

Involvement of Leptin in the Progression of Experimentally Induced Peritoneal Fibrosis in Mice

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Leptin is a hormone mainly produced by white adipose cells, and regulates body fat and food intake by acting on hypothalamus. Leptin receptor is expressed not only in the hypothalamus but in a variety of peripheral tissues, suggesting that leptin has pleiotropic functions. In this study, we investigated the effect of leptin on the progression of peritoneal fibrosis induced by intraperitoneal injection of chlorhexidine gluconate (CG) every other day for 2 or 3 weeks in mice. This study was conducted in male C57BL/6 mice and leptin-deficient *ob/ob* mice. Peritoneal fluid, blood, and peritoneal tissues were collected 15 or 22 days after CG injection. CG injection increased the level of leptin in serum and peritoneal fluid with thickening of submesothelial compact zone in wild type mice, but CG-injected *ob/ob* mice attenuate peritoneal fibrosis, and markedly reduced the number of myofibroblasts, infiltrating macrophages, and blood vessels in the thickened submesothelial area. The 2-week leptin administration induced a more thickened peritoneum in the CG-injected C57BL/6 mice than in the PBS group. Our results indicate that an upregulation of leptin appears to play a role in fibrosis and inflammation during peritoneal injury, and reducing leptin may be a therapeutically potential for peritoneal fibrosis.

Key words: leptin, peritoneal fibrosis, peritoneal dialysis, angiogenesis, macrophage infiltration

I. Introduction

Peritoneal dialysis (PD) is a beneficial therapy for end-stage renal disease, but long-term use of PD induces histopathologic changes in the peritoneum, such as peritoneal fibrosis with increased submesothelial collagen deposition and loss of mesothelial cells [4, 7, 18, 34]. In particular, some patients develop encapsulating peritoneal sclerosis (EPS) associated with high mortality, which is one of the most serious complications in PD patients [9]. The mechanism of peritoneal fibrosis in PD patients remains

poorly understood and no effective therapy is available at present.

Leptin is mainly produced by white adipose cells [37] and plays an important role in body weight regulation by inhibiting food intake via hypothalamic effects and stimulating energy expenditure in skeletal muscle cells [6]. However, the leptin receptor is expressed not only in the hypothalamus but in a variety of peripheral tissues, suggesting that leptin has pleiotropic functions. To date, the peripheral actions of leptin have been reported such as activation of platelet aggregation, modulation of immune functions, and acceleration of angiogenesis [14, 19, 23, 27]. These findings suggest that leptin acts as a pro-inflammatory cytokine in peripheral tissues. Recent studies have suggested an essential fibrogenic role for leptin in experimental

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liver fibrosis [8, 20, 29]. In addition, previous reports demonstrated that leptin-deficient (*ob/ob*) mice, which lack hypothalamic inhibition of appetite, leading to obesity, insulin resistance, and type II diabetes [2], are protected from inflammation and fibrosis in various disease models, including autoimmune encephalomyelitis [15], liver fibrosis [21], nephrotoxic nephritis [31], and interstitial fibrosis by unilateral ureteral obstruction (UUO) [11]. In fact, PD patients showed high levels of leptin in their serum or peritoneal dialysate [5, 33]. However, the role of leptin in peritoneal fibrosis has not been elucidated.

Thus, in the present study, we investigated the effect of leptin using leptin-deficient (*ob/ob*) mice in the development of peritoneal fibrosis induced by chlorhexidine gluconate (CG).

II. Material and Methods

Animals

The experiments described in this study were conducted in male C57BL/6 mice weighing 22.6 ± 1.1 g body weight, and leptin-deficient *ob/ob* mice (C57BL/6J-Lep^{ob}/Lep^{ob}) weighing 37.3 ± 1.3 g body weight (Japan Charles River Inc., Yokohama, Japan). They were housed in a light- and temperature-controlled room in Biomedical Research Center, Center for Frontier Life Sciences, Nagasaki University. They had free access to laboratory chow and tap water in standard rodent cages. The experimental protocol was inspected by Animal Care and Use Committee of Nagasaki University, and approved by the President of Nagasaki University (approval number: 1004050846-5).

Experimental protocol

Peritoneal fibrosis was induced by intraperitoneal injection of 0.05% CG in 15% ethanol dissolved in saline, as described previously with a slight modification [3, 36]. Mice received injections of CG into the peritoneal cavity at a volume of 10 ml/kg body weight 3 times a week for 3 weeks. Control group of mice received an equal volume of vehicle intraperitoneally. Mice were divided into four groups: (i) wild type C57BL/6 mice injected vehicle intraperitoneally defined as the WT-control group, (ii) *ob/ob* mice injected vehicle intraperitoneally defined as *ob/ob*-control group, (iii) wild type C57BL/6 mice injected CG intraperitoneally defined as the WT-CG group, (iv) *ob/ob* mice injected CG intraperitoneally defined as the *ob/ob*-CG group. Each group consisted of 5 mice. At days 22 after the first CG injection, mice were infused with 2 ml of saline into the abdominal cavity under anesthesia. 30 minutes after infusion, peritoneal fluid was collected for the examination of leptin level. Blood samples were drawn from all animals by transthoracic cardiac puncture under anesthesia prior to sacrifice. Then, mice were sacrificed, and peritoneal tissues were dissected out carefully. To avoid direct damage to the peritoneum caused by repeated injections, injections of CG were made at the lower part of

the peritoneum, while the upper portion of the parietal peritoneum was used for the following examination. Tissues were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.4). In addition, frozen tissues were also prepared by mounting in optimal cutting temperature compound (Miles, Inc., Elkhart, IN, USA) and rapidly frozen in dry ice and stored at -80°C until use. Fresh frozen tissues were cut into 4- μm -thick sections using a microtome and placed onto aminopropyltriethoxysilane-coated slide glasses.

Leptin administration

Recombinant mouse leptin (498-OB, R&D Systems, Inc., Minneapolis, MN, USA) was diluted in a vehicle (15 mM HCL and 7.5 mM NaOH) according to the manufacturer's protocol and adjusted to 1.25 mg/ml. The mice received CG injections into the peritoneal cavity at a volume of 10 ml/kg of body weight, 3 times a week for 2 weeks (WT-CG group). During the first CG injection, we implanted an ALZET[®] osmotic pump (1002, DURECT Corp., Cupertino, CA, USA) containing recombinant leptin (0.45 g/kg/day), which was released in a sustained manner for 2 weeks, in the mice subcutaneously injected with CG. The WT-CG group was divided into (1) an experimental group, which comprised mice administered recombinant leptin and defined as the leptin group ($n=3$), and (2) a control group, which comprised mice administered PBS instead of leptin and defined as the PBS group ($n=3$). At day 15 after the first CG injection, the mice were killed and peritoneal tissues were dissected out carefully, as described previously.

Histologic and immunohistochemical examination

For morphological examination, 4- μm thick paraffin-embedded tissues were stained with hematoxylin and eosin (HE). The immunohistochemical procedure used in the present study was described previously [1, 3, 25]. The first antibodies used for immunohistochemistry in this study are listed in Table 1. We used fresh frozen sections for CD31 staining, and paraffin-embedded sections for detection of all the rest of factors. Indirect immunohistochemical technique was used for collagen III, F4/80, and CD31. Deparaffinized tissue sections were incubated for 30 min with a blocking buffer containing 5% normal goat serum, 5% fetal calf serum, 5% bovine serum albumin, and 20% normal swine serum in PBS. The sections were then reacted with the primary antibody, and diluted in the same blocking buffer. After reacting with anti-collagen III antibody for 1 hr at room temperature, sections were reacted with horseradish peroxidase (HRP)-conjugated swine anti-rabbit immunoglobulin antibody (P0399; Dako) diluted 1/50 for 30 min at room temperature, and a complex of rabbit anti-HRP antibody and HRP (Z0113; Dako) diluted 1/100 for 30 min at room temperature. For F4/80 and CD31, sections were reacted with HRP-conjugated rabbit anti-rat immunoglobulin antibody (Z0147; Dako) and HRP-conjugated swine anti-rabbit immunoglobulin anti-

Table 1. The list of primary antibodies for immunohistochemistry

Primary Antibodies	Host	Dilution	Company
leptin receptor	goat	1:50	GT15012; Neuromics, Northfield, MN, USA
leptin	goat	1:100	GT15011; Neuromics, Northfield, MN, USA
collagen III	rabbit	1:400	LB-1393; LSL Co., Tokyo, Japan
α -SMA (myofibroblasts)	mouse	EPOS	U7033; Dako, Glostrup, Denmark
TGF- β	rabbit	1:200	sc-146; Santa Cruz Biotechnology, Santa Cruz, CA, USA
F4/80 (macrophages)	rat	1:100	MCA497; Serotec, Oxford, UK
MCP-1	goat	1:100	sc-1784; Santa Cruz Biotechnology, Santa Cruz, CA, USA
CD31 (endothelial cells)	rat	1:50	1625-01; Southern Biotechnology, Birmingham, AL, USA
VEGF	rabbit	1:100	sc-507; Santa Cruz Biotechnology, Santa Cruz, CA, USA

α -SMA; α -smooth muscle actin, EPOS; enhanced polymer one-step staining, TGF- β ; transforming growth factor-beta, MCP-1; monocyte chemotactic protein-1, VEGF; vascular endothelial growth factor.

body after reacting with primary antibodies for 1 hr at room temperature. We used direct immunohistochemical technique for detection of α -smooth muscle actin (SMA). After deparaffinization, the sections were incubated for 30 min with a blocking buffer similar to that described above, and then reacted with anti- α -SMA antibody for 1 hr at room temperature. For leptin, leptin receptor, transforming growth factor-beta (TGF- β), monocyte chemotactic protein-1 (MCP-1), vascular endothelial growth factor (VEGF) staining, deparaffinized tissues were stained with avidin-biotin complex technique using Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) after reacting with first antibody for 1 hr at room temperature. Reaction products were visualized by treating sections with H₂O₂ and 3,3'-diaminobenzidine tetrahydrochloride. Finally, the sections were counterstained with methyl green and mounted. For all specimens, negative controls were prepared with irrelevant mouse monoclonal antibody, rat monoclonal antibody, or the normal rabbit IgG in place of the primary antibody.

Morphometric analysis

To assess the extent of peritoneal thickening, we used digitized images and image analysis software (Win ROOF MITANICORP, Chiba, Japan). The image was transformed into a matrix of 1280×1024 pixels and viewed at ×200 magnification. We selected a width of 421 μ m in the examined field under the microscope and measured the area of the submesothelial layer within the selected width of 421 μ m. For each sample, 10 areas were selected and the average area of the submesothelial compact zone or collagen III-positive area was determined. In each peritoneal sample, the numbers of α -SMA-expressing cells, TGF- β -expressing cells, F4/80-positive macrophages, MCP-1-expressing cells, CD31-positive vessels, and VEGF-expressing cells and were counted in 10 fields at ×400 magnification.

Leptin measurements

Leptin concentration of serum and peritoneal fluid were measured with a commercially available ELISA kit

(MOB00; R&D systems). Blood samples were drawn from all animals by transthoracic cardiac puncture under anesthesia prior to sacrifice. Serum was obtained by centrifuging whole blood at 3000 rpm, 4°C, and for 5 min.

Statistical analysis

Data are expressed as mean±standard deviation (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 difference.

III. Results

Leptin level of serum and peritoneal fluid

We examined the concentration of leptin in serum and peritoneal fluid of CG-treated wild-type mice and untreated one. In both serum and peritoneal fluid, leptin levels were increased by CG injection (Table 2). Similarly, we confirmed the increased expression of leptin in the peritoneum of CG-injected C57BL/6 mice by immunohistochemical examination (Fig. 1a and b). In *ob/ob* mice, leptin-deficient mice, the leptin was not detected with or without CG injection (data not shown).

Immunohistochemical analysis for leptin receptor in peritoneum

In peritoneum of WT-control group, leptin receptor was only localized in mesothelial cells (Fig. 2a). In the *ob/ob*-control group, a pattern of leptin receptor expression

Table 2. Leptin concentration of serum and peritoneal fluid in wild type mice and *ob/ob* mice

	wild type		<i>ob/ob</i>	
	untreated	CG-treated	untreated	CG-treated
Serum (ng/ml)	1.08±0.59	4.74±1.81*	0	0
Peritoneal fluid (ng/ml)	0.07±0.04	0.54±0.35*	0	0

* *P*<0.01 vs untreated wild type mice.

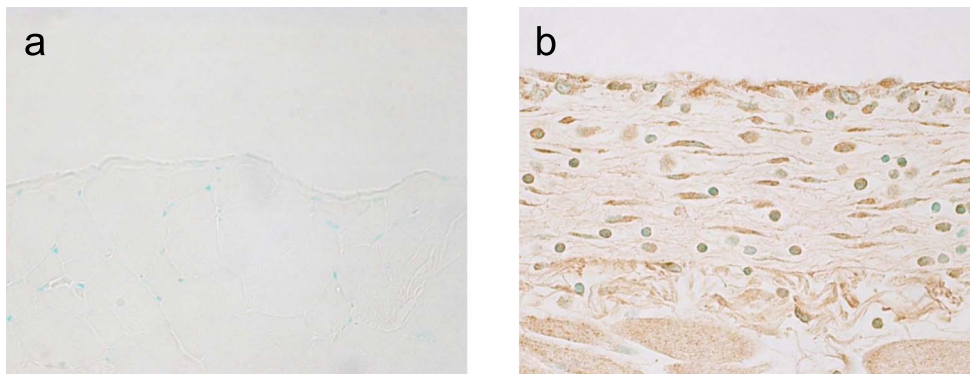


Fig. 1. Immunohistochemistry for leptin. In the WT-control group, leptin expression was not observed in the peritoneum (a). In contrast, an increase in leptin expression was observed in the CG-injected C57BL/6 mice (b).

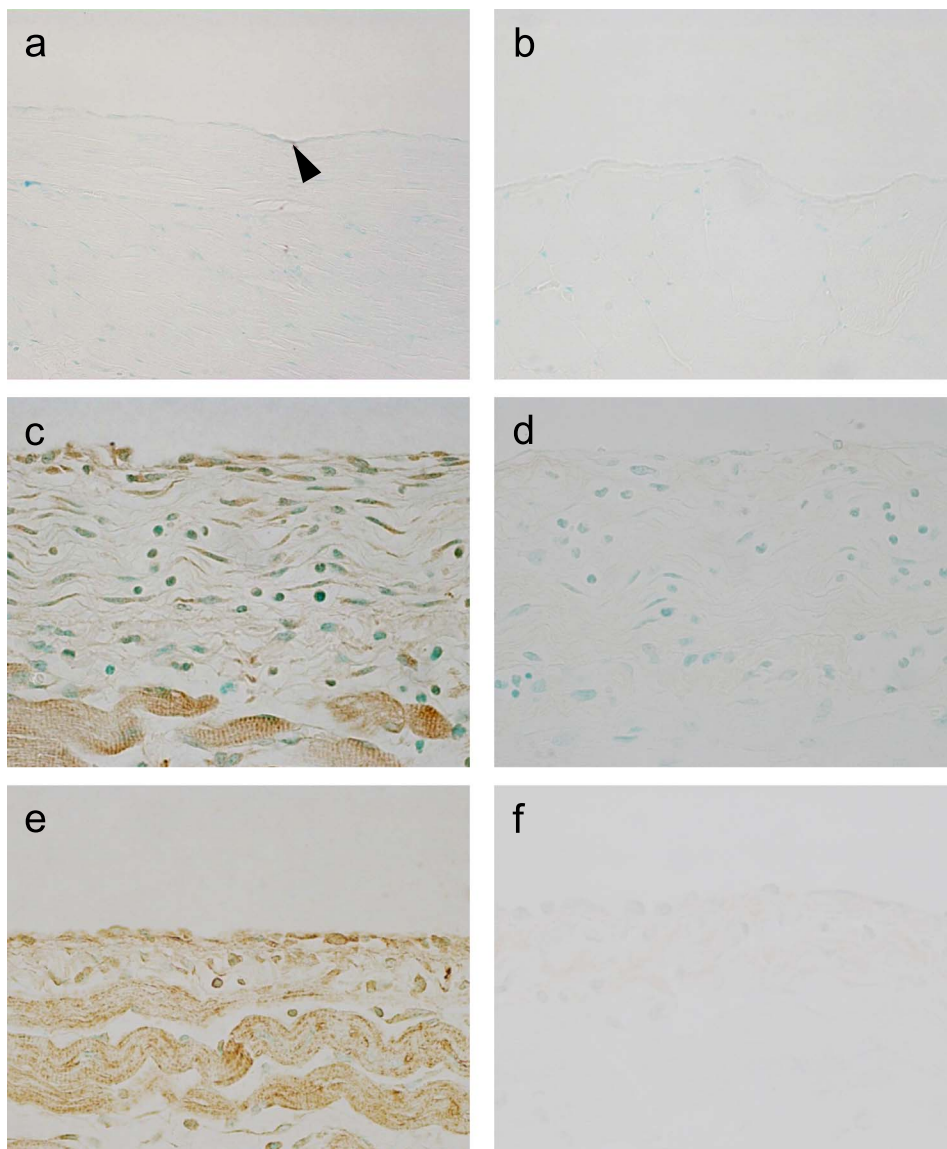


Fig. 2. Immunohistochemistry for leptin receptor. Leptin receptor expression was observed in the peritoneal tissues of the WT-control group (a), CG-treated C57BL/6 mice (c), and CG-injected *ob/ob* mice (e). The peritoneal tissue sections of the WT-control group (b), CG-treated C57BL/6 mice (d), and CG-injected *ob/ob* mice (f) were incubated with normal goat IgG instead of leptin receptor antibody as negative controls. The black arrowhead shows the leptin receptor-positive mesothelium. Magnification, $\times 400$.

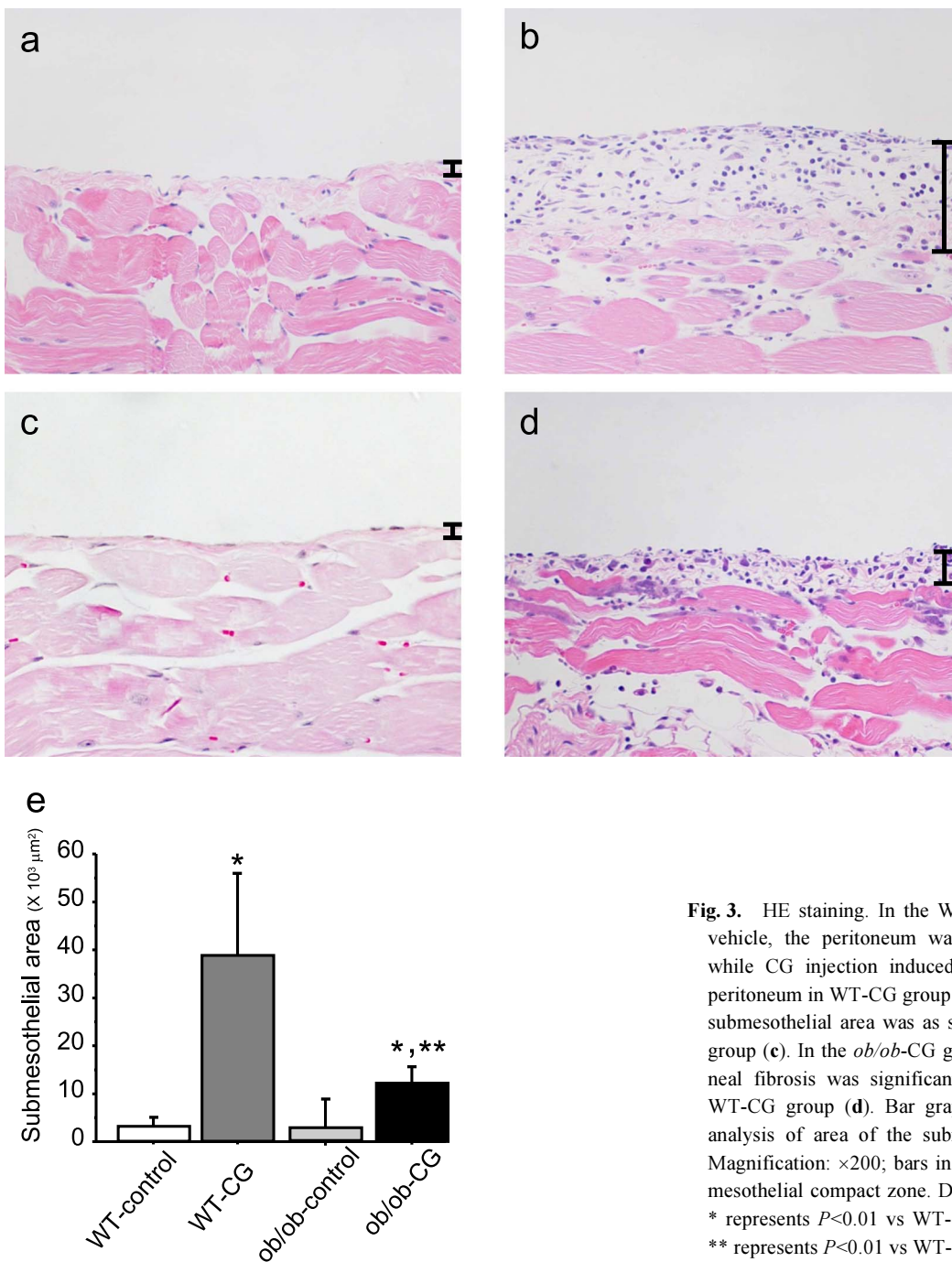


Fig. 3. HE staining. In the WT-control group, injected with vehicle, the peritoneum was only slightly thickened (a), while CG injection induced significant thickening of the peritoneum in WT-CG group (b). In the *ob/ob*-control group, submesothelial area was as same as that in the WT-control group (c). In the *ob/ob*-CG group, the progression of peritoneal fibrosis was significantly suppressed compared with WT-CG group (d). Bar graph shows the semiquantitative analysis of area of the submesothelial compact zone (e). Magnification: $\times 200$; bars indicate the thickness of the submesothelial compact zone. Data are expressed as mean \pm SD; * represents $P < 0.01$ vs WT-control or *ob/ob*-control group, ** represents $P < 0.01$ vs WT-CG group.

in the peritoneum was shown similar to that of WT-control group (data not shown). Meanwhile, the expression of leptin receptor in peritoneum of CG-injected mice was increased compared to that in control group and the leptin receptor in peritoneum of CG-injected wild type mice was identified at myofibroblasts, macrophages, and mesothelial cells (Fig. 2c). Moreover, CG-injected *ob/ob* mice showed a pattern of leptin receptor expression in the peritoneum similar to that of CG-injected wild type mice (Fig. 2e). No staining was found in sections that were reacted with goat IgG as negative controls (Fig. 2b, d, and f).

Morphologic examination

We next investigated the degree of peritoneal fibrosis induced by CG in wild-type mice and *ob/ob* mice. In WT-control mice and *ob/ob*-control mice, a monolayer of mesothelial cells was observed covering the surface without any thickening of the peritoneum (Fig. 3a and c). CG injection induced significant thickening of submesothelial compact zone in WT-CG group (Fig. 3b). Meanwhile, the area of submesothelial compact zone in *ob/ob*-CG group was significantly smaller than that in the WT-CG group (Fig. 3d).

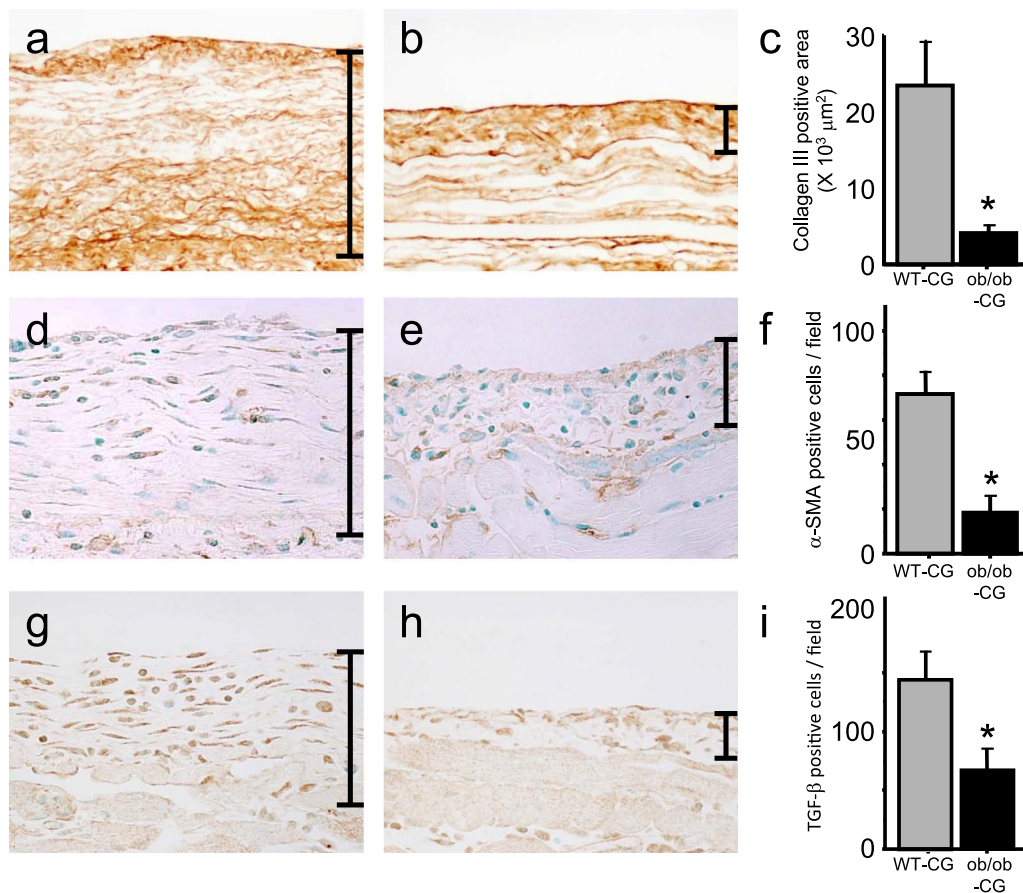


Fig. 4. Immunohistochemistry for collagen III, α -SMA, and TGF- β . In the WT-CG group, collagen III was strongly expressed in the submesothelial compact zone (a), while the expression of collagen III in *ob/ob*-control group was less than that in WT-CG group (b). Bar graph shows the semiquantitative analysis of collagen III-positive area (c). Note the presence of numerous α -SMA-expressing cells in the thickened submesothelial compact zone of the WT-CG group (d). In the *ob/ob*-CG group, the number of α -SMA-expressing cells was reduced (e). Bar graph shows the quantitative analysis of α -SMA-expressing cells (f). A large number of TGF- β -positive cells were observed in the thickened submesothelial compact zone of the WT-CG group (g). In the *ob/ob*-CG group, the number of TGF- β -positive cells was decreased (h). Bar graph shows the quantitative analysis of TGF- β -positive cells (i). Magnification, $\times 400$; bars indicate the thickness of the submesothelial compact zone. Data are expressed as mean \pm SD; * represents $P < 0.01$ vs WT-CG group.

Expression of collagen III, α -SMA, and TGF- β

The results of immunohistochemical analysis for collagen III, α -SMA and TGF- β at day 21 are shown in Fig. 4. CG injection significantly increased the accumulation of collagen III in the submesothelial area (Fig. 4a), while the positive area of collagen III was significantly reduced in *ob/ob*-CG group (Fig. 4b and c).

We examined the expression of α -SMA, a marker of myofibroblasts, which are major source of collagen-producing cells. In the WT-CG group, numerous α -SMA-positive myofibroblasts were identified in the thickened peritoneal tissues (Fig. 4d). Compared with WT-CG group (Fig. 4d), the number of α -SMA-expressing cells was markedly reduced in *ob/ob*-CG group (Fig. 4e and f). We also investigated the expression of profibrotic cytokine TGF- β in the thickened peritoneum. In the WT-CG group, the large number of TGF- β -expressing cells was presented in the peritoneum (Fig. 4g). On the contrary, *ob/ob*-CG

group showed a significant decrease of the number of TGF- β -positive cells in the peritoneum (Fig. 4h and i).

Expression of F4/80 and MCP-1

To examine the effect of leptin on the macrophage infiltration in the peritoneum, we performed immunohistochemistry for F4/80 as a marker for mouse macrophages on peritoneal tissues. In the WT-CG group, a large number of F4/80-positive macrophages was observed in the thickened peritoneum (Fig. 5a), while the number of F4/80-positive macrophages was markedly reduced in the *ob/ob*-CG group (Fig. 5b and c). We also investigated the expression of MCP-1, which exerts an important role on macrophage infiltration. The number of MCP-1-expressing cells was increased in the thickened submesothelial compact zone (Fig. 5d). In the *ob/ob*-CG group (Fig. 5e), the number of MCP-1-expressing cells was significantly less than that in WT-CG group (Fig. 5f).

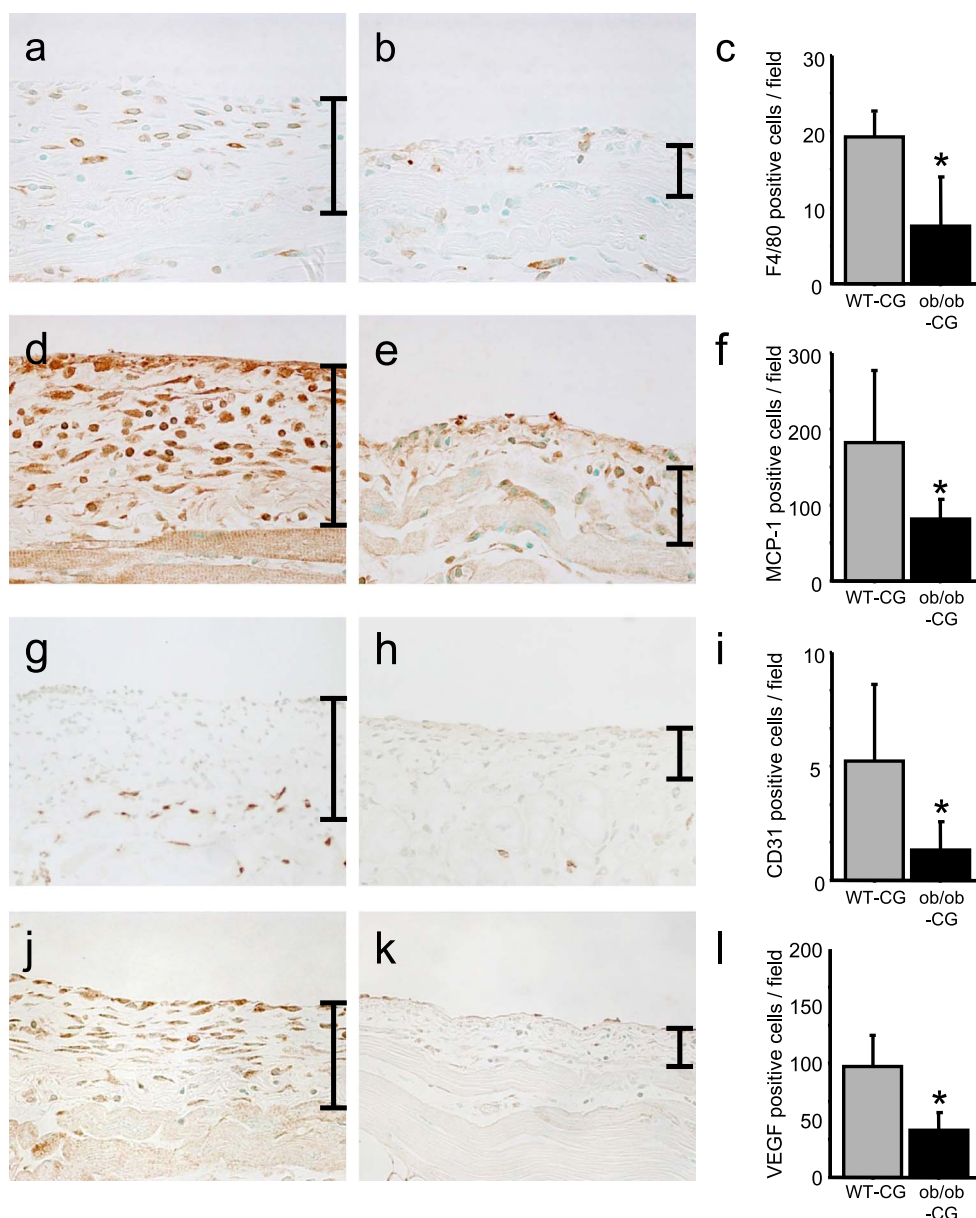


Fig. 5. Immunohistochemistry for F4/80, MCP-1, CD31, and VEGF. Numerous F4/80-positive macrophages were present in thickened peritoneal tissues of the WT-CG group (a). In the *ob/ob*-CG group, the number of F4/80-positive macrophages was reduced (b). Bar graph shows the quantitative analysis of F4/80-positive macrophages (c). Note the large number of MCP-1-positive cells in the thickened submesothelial compact zone of the WT-CG group (d). In the *ob/ob*-CG group, the number of MCP-1-positive cells in the submesothelial compact zone was significantly less than that in the WT-CG group (e). Bar graph shows the quantitative analysis of MCP-1-positive cells (f). Note the presence of numerous CD31-positive vessels in the WT-CG group (g). On the contrary, a few number of CD31-positive vessels in the *ob/ob*-control group (h). Bar graph shows the quantitative analysis of CD31-positive vessels (i). In the WT-CG group, the number of VEGF-positive cells was increased in the thickened submesothelial compact zone (j), while it was markedly decreased in *ob/ob*-control group compared with WT-CG group (k). Bar graph shows the quantitative analysis of VEGF-positive cells (l). Magnification, $\times 400$; bars indicate the thickness of the submesothelial compact zone. Data are expressed as mean \pm SD; * represents $P < 0.01$ vs WT-CG group.

Expression of CD31 and VEGF

We investigated the number of blood vessels using immunohistochemistry for CD31. The large number of CD31-positive vessels was identified in the WT-CG group (Fig. 5g). The number of CD31-expressing vessels was significantly decreased in the peritoneum of *ob/ob*-CG

group (Fig. 5h and i) compared to that of WT-CG group. We also examined the expression of VEGF, a potent stimulator of angiogenesis, in the submesothelial zone in our model. The mice in the WT-CG group exhibited a large number of VEGF-positive cells in the thickened peritoneum (Fig. 5j). Peritoneal tissues in the *ob/ob*-CG group con-

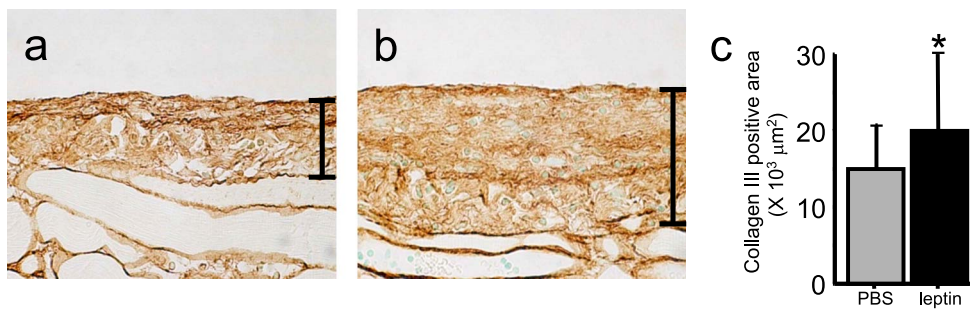


Fig. 6. Effects of leptin administration on peritoneal fibrosis. Immunohistochemistry for collagen III in the peritoneal tissues of the mice infused with PBS (a) or leptin (b) via osmotic pump for 14 days. Compared with the PBS group (a), the leptin-infused mice indicated significant accumulation of collagen III in the submesothelial compact zone (c). The bar graph shows the result of the semiquantitative analysis of the collagen III-positive area (d). Magnification, $\times 400$. The bars indicate the thickness of the submesothelial compact zone. The data are expressed as mean \pm SD; * represents $P < 0.01$ vs PBS group.

tained a significant smaller number of VEGF-expressing cells in the submesothelial area than that in the WT-CG group (Fig. 5k and l).

Effects of leptin administration on peritoneal fibrosis

To confirm the effect of leptin on the progression of peritoneal fibrosis, we administered recombinant leptin through an osmotic pump to the CG-injected WT mice. CG injection significantly increased the accumulation of collagen III in the submesothelial area (Fig. 6a). In particular, the positive area for collagen III was found to be significantly more increased in the leptin group than in the PBS group (Fig. 6b and c).

IV. Discussion

In the present study, we showed that CG injection increased the level of leptin in serum and peritoneal fluid with thickening of submesothelial compact zone in wild type mice. Similarly, we confirmed the increased expression of leptin in the peritoneum of CG-injected C57BL/6 mice by immunohistochemical examination. We also demonstrated increased expression of leptin receptors by CG injection, independent of leptin expression. Moreover, we demonstrated that leptin-deficient mice, *ob/ob* mice, attenuate peritoneal fibrosis induced by CG in the experimental mouse model. In addition, CG-injected *ob/ob* mice revealed markedly reduced the number of myofibroblasts, infiltrating macrophages, and blood vessels in the thickened submesothelial area. Meanwhile, leptin administration induced a more severe peritoneal fibrosis in the WT-CG group than in the PBS group.

Although the pathogenesis of peritoneal fibrosis in PD patients is still unclear, many factors are considered to be involved in the development of peritoneal fibrosis. In the present study, we used a mouse experimental model of peritoneal fibrosis induced by CG. Suga *et al.* [26] and Ishii *et al.* [9] developed experimental models for peritoneal fibrosis in rats and EPS in mice, respectively, by peritoneal injection of CG. Whether this model is relevant to the

peritoneal sclerosis and encapsulating peritoneal sclerosis seen in PD 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 PD patients. In fact, following injection of CG as a nonspecific chemical to induce peritoneal fibrosis, many pathologic findings in the peritoneum of PD patients, including increased expression of collagen III, α -SMA, macrophages infiltration, were also observed in our model. These similarities in alterations of the peritoneal membranes between experimental CG models and human PD patients strongly suggest that the CG model is a reasonable candidate model for examining the efficacy of various potential therapeutic reagents for regulating peritoneal sclerosis.

Adipose tissue is ubiquitously present in peritoneal tissue and it is known that patients on PD have increased fat mass due to glucose absorption from the PD fluid. Recently, peritoneal adipocytes have been suggested to play an important role in chronic inflammation through the release of adipokines [24, 32]. Among these adipokines, a marked increase in leptin concentration from serum and dialysate of patients undergoing PD has been reported [33]. In patients with PD, the serum level of leptin is increased because of decreased excretion from kidney. In addition, it has been known that there are some specific factors for patients undergoing PD, such as unphysiological high glucose PD fluid or peritonitis, which promote local synthesis of leptin through IL-1 β and TNF- α [12, 13]. Since CG injection causes chemical inflammation and increases proinflammatory cytokines including IL-1 β and TNF- α [35], we considered that these cytokines stimulate leptin synthesis in the peritoneal cavity in CG-injected mice. Moreover, the leptin receptor expression was also up-regulated in the CG-injected mice, independent of leptin expression. Matsui *et al.* previously reported that leptin and leptin receptor expressions were induced after ischemia/reperfusion [16]. Although the precise mechanism of these findings is unknown, leptin and leptin receptor expressions have been suggested to be regulated by various mediators

such as inflammatory cytokines and oxidative stress. Given that the pathophysiological mechanism was reported to be involved in inflammation or oxidative stress in a CG-induced peritoneal fibrosis model [10, 35], these factors might upregulate the expression of not only leptin but also the leptin receptor in our study.

TGF- β is known to play an essential role on the progression of fibrosis. Leung *et al.* also demonstrated that leptin induces TGF- β synthesis through the functional leptin receptor expressed by human mesothelial cells [13]. Moreover, Kumpers *et al.* showed that leptin can serve as a cofactor of TGF- β activation [11]. Thus, leptin deletion might lead to a significant decrease of the number of TGF- β -positive cells and fail to activate TGF- β signaling in the peritoneum of CG-injected *ob/ob* mice in our study. Based on the previous data [17, 22], macrophage infiltration also may play an important role in the development of peritoneal fibrosis. Recently, Tanaka *et al.* demonstrated that leptin administration in *ob/ob* mice accelerated renal macrophage infiltration through the melanocortin system in unilateral ureteral obstruction model [28]. Therefore, it is possible that leptin deletion led to suppression of macrophage infiltration and prevention of peritoneal fibrosis in our model. Furthermore, leptin promotes angiogenesis via upregulation of VEGF [30]. Similarly, we confirmed the upregulation of VEGF and angiogenesis in CG mice and a decreased number of VEGF-positive cells and vessels in CG injected-*ob/ob* mice. Taken together, these findings seemed to support that increased leptin contribute to the progression of fibrosis through various pathways.

In conclusion, we found that in wild-type mice, CG treatment cause the progression of peritoneal fibrosis through increase of TGF- β expressing cells, infiltrating macrophages, and angiogenesis. All of these parameters were decreased in peritoneal tissues of CG-injected *ob/ob* mice. In addition, we also demonstrated that leptin administration in the CG-injected mice aggravated peritoneal fibrosis. Thus, an upregulation of leptin appears to play a role in fibrosis and inflammation during peritoneal injury. Our results indicate the involvement of leptin in the progression of peritoneal fibrosis and treatment strategy for reducing leptin may be a therapeutically potential for peritoneal fibrosis.

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VI. References

1. Abo, T., Nagayasu, T., Hishikawa, Y., Tagawa, T., Nanashima, A., Yamayoshi, T., Matsumoto, K., An, S. and Koji, T. (2010) Expression of keratinocyte growth factor and its receptor in rat tracheal cartilage: possible involvement in wound healing of the damaged cartilage. *Acta Histochem. Cytochem.* 43; 89–98.
2. Ahima, R. S., Prabakaran, D., Mantzoros, C., Qu, D., Lowell, B., Maratos-Flier, E. and Flier, J. S. (1996) Role of leptin in the neuroendocrine response to fasting. *Nature* 382; 250–252.
3. Arai, H., Furusu, A., Nishino, T., Obata, Y., Nakazawa, Y., Nakazawa, M., Hirose, M., Abe, K., Koji, T. and Kohno, S. (2011) Thalidomide prevents the progression of peritoneal fibrosis in mice. *Acta Histochem. Cytochem.* 44; 51–60.
4. Coles, G. A. and Topley, N. (2000) Long-term peritoneal membrane changes. *Adv. Ren. Replace Ther.* 7; 289–301.
5. Dagogo-Jack, S., Ovalle, F., Landt, M., Gearing, B. and Coyne, D. W. (1998) Hyperleptinemia in patients with end-stage renal disease undergoing continuous ambulatory peritoneal dialysis. *Perit. Dial. Int.* 18; 34–40.
6. Friedman, J. M. and Halaas, J. L. (1998) Leptin and the regulation of body weight in mammals. *Nature* 395; 763–770.
7. Hauglustaine, D., van Meerbeek, J., Monballyu, J., Goddeeris, P., Lauwerijns, J. and Michielsens, P. (1984) Sclerosing peritonitis with mural bowel fibrosis in a patient on long-term CAPD. *Clin. Nephrol.* 22; 158–162.
8. Ikejima, K., Takei, Y., Honda, H., Hirose, M., Yoshikawa, M., Zhang, Y. J., Lang, T., Fukuda, T., Yamashina, S., Kitamura, T. and Sato, N. (2002) Leptin receptor-mediated signaling regulates hepatic fibrogenesis and remodeling of extracellular matrix in the rat. *Gastroenterology* 122; 1399–1410.
9. Ishii, Y., Sawada, T., Shimizu, A., Tojimbara, T., Nakajima, I., Fuchinoue, S. and Teraoka, S. (2001) An experimental sclerosing encapsulating peritonitis model in mice. *Nephrol. Dial. Transplant.* 16; 1262–1266.
10. Kitamoto, M., Kato, K., Sugimoto, A., Kitamura, H., Uemura, K., Takeda, T., Wu, C., Nogaki, F., Morimoto, T. and Ono, T. (2011) Sairei-to ameliorates rat peritoneal fibrosis partly through suppression of oxidative stress. *Nephron Exp. Nephrol.* 117; e71–81.
11. Kumpers, P., Gueler, F., Rong, S., Mengel, M., Tossidou, I., Peters, I., Haller, H. and Schiffer, M. (2007) Leptin is a co-activator of TGF-beta in unilateral ureteral obstructive kidney disease. *Am. J. Physiol. Renal Physiol.* 293; F1355–1362.
12. Lam, M. F., Leung, J. C., Lo, W. K., Tam, S., Chong, M. C., Lui, S. L., Tse, K. C., Chan, T. M. and Lai, K. N. (2007) Hyperleptinaemia and chronic inflammation after peritonitis predicts poor nutritional status and mortality in patients on peritoneal dialysis. *Nephrol. Dial. Transplant.* 22; 1445–1450.
13. Leung, J. C., Chan, L. Y., Tang, S. C., Chu, K. M. and Lai, K. N. (2006) Leptin induces TGF-beta synthesis through functional leptin receptor expressed by human peritoneal mesothelial cell. *Kidney Int.* 69; 2078–2086.
14. Lord, G. M., Matarese, G., Howard, J. K., Baker, R. J., Bloom, S. R. and Lechler, R. I. (1998) Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppression. *Nature* 394; 897–901.
15. Matarese, G., Di Giacomo, A., Sanna, V., Lord, G. M., Howard, J. K., Di Tuoro, A., Bloom, S. R., Lechler, R. I., Zappacosta, S. and Fontana, S. (2001) Requirement for leptin in the induction and progression of autoimmune encephalomyelitis. *J. Immunol.* 166; 5909–5916.
16. Matsui, H., Motooka, M., Koike, H., Inoue, M., Iwasaki, T., Suzuki, T., Kurabayashi, M. and Yokoyama, T. (2007) Ischemia/reperfusion in rat heart induces leptin and leptin receptor gene expression. *Life Sci.* 80; 672–680.
17. Mishima, Y., Miyazaki, M., Abe, K., Ozono, Y., Shiohita, K., Xia, Z., Harada, T., Taguchi, T., Koji, T. and Kohno, S. (2003) Enhanced expression of heat shock protein 47 in rat model of peritoneal fibrosis. *Perit. Dial. Int.* 23; 14–22.
18. Nakamoto, M. (1996) Pathogenesis of peritoneal fibrosis and peritoneal small vessel changes. *Perit. Dial. Int.* 16 Suppl 1; S39–41.
19. Nakata, M., Yada, T., Soejima, N. and Maruyama, I. (1999) Leptin promotes aggregation of human platelets via the long form of its receptor. *Diabetes* 48; 426–429.

20. Sahai, A., Malladi, P., Pan, X., Paul, R., Melin-Aldana, H., Green, R. M. and Whittington, P. F. (2004) Obese and diabetic db/db mice develop marked liver fibrosis in a model of nonalcoholic steatohepatitis: role of short-form leptin receptors and osteopontin. *Am. J. Physiol. Gastrointest. Liver Physiol.* 287; G1035–1043.
21. Saxena, N. K., Ikeda, K., Rockey, D. C., Friedman, S. L. and Anania, F. A. (2002) Leptin in hepatic fibrosis: evidence for increased collagen production in stellate cells and lean littermates of ob/ob mice. *Hepatology* 35; 762–771.
22. Shiohita, K., Miyazaki, M., Ozono, Y., Abe, K., Taura, K., Harada, T., Koji, T., Taguchi, T. and Kohno, S. (2000) Expression of heat shock proteins 47 and 70 in the peritoneum of patients on continuous ambulatory peritoneal dialysis. *Kidney Int.* 57; 619–631.
23. Sierra-Honigmann, M. R., Nath, A. K., Murakami, C., Garcia-Cardena, G., Papapetropoulos, A., Sessa, W. C., Madge, L. A., Schechner, J. S., Schwabb, M. B., Polverini, P. J. and Flores-Riveros, J. R. (1998) Biological action of leptin as an angiogenic factor. *Science* 281; 1683–1686.
24. Sommer, G., Kralisch, S., Stangl, V., Vietzke, A., Kohler, U., Stepan, H., Faber, R., Schubert, A., Lossner, U., Bluher, M., Stumvoll, M. and Fasshauer, M. (2009) Secretory products from human adipocytes stimulate proinflammatory cytokine secretion from human endothelial cells. *J. Cell Biochem.* 106; 729–737.
25. Song, N., Liu, J., An, S., Nishino, T., Hishikawa, Y. and Koji, T. (2011) Immunohistochemical analysis of histone H3 modifications in germ cells during mouse spermatogenesis. *Acta Histochem. Cytochem.* 44; 183–190.
26. Suga, H., Teraoka, S., Ota, K., Komemushi, S., Furutani, S., Yamauchi, S. and Margolin, S. (1995) Preventive effect of pirfenidone against experimental sclerosing peritonitis in rats. *Exp. Toxicol. Pathol.* 47; 287–291.
27. Suganami, E., Takagi, H., Ohashi, H., Suzuma, K., Suzuma, I., Oh, H., Watanabe, D., Ojima, T., Suganami, T., Fujio, Y., Nakao, K., Ogawa, Y. and Yoshimura, N. (2004) Leptin stimulates ischemia-induced retinal neovascularization: possible role of vascular endothelial growth factor expressed in retinal endothelial cells. *Diabetes* 53; 2443–2448.
28. Tanaka, M., Suganami, T., Sugita, S., Shimoda, Y., Kasahara, M., Aoe, S., Takeya, M., Takeda, S., Kamei, Y. and Ogawa, Y. (2010) Role of central leptin signaling in renal macrophage infiltration. *Endocr. J.* 57; 61–72.
29. Tang, M., Potter, J. J. and Mezey, E. (2002) Leptin enhances the effect of transforming growth factor beta in increasing type I collagen formation. *Biochem. Biophys. Res. Commun.* 297; 906–911.
30. Tanigawa, T., Watanabe, T., Otani, K., Nadatani, Y., Machida, H., Okazaki, H., Yamagami, H., Watanabe, K., Tominaga, K., Fujiwara, Y., Oshitani, N., Higuchi, K. and Arakawa, T. (2010) Leptin promotes gastric ulcer healing via upregulation of vascular endothelial growth factor. *Digestion* 81; 86–95.
31. Tarzi, R. M., Cook, H. T., Jackson, I., Pusey, C. D. and Lord, G. M. (2004) Leptin-deficient mice are protected from accelerated nephrotoxic nephritis. *Am. J. Pathol.* 164; 385–390.
32. Tilg, H. and Moschen, A. R. (2006) Adipocytokines: mediators linking adipose tissue, inflammation and immunity. *Nat. Rev. Immunol.* 6; 772–783.
33. Tsujimoto, Y., Shoji, T., Tabata, T., Morita, A., Emoto, M., Nishizawa, Y. and Morii, H. (1999) Leptin in peritoneal dialysate from continuous ambulatory peritoneal dialysis patients. *Am. J. Kidney Dis.* 34; 832–838.
34. Williams, J. D., Craig, K. J., Topley, N., Von Ruhland, C., Fallon, M., Newman, G. R., Mackenzie, R. K. and Williams, G. T. (2002) Morphologic changes in the peritoneal membrane of patients with renal disease. *J. Am. Soc. Nephrol.* 13; 470–479.
35. Yokoi, H., Kasahara, M., Mori, K., Ogawa, Y., Kuwabara, T., Imamaki, H., Kawanishi, T., Koga, K., Ishii, A., Kato, Y., Mori, K. P., Toda, N., Ohno, S., Muramatsu, H., Muramatsu, T., Sugawara, A., Mukoyama, M. and Nakao, K. (2012) Pleiotrophin triggers inflammation and increased peritoneal permeability leading to peritoneal fibrosis. *Kidney Int.* 81; 160–169.
36. Yoshio, Y., Miyazaki, M., Abe, K., Nishino, T., Furusu, A., Mizuta, Y., Harada, T., Ozono, Y., Koji, T. and Kohno, S. (2004) TNP-470, an angiogenesis inhibitor, suppresses the progression of peritoneal fibrosis in mouse experimental model. *Kidney Int.* 66; 1677–1685.
37. Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L. and Friedman, J. M. (1994) Positional cloning of the mouse obese gene and its human homologue. *Nature* 372; 425–432.

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