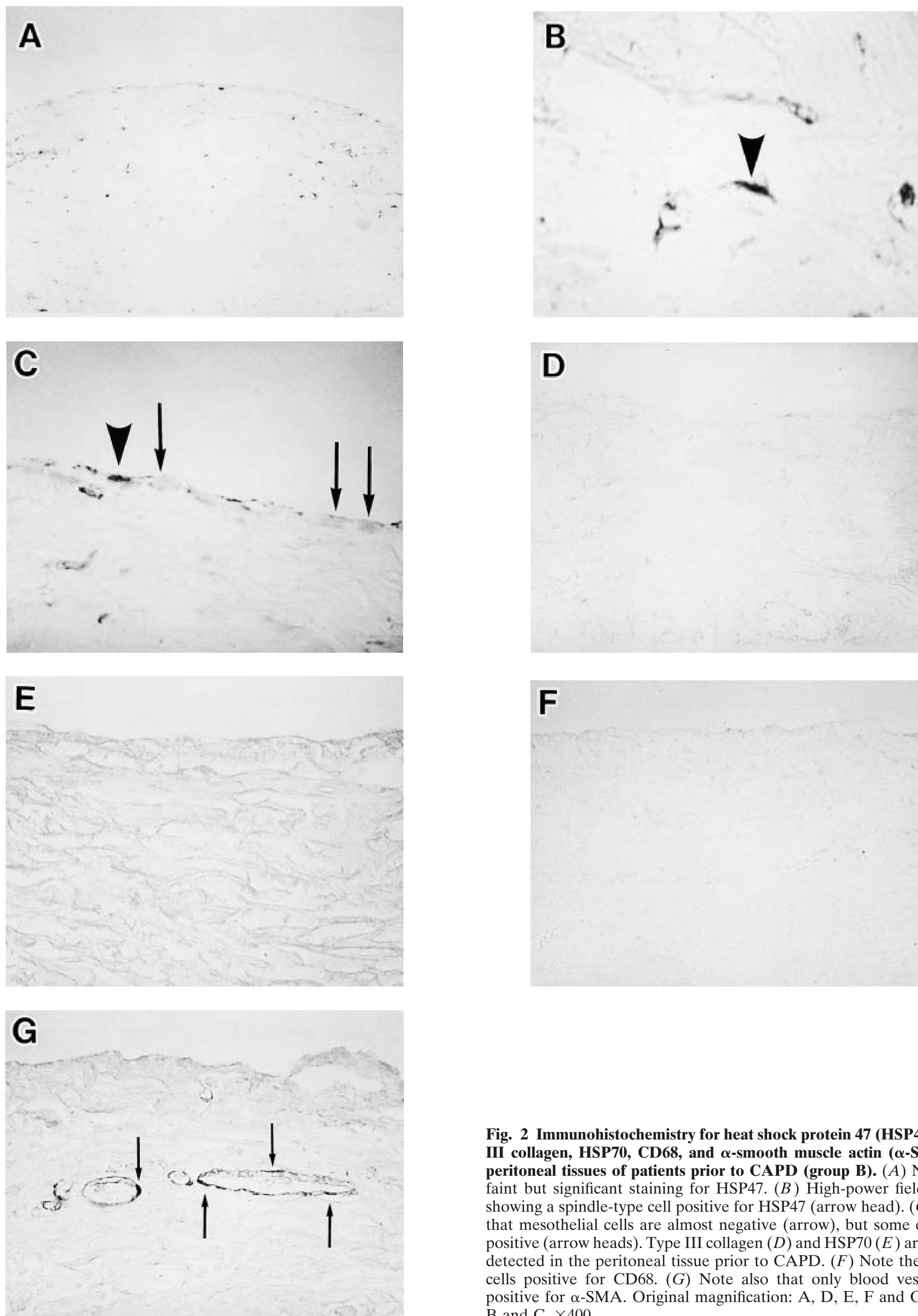

Fig. 2 Immunohistochemistry for heat shock protein 47 (HSP47), type III collagen, HSP70, CD68, and α-smooth muscle actin (α-SMA) in peritoneal tissues of patients prior to CAPD (group B). (*A*) Note the faint but significant staining for HSP47. (*B*) High-power field of 2A showing a spindle-type cell positive for HSP47 (arrow head). (*C*) Note that mesothelial cells are almost negative (arrow), but some cells are positive (arrow heads). Type III collagen (*D*) and HSP70 (*E*) are hardly detected in the peritoneal tissue prior to CAPD. (*F*) Note the lack of cells positive for CD68. (*G*) Note also that only blood vessels are positive for α-SMA. Original magnification: A, D, E, F and G, ×100; B and C, ×400.

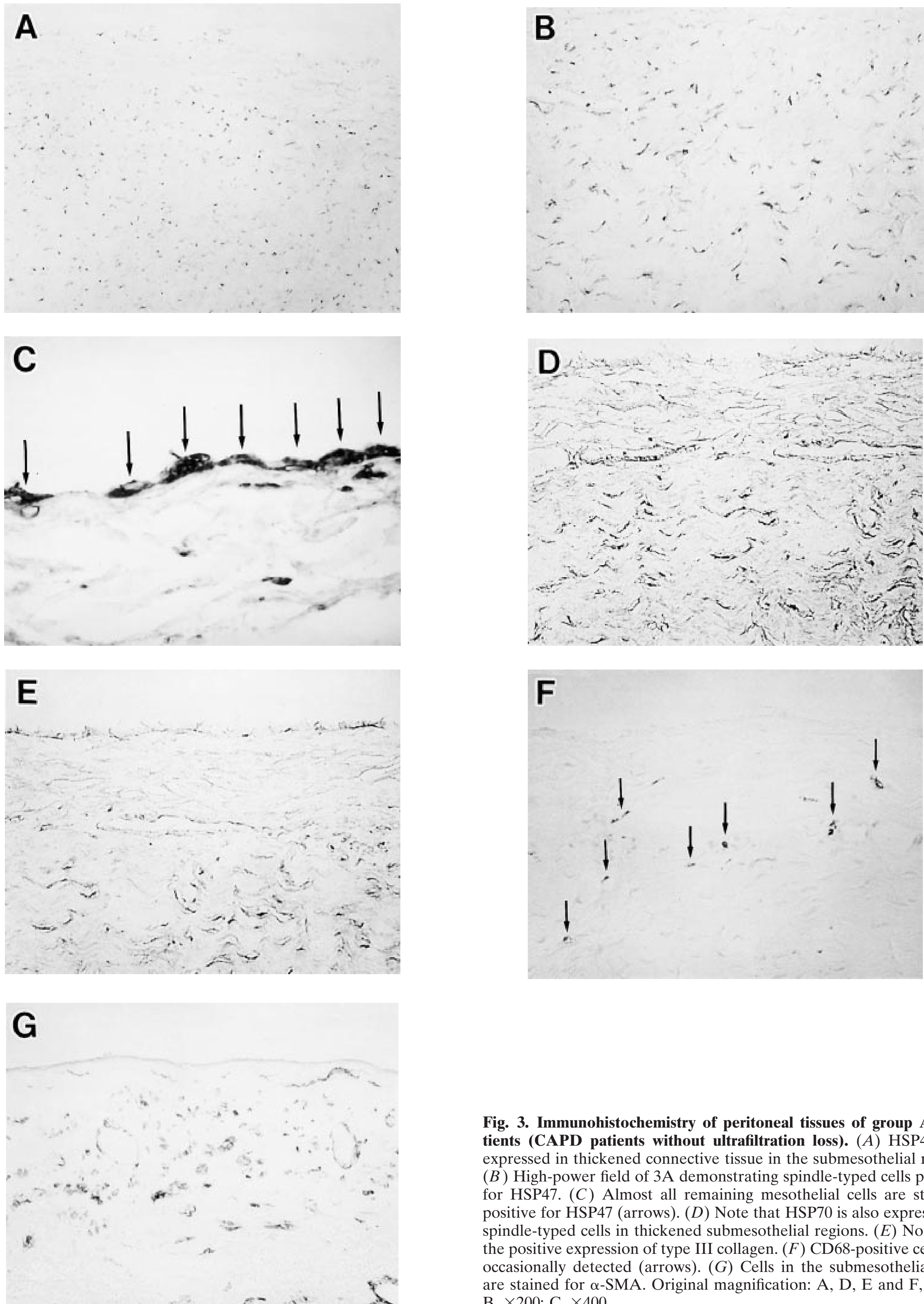

Fig. 3. Immunohistochemistry of peritoneal tissues of group A1 patients (CAPD patients without ultrafiltration loss). (*A*) HSP47 was expressed in thickened connective tissue in the submesothelial region. (*B*) High-power field of 3A demonstrating spindle-typed cells positive for HSP47. (*C*) Almost all remaining mesothelial cells are strongly positive for HSP47 (arrows). (*D*) Note that HSP70 is also expressed in spindle-typed cells in thickened submesothelial regions. (*E*) Note also the positive expression of type III collagen. (*F*) CD68-positive cells are occasionally detected (arrows). (*G*) Cells in the submesothelial area are stained for α-SMA. Original magnification: A, D, E and F, ×100; B, ×200; C, ×400.

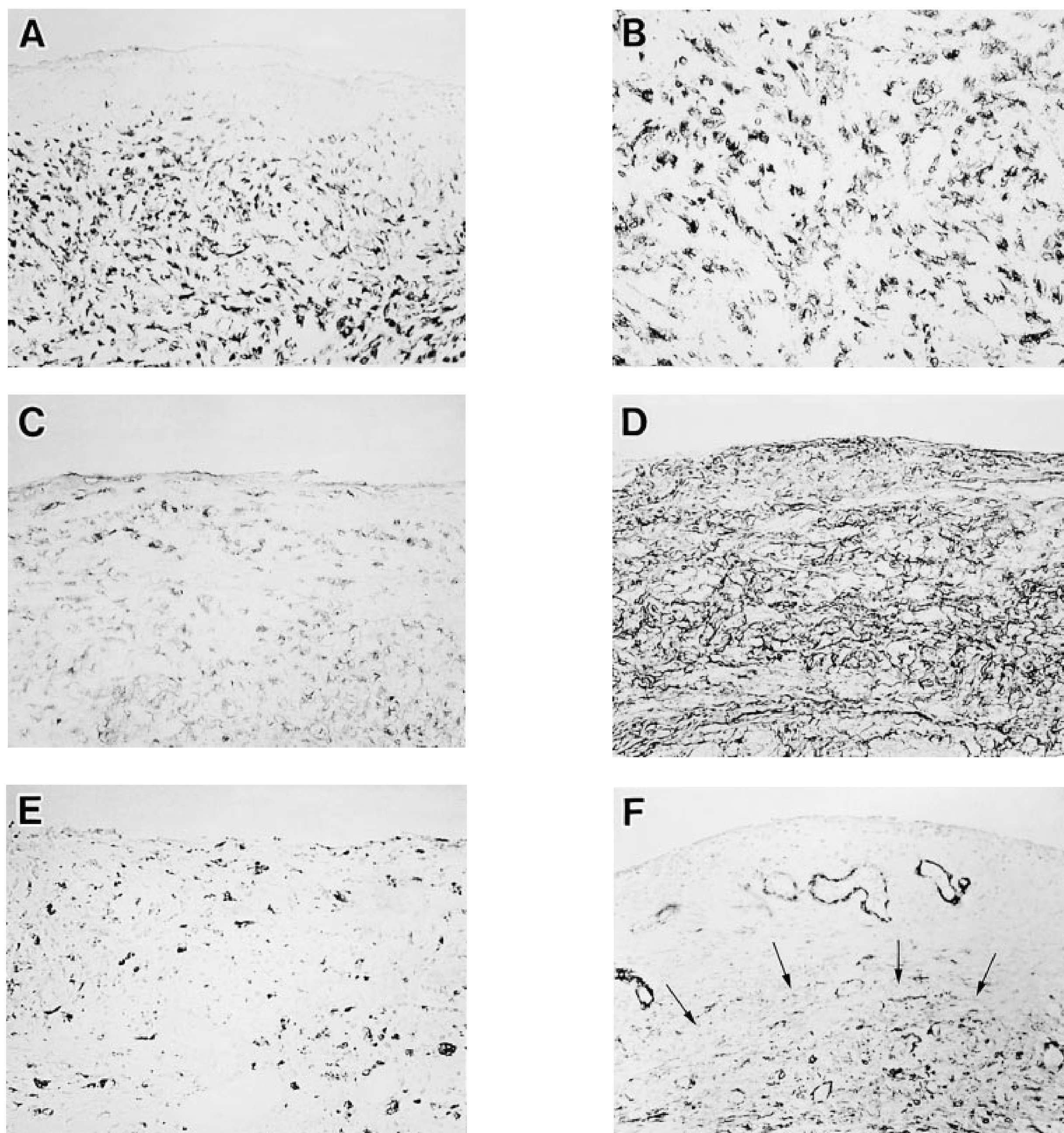

Fig. 4. Immunohistochemistry of peritoneal tissues of group A2 patients (CAPD patients with ultrafiltration loss). Peritoneal tissues show intense expression of HSP47 and HSP70 (*A–C*). Type III collagen is also strongly stained in the area (*D*). (*E*) Many cells positive for CD68 are present in the markedly thickened area with proliferation of collagen fibers. α-SMA is abundantly expressed in the markedly thickened submesothelial area (*F*, shown by arrows) as well as blood vessels. Original magnification: A, C, D, E, and F, ×100; B, ×200.

sion level of HSP47, HSP70, and collagen III was significantly higher in groups A1 and A2 than in group B ($P < 0.01$; Table 4). Furthermore, the expression levels of HSP47, HSP70, collagen III, and α-SMA in group A2 were significantly higher than in group A1, as shown in Figure 4. On the other hand, a significantly higher level of CD68 expression was present in the peritoneal tissue of group A2 than in groups A1 and B.

Because peritonitis may stress the peritoneum, we also examined the effect of peritonitis present at the time of peritoneal collection on histochemical alteration. The levels of expression of HSP47 and CD68 in peritoneal tissues from 6 patients with recurrent or refractory peritonitis episodes were significantly higher than in tissues from 14 patients without such episodes ($P < 0.01$ and $P < 0.05$, respectively), whereas the expressions of HSP70,

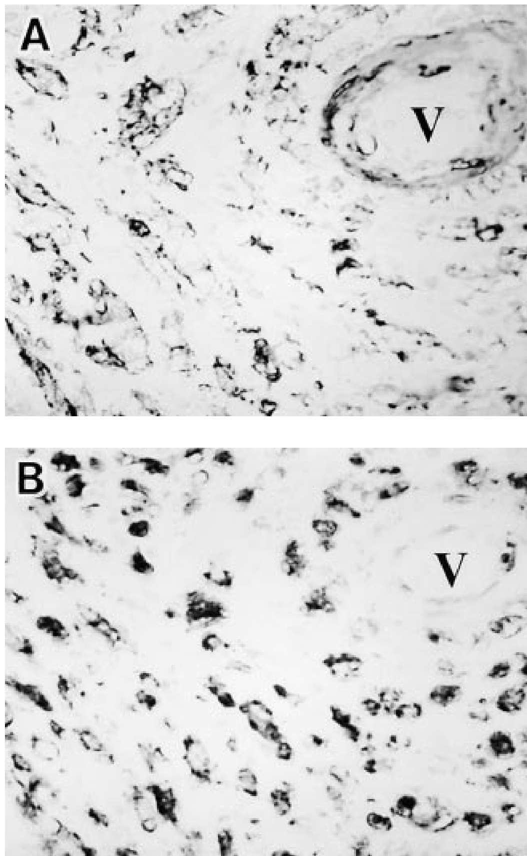

Fig. 5. Immunohistochemistry for HSP47 and α-SMA in serial sections from group A2 patients. To identify the relationship between α-SMA–positive cells (myofibroblasts) and cells positive for HSP47, immunohistochemistry for HSP47 (*A*) and α-SMA (*B*) was performed in serial sections. Note that the majority of cells that express α-SMA are also positive for HSP47, suggesting that HSP47 is mainly expressed in myofibroblasts. Blood vessel wall (V) is stained for α-SMA, not HSP47. Original magnification: A and B, ×400.

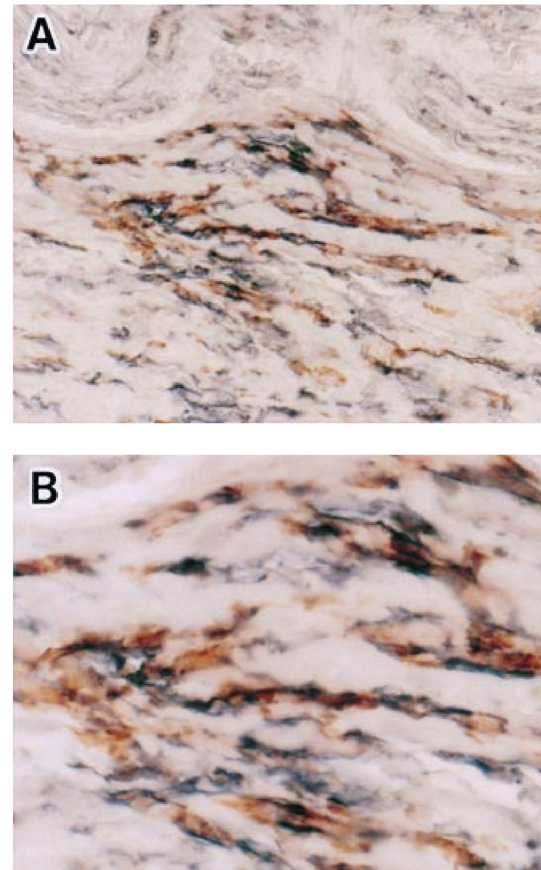

Fig. 6. Double immunostaining demonstrating HSP47 (brown) and α-SMA (blue) in the same section. To determine the presence of α-SMA–positive cells (myofibroblasts) among cells positive for HSP47, double immunolabeling for HSP47 and α-SMA was performed in the same section. Note that α-SMA–positive cells in the peritoneal tissue are also stained with anti-HSP47 antibody. Original magnification: A, ×200; and B, ×400.

α-SMA, and collagen III were not significantly different in the two groups. Furthermore, the expression of HSP47, collagen III, and CD68, but not HSP70 nor α-SMA, correlated with the frequency of peritonitis episodes [$r = 0.615$, 0.646 (Fig. 9), 0.547, respectively, $P < 0.05$].

Relationship between expression of heat shock proteins and cells positive for collagen type III, α-smooth muscle actin, and CD68 in peritoneal specimens

There was a positive correlation between the expression of HSP47 and type III collagen in peritoneal specimens from the three groups ($r = 0.819$; Table 5). The expression of HSP47 also positively correlated with that of HSP70 and α-SMA ($r = 0.676$ and 0.765, respectively; Table 5). In addition, the level of HSP47 in the peritoneum also correlated with CD68-positive cell counts ($r = 0.600$). Furthermore, the expression of HSP70 correlated with that of collagen III, α-SMA, and CD68 in peritoneal specimens of all groups ($r = 0.639$, 0.743, and 0.496, respectively; Table 5). Finally, we examined the relationship between the duration of CAPD therapy and the expression of HSP47, HSP70, α-SMA, CD68, and collagen III in peritoneal tissues. Because the mean duration from the beginning to the development of EPS is about 60 months in Japanese patients with EPS [21], we divided our patients into those with CAPD therapy for more or less than 60 months. In peritoneal tissue from patients with more than 60 months of CAPD therapy, the degree of HSP47 and collagen III expression was significantly higher than in patients with less than 60 months of CAPD ($P = 0.026$ and $P = 0.043$, respectively; Fig. 10). The expression of HSP70 and α-SMA did not correlate with the duration of CAPD therapy.

DISCUSSION

Using immunohistochemistry, we have demonstrated in this study that HSP47 and HSP70 are expressed in

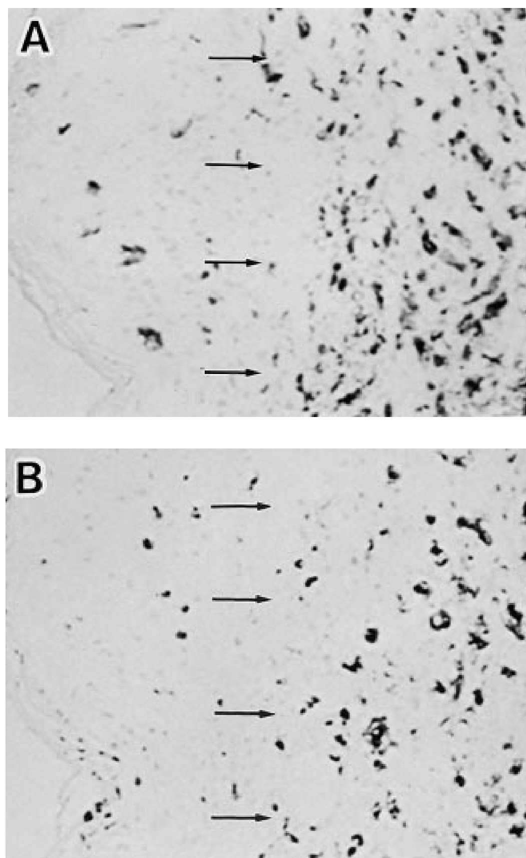

Fig. 7. Immunohistochemistry for HSP47 (*A*) and CD68 (*B*) in serial sections. HSP47 is expressed in the area where many cells positive for CD68 have infiltrated the peritoneum (arrows). Original magnification: A and B, ×200.

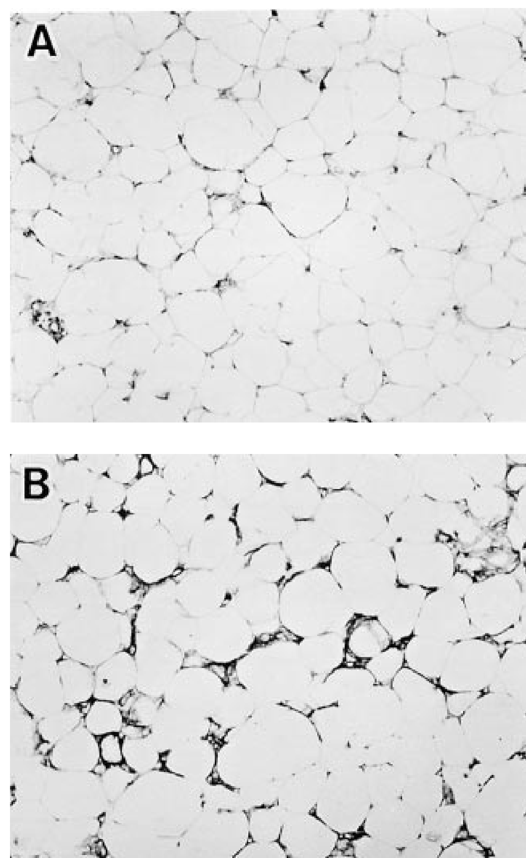

Fig. 8. Immunohistochemistry for HSP47 and collagen III in peritoneal adipose tissues from group A2 patients treated with CAPD. (*A*) Adipocytes are stained for HSP47. (*B*) Note that adipocytes in fat tissues are positive for collagen III. Original magnification: A and B, ×100.

areas of thickened connective tissue in sclerotic peritoneal specimens of CAPD patients. The expression of HSP47, a collagen-specific stress protein, correlated with the frequency of peritonitis episodes. To our knowledge, this is the first report that demonstrates the expression of HSPs in human peritoneal specimens obtained from CAPD patients.

Various stress factors are known to induce the expression of HSPs. In this study, we examined two types of HSPs: HSP47, which is associated with collagen synthesis, and HSP70, which is not associated with collagen synthesis. The expression of HSP70 as well as that of HSP47 in CAPD patients (groups A1 and A2) was higher than in patients with chronic renal failure before CAPD treatment (group B). This finding suggests that CAPD itself may cause some stress on the peritoneal tissue of CAPD patients. In fact, our results showed that HSP47 and HSP70 were also expressed even in the peritoneum of a patient without peritonitis episode who had been treated with CAPD for only 16 months. Previous studies indicated that many factors might be involved in the development of peritoneal sclerosis, including endotoxin contamination in the dialysate [2, 3], acetate dialysate [1–3], and the use of antiseptics [3, 22]. Moreover, glycation [23] and carbonyl modification (abstract; Horie et al, *J Am Soc Nephrol* 9:1444A, 1998) of peritoneal tissue were recently reported in CAPD patients. In this regard, it is possible that the long-term use of CAPD therapy

Table 4. Comparison of signal density in the three groups examined in the present study

Antibody	Group B	Group A1	Group A2
HSP47	2.13 ± 1.75	7.67 ± 4.55[a]	19.59 ± 6.50[ab]
HSP70	2.34 ± 1.29	7.12 ± 4.35[a]	12.05 ± 6.70[ab]
Collagen III	2.00 ± 1.61	13.44 ± 4.90[a]	24.58 ± 5.54[ab]
α-SMA	0.22 ± 0.10	1.82 ± 1.49	8.34 ± 6.96[ab]
CD68	0.70 ± 0.17	3.96 ± 0.75	21.47 ± 3.24[ab]

Images were transformed into a matrix of 1280 × 1000 pixels, and the voltage signal at each pixel was converted to 1 of 256 intensity gray levels in proportion to the colorimetric staining. Data are mean ± SD ($\times 10^6$) of the sum pixel values for HSP47, HSP70, α-SMA and type III collagen. The number of CD68 positive cells was counted per 0.14 mm^2 at a × 200 magnification. Differences between different groups were tested for statistical significance using one-way analysis of variance with Scheffe's *F*-test.

[a] $P < 0.01$, compared with group B
[b] $P < 0.01$, compared with group A1

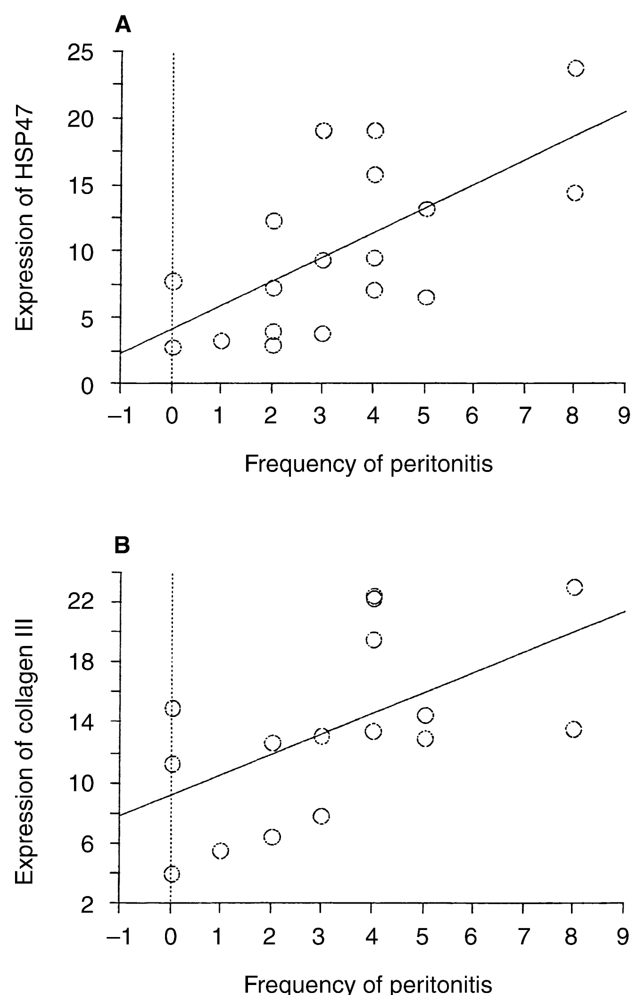

Fig. 9. Correlation between expression of HSP47, collagen III, and frequency of peritonitis. Ordinate data represent the sum of pixel values of ($\times 10^6$) HSP47 (*A*) and collagen III (*B*). Spearman rank correlation. Note the correlation positive between expression of HSP47 and collagen III with the frequency of peritonitis episodes (r = 0.615, 0.646, respectively, $P < 0.05$).

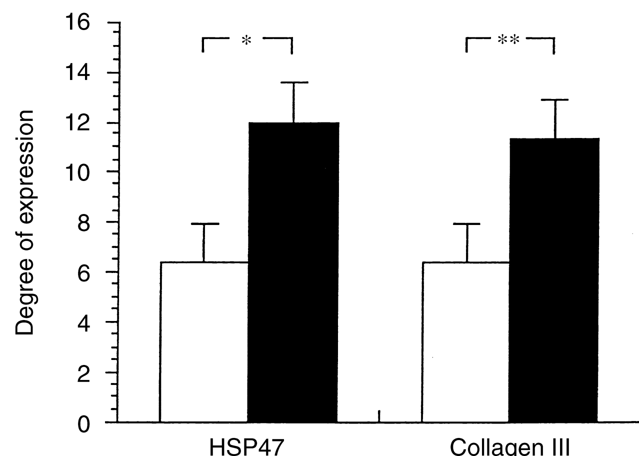

Fig. 10. Expression levels of HSP47 and collage III are influenced by the duration of CAPD therapy. Data are mean ± SD of the sum of pixel values of ($\times 10^6$) HSP47 and collagen III. The degree of HSP47 and collagen III expression was significantly higher in peritoneal tissues from patients with more than 60 months of CAPD therapy (■) compared with that in patients with less than 60 months of CAPD (□). Differences between data of different groups were tested for statistical significance using one-way analysis of variance with Scheffe's *F*-test. *P = 0.026; **P = 0.043.

Table 5. Correlation between the expression of HSPs and that of type III collagen, α-SMA and the number of CD68-positive cells

	HSP47	HSP70	Collagen III	α-SMA	CD68
HSP47	—	0.676[a]	0.819[a]	0.765[a]	0.600[a]
HSP70	0.676[a]	—	0.639[b]	0.743[a]	0.496[b]

Data represent the Spearman rank correlation coefficients between paired variables. The expression of HSP47 correlated positively with that of HSP70, collagen III, α-SMA, and number of CD68 positive cells in peritoneal specimens. The expression of HSP70 also correlated positively with that of collagen III, α-SMA, and number of CD68-positive cells.

[a] $P < 0.01$, [b] $P < 0.05$

represents a risk factor for the development of peritoneal sclerosis [2, 4, 21, 24]; however, we could not identify the exact effect of CAPD therapy itself on the progression of peritoneal sclerosis because the number of patients without peritonitis was too small in this study. The effects of high glucose concentration in dialysate and acidic dialysate on HSP47 and HSP70 expression are also important issues. Further studies are warranted to investigate the pathophysiological effects of CAPD *per se*, including new peritoneal dialysis solution and long-term duration of CAPD therapy on the peritoneum.

Our results showed a high level of HSP47 expression, but not that of HSP70, in peritoneal tissues of CAPD patients with recurrent or refractory peritonitis episodes. Moreover, the level of HSP47 expression correlated with the frequency of peritonitis episodes. Although the number of patients in this study is too small to draw a definite conclusion, our results indicate that peritonitis is a strong inducer of HSP47. Acute inflammation may induce HSPs in the peritoneum; however, the serum C-reactive protein was negative in all patients, and no polymorphonuclear cells were present in the peritoneum, irrespective of the history of peritonitis. Therefore, we suggest that peritonitis may exert a chronic effect on the peritoneum, ultimately leading to peritoneal sclerosis. The precise mechanism of the induction of HSP47 is uncertain, but in our study, more macrophage infiltration was observed in the peritoneum of patients with recurrent or refractory peritonitis episodes. It is possible that increased levels of cytokines and growth factors secreted by macrophages may be involved in HSP47 expression [25, 26]. Clinically, Rubin, Herrera, and Collins also demonstrated a good correlation between the number of peritonitis episodes and histologic changes in the peritoneum of CAPD autopsies [27]. With regard to the factors associated with induction of HSP47, a few reports have suggested that

malnutrition is involved in the expression of some HSPs, such as HSP60 and HSP70 [28, 29]. Because serum albumin concentrations in patients of group A2 were lower than in groups A1 and B, it is also possible that malnutrition or underdialysis may have some effect on HSP47 expression. Further studies are necessary to identify the precise mechanism of HSP47 induction in the peritoneal tissue of CAPD patients.

What are the functional consequences of progression of peritoneal sclerosis? Only a few studies have examined the effect of structural changes in the peritoneum on ultrafiltration loss. Honda et al reported three CAPD patients with ultrafiltration loss who exhibited a variety of vascular changes, including deposition of collagen IV and laminin and severe fibrosis and hyalinization [30]. In the present study, CAPD patients with ultrafiltration loss showed more severe hyalinization of the thickened peritoneum, overexpression of HSP47, collagen III and macrophage infiltration than those without functional loss. The high degree of macrophage infiltration in the peritoneum of patients with ultrafiltration loss suggests that inflammation as well as peritoneal sclerosis may be associated with the loss of peritoneal ultrafiltration properties. Moreover, considering the fact that macrophage-derived cytokines and growth factors, including transforming growth factor-β [31, 32] and platelet-derived growth factor [33], enhance the differentiation of fibroblasts into myofibroblasts, it is possible that infiltrated macrophages in the peritoneum are closely involved with increased number of myofibroblasts, leading to the progression of peritoneal sclerosis via HSP47 expression. It is possible that the progression of peritoneal sclerosis may be associated with ultrafiltration loss in patients on CAPD.

In the present study, we demonstrate that cells positive for HSP47 are α-SMA–positive myofibroblasts, mesothelial cells, and adipocytes. Although we did not provide a direct evidence that cells positive for HSP47 synthesized collagen by *in situ* hybridization, taken together with the results of *in situ* hybridization reported by Masuda et al [8], where the distribution of HSP47 mRNA was similar to that of collagen type I and type III mRNAs and other previous studies [6, 7], our results suggest that HSP47-positive cells are closely associated with collagen production. This is consistent with the results of *in vitro* studies showing that peritoneal fibroblasts [34], mesothelial cells [35], and adipocytes [36] are capable of producing collagen. Because mesothelial cells are detached from sclerotic peritoneal tissue and the location of these three cells is different, we believe that these cells may be involved in the progression of peritoneal sclerosis through a complex network. In this regard, the expression of HSP47 detected by immunohistochemistry does not always indicate protein synthesis, and hence, examination of HSP47 mRNA in the peritoneum of CAPD patients should be performed in future studies.

In conclusion, the results of this study demonstrate that the sclerotic peritoneal tissues of CAPD patients overexpress HSP47 and HSP70 in α-SMA–positive myofibroblasts and mesothelial cells, as well as adipocytes. We also show that the progression of peritoneal sclerosis correlates with deterioration of peritoneal ultrafiltration function in such patients. We propose that peritonitis and long-term CAPD therapy are associated with the progression of peritoneal sclerosis, at least through HSP47 expression. Understanding of the mechanisms that regulate HSPs expression may be of potential therapeutic value in patients with peritoneal sclerosis.

ACKNOWLEDGMENTS

Part of this study was supported by a Research Grant for Progressive Renal Disease from the Specially Selected Disease by the Ministry of Health and Welfare Research Project and a Baxter PD Research Fund. The authors thank Mr. Masahiro Harada for technical assistance.

Reprint requests to Masanobu Miyazaki, M.D., The Second Department of Internal Medicine, Nagasaki University School of Medicine, 1-7-1 Sakamoto, Nagasaki 852-8521, Japan.
E-mail: msnbmiya-ngs@umin.ac.jp

REFERENCES

1. Gandhi VC, Humayun HM, Ing TS, Daugirdas JT, Jablokow VR, Iwaysuki S, Geis WP, Hano JE: Sclerotic thickening of the peritoneal membrane in maintenance peritoneal dialysis patients. *Arch Intern Med* 140:1201–1203, 1980
2. Afthentopouls IE, Passadakis P, Oreopoulos DG: Sclerosing peritonitis in continuous ambulatory peritoneal dialysis patients: One center's experience and review of the literature. *Adv Ren Replace Ther* 5:157–167, 1998
3. Dobbie JW: Pathogenesis of peritoneal fibrosing syndromes (sclerosing peritonitis) in peritoneal dialysis. *Perit Dial Int* 12:14–27, 1992
4. Oules R, Challah S, Brunner P: Case-control study to determine the cause of sclerosing peritoneal disease. *Nephrol Dial Transplant* 3:66–69, 1988
5. Morimoto RI, Tissieres A, Georgopoulos C: *The Biology of Heat Shock Proteins and Molecular Chaperones.* Cold Spring Harbor, Cold Spring Harbor Laboratory Press, 1994, pp 1–30
6. Nagata K, Saga S, Yamada KM: A major collagen-binding protein of chick embryo fibroblasts is a novel heat shock protein. *J Cell Biol* 103:223–229, 1986
7. Nakai A, Satoh M, Hirayoshi K, Nagata K: Involvement of the stress protein HSP47 in procollagen processing in the endoplasmic reticulum. *J Cell Biol* 117:903–914, 1992
8. Masuda H, Fukumoto M, Hirayoshi K, Nagata K: Coexpression of the collagen-binding stress protein HSP47 gene and the a1 (I) and a1 (III) collagen genes in carbon tetrachloride-induced rat liver fibrosis. *J Clin Invest* 94:2481–2488, 1994
9. Moriyama T, Kawada N, Ando A, Yamauchi A, Horio M, Nagata K, Imai E, Hori M: Up-regulation of HSP47 in the mouse kidneys with unilateral ureteral obstruction. *Kidney Int* 54:110–119, 1998
10. Razzaque MS, Taguchi T: Collagen-binding heat shock protein (HSP) 47 expression in anti-thymocyte serum (ATS)-induced glomerulonephritis. *J Pathol* 183:24–29, 1997
11. Razzaque MS, Shimokawa I, Nazneen A, Higami Y, Taguchi T: Age-related nephropathy in the Fischer 344 rat is associated with overexpression of collagens and collagen-binding heat shock protein 47. *Cell Tissue Res* 293:471–478, 1998
12. Cheng M, Razzaque MS, Nazneen A, Taguchi T: Expression of

the heat shock protein 47 in gentamicin-treated rat kidneys. *Int J Exp Pathol* 79:125–132, 1998

13. Sunamoto M, Kuze K, Tsuji H, Ohishi N, Yagi K, Nagata K, Kita T, Doi T: Antisense oligonucleotides against collagen-binding stress protein HSP47 suppress collagen accumulation in experimental glomerulonephritis. *Lab Invest* 78:967–972, 1998
14. Welch WJ: Mammalian stress response: Cell physiology, structure/function of stress proteins, and implications for medicine and disease. *Physiol Rev* 72:1063–1081, 1992
15. Lete Z, Engel S, Krone PH: Hsp47 and hsp70 gene expression is differentially regulated in a stress- and tissue-specific manner in Zebrafish embryos. *Dev Genet* 21:123–133, 1997
16. Piraino B: Peritonitis as a complication of peritoneal dialysis. *J Am Soc Nephrol* 9:1956–1964, 1998
17. Suassuna JH, Neves FC, Hartney RB, Ogg CS, Cameron JS: Immunohistochemical studies of the peritoneal membrane and infiltrating cells in normal subjects and in patients on CAPD. *Kidney Int* 46:443–454, 1994
18. Sauk JJ, Norris K, Moehring J, Foster RA, Somerman MJ: The expression of colligin/hsp47 after stress in human periodontal fibroblast in vitro. *Arch Oral Biol* 35:645–651, 1990
19. Darby I, Skalli O, Gabbiani G: α-smooth muscle actin is transiently expressed by myofibroblasts during experimental wound healing. *Lab Invest* 63:21–29, 1990
20. Furusu A, Miyazaki M, Abe K, Tsukasaki S, Shioshita K, Sasaki O, Miyazaki K, Ozono Y, Koji T, Harada T, Sakai H, Kohno S: Expression of endothelial and inducible nitric oxide synthase in human glomerulonephritis. *Kidney Int* 53:1760–1768, 1998
21. Nomoto Y, Kawaguchi Y, Kubo H, Hirano H, Sakai S, Kurokawa K: Sclerosing encapsulating peritonitis in patients undergoing continuous ambulatory peritoneal dialysis: A report of the Japanese sclerosing encapsulating peritonitis study group. *Am J Kidney Dis* 28:420–427, 1996
22. Lo WK, Chan KT, Leung ACT, Pang SW, Tse CY: Sclerosing peritonitis complicating prolonged use of chlorhexidine in alcohol in the connection procedure for continuous ambulatory peritoneal dialysis. *Perit Dial Int* 2:166–172, 1991
23. Nakayama M, Kawaguchi Y, Yamada K, Hasegawa T, Takazoe K, Katoh N, Hayakawa H, Osaka N, Yamamoto H, Ogawa A, Kubo H, Shigematsu T, Sakai O, Horiuchi S: Immunohistochemical detection of advanced glycosylation end-products in the peritoneum and its possible pathophysiological role in CAPD. *Kidney Int* 51:182–186, 1997
24. Rigby RJ, Hawley CM: Sclerosing peritonitis: The experience in Australia. *Nephrol Dial Transplant* 13:154–159, 1998
25. Yamamura I, Hirata H, Hosokawa N, Nagata K: Transcriptional activation of the mouse HSP47 gene in mouse osteoblast MC3T3-E1 cells by TGF-beta 1. *Biochem Biophys Res Commun* 244:68–74, 1998
26. Takahashi K, Kubo T, Goomer RS, Amiel D, Kobayashi K, Imanishi J, Teshima R, Hirasawa Y: Analysis of heat shock proteins and cytokines expressed during early stages of osteoarthritis in a mouse model. *Osteoarthritis Cartilage* 5:321–329, 1997
27. Rubin J, Herrera GA, Collins D: An autopsy study of the peritoneal cavity from patients on continuous ambulatory peritoneal dialysis. *Am J Kidney Dis* 18:97–102, 1991
28. Ehrenfried JA, Evers BM, Chu KU, Townsend CM Jr, Thompson JC: Caloric restriction increases the expression of heat shock protein in the gut. *Ann Sung* 223:592–597, 1996
29. Heydari AR, You S, Takahashi R, Gutsmann A, Sarge KD, Richardson A: Effect of caloric restriction on the expression of heat shock protein 70 and the activation of heat shock transcription factor 1. *Dev Genet* 18:114–124, 1996
30. Honda K, Nitta K, Horita S, Yumura W, Nihei H: Morphological changes in the peritoneal vasculature of patients on CAPD with ultrafiltration failure. *Nephron* 72:171–176, 1996
31. Desmouliere A, Geinoz A, Gabbiani F, Gabbiani G: Transforming growth factor-β-induced α-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. *J Cell Biol* 122:103–111, 1993
32. Mattey DL, Dawes PT, Nixon NB, Slater H: Transforming growth factor beta 1 and interleukin 4 induced alpha smooth muscle actin expression and myofibroblast-like differentiation in human synovial fibroblasts in vitro: Modulation by basic fibroblast growth factor. *Ann Rheum Dis* 56:426–431, 1997
33. Tang WW, Van GY, Qi M: Myofibroblast and alpha 1 (III) collagen expression in experimental tubulointerstitial nephritis. *Kidney Int* 51:926–931, 1997
34. Breborowicz A, Patrikarea A, Martis L, Oreopoulos DG: Effect of the dialysate effluent from CAPD patients on peritoneal mesothelial cells and fibroblasts. *Adv Perit Dial* 10:230–234, 1994
35. Stylianou E, Jenner LA, Davis M, Coles GA, Williams JD: Isolation, culture and characterization of human peritoneal mesothelial cells. *Kidney Int* 37:1563–1570, 1990
36. Cryer A, Van RL: Characterization of the collagen types synthesized by human and rat adipocyte precursors in vitro. *Eur J Clin Invest* 12:235–238, 1982