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<td>Author(s)</td>
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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
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-Lepob/Lepob) 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 ob/ob-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-
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 μm in the examined field under the microscope and measured the area of the submesothelial layer within the selected width of 421 μm. 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 was detected in peritoneal macrophages (Fig. 2b). In ob/ob mice, leptin receptor was localized in mesothelial cells and peritoneal macrophages, but not in submesothelial myofibroblasts (Fig. 2c and d).

**Table 1. The list of primary antibodies for immunohistochemistry**

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

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

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<tr>
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<th>ob/ob</th>
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<tr>
<td>Serum (ng/ml)</td>
<td>1.08±0.59</td>
<td>0.07±0.04</td>
</tr>
<tr>
<td>Peritoneal fluid (ng/ml)</td>
<td>4.74±1.81*</td>
<td>0.54±0.35*</td>
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*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, ×400.
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).
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 compact zone (Fig. 4a), while the positive area of collagen III was significantly reduced in ob/ob-control group (Fig. 4b and c). Bar graph shows the semiquantitative analysis of collagen III-positive area (e). 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, ×400; bars indicate the thickness of the submesothelial compact zone. Data are expressed as mean±SD; * represents P<0.01 vs WT-CG group.

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 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).
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-
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-1β and TNF-α [12, 13]. Since CG injection causes chemical inflammation and increases proinflammatory cytokines including IL-1β 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 upregulated 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.

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 (e). The bar graph shows the result of the semiquantitative analysis of the collagen III-positive area (f). Magnification, ×400. The bars indicate the thickness of the submesothelial compact zone. The data are expressed as mean±SD; * represents P<0.01 vs PBS group.
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.

V. Acknowledgments

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VI. References


