Attenuation of methylglyoxal-induced peritoneal fibrosis: immunomodulation by interleukin-10

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Peritoneal fibrosis (PF), a serious pathophysiology of peritoneal dialysis (PD), is implicated in various types of chronic inflammation. In the present study, we examined the benefits of interleukin (IL)-10, which exerts anti-inflammatory effects, in an experimental rat model of methylglyoxal (MGO)-induced PF. We injected an adeno-associated virus (AAV) vector encoding rat IL-10 or enhanced green fluorescent protein (GFP) into male Sprague-Dawley rats at 6 weeks of age. Four weeks later, the rats received continuous peritoneal injections of conventional PD fluid (PDF) with MGO for 3 weeks. Then, the peritoneal histology and the expression levels of fibrogenic mediators and proinflammatory cytokines were analyzed. The rats demonstrating persistent IL-10 expression showed significantly reduced fibrous peritoneal thickening compared with those with GFP expression. The infiltration of macrophages, the expression of tumor necrosis factor- α , IL-1 β , IL-6, transforming growth factor- β 1, Snail, and matrix metalloproteinase 2 genes as well as the proliferation of mesenchymal-like mesothelial cells augmented by MGO were all significantly suppressed by IL-10 expression. IL-10 also abrogated the extent of MGO-induced bowel adhesions mimicking a cocoon-like mass. Our findings provide valuable insight into the potential benefit of immunomodulation with IL-10 as one potentially effective therapeutic strategy for preventing the onset of peritoneal injury resulting in PF.

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Peritoneal dialysis (PD) is a leading mode of renal replacement therapy for patients with advanced chronic kidney disease.^{1,2} The occurrence of peritoneal fibrosis (PF) has been the focus of serious concern because it may lead to functional failure, including a loss of ultrafiltration capacity and an increased rate of peritoneal transport, thereby attenuating the efficiency of the peritoneum as a biological dialyzing membrane.^{3,4} Although the cause of PF has not yet been elucidated, a main focus of research concerns the fibrogenic responses of the peritoneal tissue to a wide range of injuries owing to chronic inflammation and the bioincompatible nature of conventional PD solutions, which contain a variety of glucose degradation products such as formaldehyde, acetaldehyde, and methylglyoxal (MGO).^{1,3,5–7} MGO is one of a series of dicarbonyl intermediates that can react rapidly with lysine residue, leading to crosslinking and denaturation of proteins.^{1,8} It may cause peritoneal injury with morphological alteration and loss of function by enhancing the accumulation of advanced glycation end-products and/or by stimulating

the production of vascular endothelial growth factor.^{1,7} Previously, a link between the intraperitoneal injection of MGO and the development of PF has been demonstrated in experimental rats models.^{1,6,7}

Interleukin (IL)-10 is a multifunctional cytokine with anti-inflammatory properties.⁹ Accumulating evidence suggests that IL-10 may have a role in suppressing the synthesis of numerous proinflammatory mediators such as tumor necrosis factor (TNF)- α , IL-1, and IL-6 in various pathophysiological settings.^{9,10} Moreover, IL-10 has been shown to reduce the formation of excessive amounts of fibrous in the tissue of various organs, including the heart, lung, liver, and kidney.^{10–13} These findings led us to propose that the administration of IL-10 may thus be a potentially useful therapeutic modality in the future management of PD subjects with PF.

In the present study, we evaluated the effects of IL-10 on the biological characteristics of the peritoneum in an experimental rat model of MGO-induced PF, which is considered to reflect the actual conditions encountered in

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clinical settings.^{1,14} The biological efficiency of the systemic administration of recombinant IL-10 at first appears to be insufficient because of its short bioactive half-life.¹⁵ To overcome this obstacle, we transferred the gene encoding IL-10 using an adeno-associated virus (AAV) vector, which has low potential for targeted integration.¹⁶ As these vectors can be used to transduce skeletal muscle, they can induce both the systemic and sustained expression of candidate therapeutic proteins following a single intramuscular administration.^{9,13,17}

MATERIALS AND METHODS

Production of AAV Type 1 Vectors

Complementary DNA (cDNA) encoding rat IL-10 has been previously described.¹⁷ For efficient transgene expression in the skeletal muscle, we constructed a recombinant AAV type 1 vector that carried either the IL-10 gene (AAV-IL-10) or green fluorescent protein (GFP) gene (AAV-GFP) and that was controlled by a cytomegalovirus (CMV) post-transcriptional regulatory element.^{9,17} The AAV vectors were prepared according to the baculovirus system with some modifications.^{18,19} Briefly, the plasmid encoding rat IL-10 with a CMV promoter (pCMV-IL-10) was digested with *NotI*, and then the CMV-IL-10 expression cassette was inserted into the *NotI* sites of a derivative¹⁸ of the pFastBac Dual (Life Technologies, Carlsbad, CA, USA) (pFBD-CMV-IL-10). The transfer of the expression cassette in pFBD-CMV-IL-10 to the baculovirus genome (bacmid DNA) was carried out in DH10Bac competent cells (Life Technologies). The original virus was amplified in *Spodoptera frugiperda* 9 (*Sf9*) cells (Life Technologies) incubated at 28 °C in shake flask cultures, and then the cultured cells were centrifuged and the supernate was collected as the viral stock. *Sf9* cells were then co-infected with the stocked virus as well as the recombinant baculoviruses expressing the AAV nonstructural replication proteins (Rep) and the type 1 AAV structural capsid proteins (VP). Finally, the AAV vectors were purified from crude cellular lysates by iodixanol step-gradient centrifugation and subsequent column chromatography as has been described previously.²⁰ The titers of the recombinant AAV vectors were determined by real-time PCR with CMV-specific primers,¹⁸ on an ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA).

Laboratory Animals and Experimental Design

All animal procedures were approved by the Jichi Medical University ethics committee and were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Six-week-old male Sprague-Dawley rats were obtained from Japan (Shizuoka, Japan). The animals were housed in a temperature- and humidity-controlled room with a 12-h light/dark cycle and provided standard rat chow (CE-2, Clea Japan, Tokyo, Japan) and water *ad libitum*. Standard PD fluid (PDF), containing 2.5% glucose, 100 mM NaCl, 35 mM sodium

lactate, 2 mM CaCl₂, and 0.7 mM MgCl₂ (Midperiq L 250, Terumo, Tokyo, Japan), and PDF, prepared by adding 10 mM MGO (Sigma-Aldrich, St Louis, MO, USA) to the standard PDF, both of which were sterilized by filtration, were used in the present study. All PDFs were prepared and adjusted to a pH of 5.0 immediately prior to injection each day.

Following an acclimatization period of 3 days, the rats were then randomly assigned to the following three groups (experimental day 0): the phosphate-buffered saline (PBS) group, the AAV-GFP group, and the AAV-IL-10 group. Under ether anesthesia, the PBS buffer or AAV vectors in PBS were injected into the bilateral anterior tibial muscle of the rats (100 µl per cite, 3 × 10¹¹ genome copies per rat). For each animal, the body weight was measured during the observation period. Twenty-eight days later, the PBS rats were further subdivided as follows (*n* = 12 in each group): group 1, PBS rats administered daily intraperitoneal injection of 100 ml/kg of standard PDF (control); and group 2, PBS rats administered daily intraperitoneal injection of 100 ml/kg of standard PDF with MGO (PBS-MGO). The AAV-GFP and AAV-IL-10 rats were also subjected to the daily intraperitoneal injection of 100 ml/kg of standard PDF with MGO and they were designated as group 3 (GFP-MGO, *n* = 12) and group 4 (IL-10-MGO, *n* = 12), respectively. The daily peritoneal injections were performed via simple needle puncture for 21 days, from days 28 to 49, under inhalation anesthesia, as previously described.^{1,6,7} Blood samples were collected by tail vein clipping under ether anesthesia on days 28, 35, and 49 to determine the serum levels of IL-10 using a commercial assay kit (Quantikine ELISA Kit; R&D Systems, Minneapolis, MN, USA). The rats (*n* = 6 in each group) were euthanized with deep anesthesia on day 35 as well as on day 49, at which point the experiment was terminated, to obtain specimens of the

Figure 1 Experimental protocol. Six-week-old Sprague-Dawley rats were randomly assigned on day 0 into the following three groups: the PBS group, the AAV-GFP group, and the AAV-IL-10 group. On day 28, the PBS rats were then further subdivided into the group treated with standard PDF (control) or with that containing MGO (PBS-MGO). The AAV-GFP and AAV-IL-10 rats were also treated with the PDF containing MGO and were designated as the GFP-MGO and the IL-10-MGO groups, respectively. AAV, adeno-associated virus; GFP, green fluorescent protein; IL, interleukin; MGO, methylglyoxal; PBS, phosphate-buffered saline; PDF, PD fluid.

parietal peritoneum for histological evaluations and quantitative reverse transcription-PCR (qRT-PCR) analyses (Figure 1).

Light Microscopy

The parietal peritoneum tissue specimens were fixed with 4% paraformaldehyde and embedded in paraffin. Sections were then created perpendicular to the peritoneal surface, each of which was subsequently stained with hematoxylin–eosin (HE) to determine the cell type. Some sections were subjected to Azan staining to visualize the intensity of collagen fibers in the peritoneum^{7,21}; the morphological changes in the peritoneum were evaluated using photomicroscopy at $\times 200$ magnification. In order to quantify the degree of PF, the thickness of the submesothelial compact zone in the peritoneum, extending from the lower limit of the mesothelial layer to the upper limit of the muscle layer,^{1,6,22} was evaluated. Peritoneal specimens were obtained from two sites in each rat and the thickness was measured at 20 points per site (at 0.5-mm intervals within a range of 1.0 cm) in each HE-stained specimen. The average thickness was then calculated using computerized morphometry (Image Pro 5.1, Media Cybernetics, Bethesda, MD, USA).

Immunofluorescence and Immunohistochemistry

Deparaffinized sections of peritoneal tissues were rehydrated and autoclaved for 15 min at 121 °C in 10 mM citrate buffer (pH 6.0) to retrieve the antigens. Next, the sections were blocked in 10% normal goat serum and washed three times with PBS. For the immunofluorescence studies, the sections were incubated overnight at 4 °C with a monoclonal antibody against cytokeratin (Acris Antibodies GmbH, Herford, Germany) at a dilution of 1:50 and a monoclonal antibody against α -smooth muscle actin (α -SMA) conjugated with Cy3 (Sigma Chemical, St Louis, MO, USA) at a dilution of 1:400, followed by incubation with goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at a dilution of 1:400 for 1 h at room temperature, in order to determine the nature of the cells within the peritoneal tissues.^{1,7,23} Finally, the sections were mounted in VECTASHIELD mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA). Fluorescence was detected by observation using a BX50 optical microscope (Olympus, Tokyo, Japan). Some sections subjected to immunostaining against cluster of differentiation 68 (CD68), which is a specific marker molecule for monocytes/macrophages,²⁴ were treated with 3% H₂O₂ deionized water prior to blocking and incubated with a mouse anti-CD68 monoclonal antibody (Clone No., ED1, AbD Serotec, Oxford, UK) at a dilution of 1:200 overnight at 4 °C. The reaction was then detected with 3,3-diaminobenzidine using the EnVision Kit (Dako, Carpinteria, CA, USA), and the nuclei were counterstained with hematoxylin. The number of CD68-positive cells per unit area were quantified in 10 arbitrary fields per specimen using the image Pro 5.1 software package (Media Cybernetics,

Bethesda, MD, USA), as described previously, with some modifications.^{1,25}

Quantitative Reverse Transcription-PCR

The gene expression levels of TNF- α , IL-1 β , IL-6, transforming growth factor- β 1 (TGF- β 1), Snail, matrix metalloproteinase-2 (MMP-2), cytokeratin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) within the peritoneal tissues and the cultured mesothelial cells were assessed using qRT-PCR. Total RNA was extracted from each specimen according to the RNeasy Kit (QIAGEN GmbH, Hilden, Germany), and the prepared total RNA concentration was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, MA, USA). A total of 0.4 μ g of isolated total RNA was reverse transcribed using the SuperScript VILO cDNA Synthesis Kit (Life Technologies), according to the manufacturer's instructions. For the quantitative analysis of the mRNA content, 6- μ l aliquots from 200 μ l of resulting cDNA were amplified using the QuantiTect SYBR Green PCR Kit (QIAGEN GmbH) on an Applied Biosystems StepOnePlus Real-Time PCR system (Applied Biosystems). All reactions were carried out in a 20- μ l reaction volume and were performed in duplicate. After an initial cycle of denaturation at 95 °C for 10 s, real-time qRT-PCR was performed for 45 cycles under the following conditions: denaturation at 95 °C for 15 s, followed by annealing and extension at 60 °C for 60 s. The sequences of the primers are listed in Table 1. Following real-time qRT-PCR, a dissociation analysis was performed to confirm the specificity of the amplification. Quantification of the target gene expression was obtained using the StepOne software program (Applied Biosystems), and the expression levels of mRNA were normalized with that of GAPDH as a constitutive control. The data are expressed as relative quantities compared with the control group.

Statistical Analysis

The data are shown as the means $\pm$ s.d. The groups were compared using a one-way analysis of variance with Fisher's

Table 1 PCR primer sequences

Primer	Forward (5' $\rightarrow$ 3')	Reverse (5' $\rightarrow$ 3')
TNF- α	ATACACTGGCCCGAGGCAAC	CCACATCTCGGATCATGCTTTC
IL-1 β	GCTGTGGCAGCTACCTATGTCTTG	AGGTCGTCATCATCCACGAG
IL-6	CCACTTCACAAGTCGGAGGCTTA	GTGCATCATCGCTGTTTCATACAATC
TGF- β 1	TGCGCCTGCAGAGATTCAAG	AGGTAACGCCAGGAATTGTTGCTA
Snail	TGCACATCCGAAGCCACA	TCTTCACATCCGAGTGGGTCTG
MMP-2	ACAGCCTGGCATGGGGCAGT	TTCTCCTCCATCCAGTGGAG
GAPDH	GGCACAGTCAAGGCTGAGAATG	ATGGTGGTGAAGACGCCAGTA

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-6, interleukin-6, IL-1 β , interleukin-1 β , MMP-2, matrix metalloproteinase-2; TGF- β 1, transforming growth factor- β 1; TNF- α , tumor necrosis factor- α .

protected least significant difference test. *P*-values of <0.05 were considered to be statistically significant. The statistical analyses were performed using the StatView software program 5 for Windows (SAS Institute, Cary, NC, USA), unless otherwise stated.

RESULTS

Body Weight and Serum Concentrations of IL-10 in PF Model Rats

The longitudinal data for the body weights of the rats in each group are shown in Table 2. The mean body weights of each group were comparable to each other on day 28. On day 49, those of the PBS-MGO, GFP-MGO, and IL-10-MGO groups were numerically lower than those of the control group; however, these differences did not reach statistical significance.

The serum concentrations of IL-10 in the rats were determined at 28, 35, and 49 days after the initial treatments, including the administration of PBS and AAV-GFP or AAV-IL-10. As shown in Figure 2, significant increases in the serum IL-10 levels were confirmed in the IL-10-MGO group, whereas the levels in the PBS-MGO, GFP-MGO, and control groups were comparable. At day 28, the serum levels of IL-10 in the IL-10-MGO group were about 10-fold higher

than in the rest of the group, and similar trends were also confirmed on days 35 and 49.

Changes in the Peritoneal Characteristics During the Observation Period

We next explored the anatomical and histological changes in the peritoneum in all the groups. As shown in Figure 3, the intraperitoneal injection of MGO induced significant peritoneal thickening. The degree of peritoneal thickening was significantly higher in the PBS-MGO and GFP-MGO groups than in the control, while the expression of IL-10 caused a significant decrease in peritoneal thickening when compared with the PBS-MGO and GFP-MGO groups. No statistically significant difference was confirmed between the control and the IL-10-MGO groups. The results of Azan staining revealed a prominent accumulation of collagen at the dependent portions of the thickened peritoneum.

Figure 4 shows the results of the immunohistochemical analyses of the peritoneal expression levels of cytokeratin and α -SMA. A monolayer of mesothelial cells with negative signals for α -SMA and weakly positive signals for cytokeratin was observed on the surface of the thin peritoneum in the control rats. The expression of α -SMA proteins appeared to be promoted and those of cytokeratin to be diminished by the peritoneal injection of MGO on day 35 regardless of the presence or absence of the AAV vector, and such findings became more prominent at day 49, while these changes seemed to be prevented or attenuated in the IL-10-MGO group. Similar trends were also noted in the amounts of gene transcripts of TGF- β 1, Snail, and MMP-2 in the peritoneum. As shown in Figure 5, the qRT-PCR analysis revealed that the transduction of IL-10 diminished the increases in the gene expression levels of TGF- β 1, Snail, and MMP-2 that were confirmed in the PBS-MGO and GFP-MGO groups. No significant differences in the amounts of these gene transcripts were noted between the control and IL-10-MGO groups.

Table 2 Changes in body weight in each group during the observation period

	Control	PBS-MGO	GFP-MGO	IL-10-MGO
Day 0 (g)	197.0 $\pm$ 11.3	191.3 $\pm$ 6.5	194.5 $\pm$ 5.7	193.5 $\pm$ 5.6
Day 28 (g)	360.8 $\pm$ 21.2	356.5 $\pm$ 15.6	363.5 $\pm$ 6.0	353.7 $\pm$ 19.8
Day 49 (g)	382.0 $\pm$ 26.8	370.2 $\pm$ 23.3	371.0 $\pm$ 20.3	375.8 $\pm$ 32.2

Abbreviations: GFP, green fluorescent protein; IL, interleukin; MGO, methylglyoxal; PBS, phosphate-buffered saline.

Data are shown as means $\pm$ s.d.

Figure 2 The serum levels of IL-10 at days 28, 35, and 49 in each group ($n=6$). Note that the IL-10-MGO group had serum IL-10 levels that were ≥ 10 times than the control and the groups of PBS-MGO and GFP-MGO had. $\#\#P < 0.01$ vs control, and PBS-MGO and GFP-MGO groups at the same point. GFP, green fluorescent protein; IL, interleukin; MGO, methylglyoxal; PBS, phosphate-buffered saline.

The Effect of IL-10 on the Inflammatory Characteristics of the Peritoneum

We further evaluated the peritoneal inflammatory characteristics. The numbers of CD68-positive cells infiltrating the peritoneum in the PBS-MGO, GFP-MGO, and IL-10-MGO groups were significantly higher than that of the control rats on day 35, while the persistent expression of IL-10 caused a significant decrease in the degree of infiltration when compared with the PBS-MGO and GFP-MGO groups. On day 49, accelerated infiltration of the cells was still noted in the PBS-MGO and GFP-MGO groups; however, the counter-vailing effect of IL-10 was no longer confirmed (Figure 6). We then assessed the gene expression levels of proinflammatory cytokines. As shown in Figure 7, the qRT-PCR analysis disclosed the inhibitory effect of a persistent expression of IL-10 on the accelerated gene expression levels of TNF- α ,

Figure 3 The results of the histological analyses (a) of the parietal peritoneum with HE (upper panel) and Azan (lower panel) staining. The scale bar is as indicated. The peritoneal thickness in each group ($n=6$) was also demonstrated (b). Note the presence of profound collagen depositions, confirmed by Azan staining, in the dependent portions of the thickened peritoneum. $^{**}P<0.01$. GFP, green fluorescent protein; HE, hematoxylin–eosin; IL, interleukin; MGO, methylglyoxal; PBS, phosphate-buffered saline.

IL-1 β , and IL-6, which were then confirmed in the PBS-MGO and GFP-MGO groups on both days 35 and 49.

Bowel Adhesion Induced by the Peritoneal Administration of MGO

The appearances of organs in the peritoneal cavity on day 49 are shown in Figure 8. Although no rat was associated with macroscopic peritoneal adhesion on day 35 (data not shown), 5 out of the 12 rats in both the PBS-MGO and GFP-MGO groups showed bowel adhesions simulating a cocoon-like mass. In contrast, no adhesive change in the bowel was observed in the rats of the IL-10-MGO group.

DISCUSSION

The present study clearly demonstrates, for the first time, that IL-10, delivered by an AAV vector, suppresses the pathophysiological alterations of the peritoneum in an experimental rat model of MGO-induced PF, including the peritoneal thickening associated with fibrogenic characteristics and elevated gene expression of several proinflammatory cytokines without affecting the body weight. These results suggest that IL-10 has a role in the overall management of PF among the subjects under PD treatment. The dosage of MGO used in the current study may be controversial. However, our previous findings indicated the amounts of MGO required to induce PF and the risk assessment for peritoneal injury by MGO prompted us to conclude that the dosage of MGO in the current experimental protocol may not necessarily be impractical.^{6,7} Indeed, there are several studies in rat experimental models that used similar amounts of MGO

which were designed to evaluate the pathologies of PF by mimicking the compromised peritoneum in the long-term PD patients.^{14,26,27}

The precise pathogenesis of PF in PD patients remains to be elucidated; however, numerous studies have focused on the alterations in peritoneal integrity owing to cross-reactions between matrix molecules and the effects of multiple receptor-dependent damaging processes as major determinants of PF.^{1,3,23,28} Resident stromal fibroblasts and inflammatory cells have been classically considered the chief cells responsible for such structural and functional changes in the peritoneum, while recent works have shown that mesothelial cells, which acquire higher migratory and invasive capacities through epithelial-to-mesenchymal transition (EMT), likely contribute to PF and ultimately lead to peritoneal membrane failure.^{1,3,23,28} In this regard, the peritoneal findings of the MGO-treated rats confirmed in the current study, including the gradual loss of epithelial phenotype, the proliferation of mesenchymal-like mesothelial cells, and the *de novo* expression of α -SMA accompanied by increased gene expression levels of TGF- β 1, Snail, and MMP-2, all of which are considered to be the histological and molecular characteristics of EMT,^{1,3,6,7,28} may not be surprising. However, the impact of the present experiment should be carefully evaluated especially regarding the fact that our research protocol may make it possible to develop new therapeutic modalities for the treatment of chronic peritoneal injury resulting in the concurrent development of PF.

Infectious episodes with microbes and repeated stimulus by PDFs with a bioincompatible nature result in a various

Figure 4 Characteristics of the cells in the peritoneal tissues at days 35 and 49 in each group. The cellular expression levels of cytokeratin (upper panel, green) and α -SMA (middle panel, red) in the peritoneum were evaluated. The presence or absence of dual positive cells (arrow heads) was also analyzed (lower panel) with nuclear counterstaining (blue, DAPI). The scattered dual-positive cells were seen on the surface of the peritoneum at day 35, while the number of the cells expressing α -SMA rather became more prominent at day 49. Note that the findings of the control and those of the IL-10-MGO group appeared to be comparable. The scale bar is as indicated. α -SMA, α -smooth muscle actin; DAPI, 4',6-diamidino-2-phenylindole; GFP, green fluorescent protein; IL, interleukin; MGO, methylglyoxal; PBS, phosphate-buffered saline.

magnitudes of inflammation, which thus cause acute and/or chronic damage to peritoneal tissue.²⁸ Inflammatory alterations, such as an elevated number of macrophages, are often seen in the peritoneum of PD patients, while this is true even in the absence of infectious peritonitis, thus indicating that the peritoneum of PD patients is a chronically inflamed

organ.^{29,30} As a matter of course, a wide number of cytokines and growth factors likely have a role in preserving the complex chronic inflammatory status of the peritoneum.^{3,5,28,30,31} Mesothelial cells as well as recruited macrophages are considered to be significantly incorporated into the cytokine network that is associated with peritoneal diseases.^{32,33} On the

Figure 5 The amount of gene transcripts of TGF-β1 (a), Snail (b), and MMP-2 (c) in the peritoneum at day 49 was quantified using qRT-PCR. The results were normalized to the level of GAPDH and are shown as relative quantities compared with the values in the control group. * $P < 0.05$, ** $P < 0.01$. $n = 6$ in each group. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; IL, interleukin; MGO, methylglyoxal; MMP-2, matrix metalloproteinase-2; PBS, phosphate-buffered saline; qRT-PCR, quantitative reverse transcription-PCR; TGF-β1, transforming growth factor-β1.

Figure 6 The infiltration of CD68-positive cells within the peritoneal tissues at days 35 (upper panel) and 49 (lower panel) were evaluated using immunohistochemical staining (a). The number of cells expressing CD68 within the peritoneum ($n = 6$ in each group) were quantified (b). ** $P < 0.01$. CD68, cluster of differentiation 68; GFP, green fluorescent protein; IL, interleukin; MGO, methylglyoxal; PBS, phosphate-buffered saline.

other hand, several kinds of MGO-mediated processes have been shown to be implicated in the accelerated productions of TNF- α , IL-1 β , and IL-6 in macrophages.^{34,35} In addition, the overexpression of TNF- α or IL-1 β in the peritoneum has been shown to be able to induce an increased expression of TGF- β

along with fibronectin expression and collagen deposition as well as an acute inflammatory response.³⁶

IL-10 was originally described as cytokine synthesis inhibitory factor.³⁷ Although the mechanism of immunomodulation by IL-10 remains to be delineated, there are several

Figure 7 The amount of gene transcripts of TNF- α (a), IL-1 β (b), and IL-6 (c) in the peritoneum at days 35 and 49 was quantified using qRT-PCR. The results were normalized to the level of GAPDH and are shown as relative quantities compared with the values in the control group at the same time point. * $P < 0.05$, ** $P < 0.01$. $n = 6$ in each group. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; IL, interleukin; MGO, methylglyoxal; PBS, phosphate-buffered saline; qRT-PCR, quantitative reverse transcription-PCR; TNF- α , tumor necrosis factor α .

Figure 8 Macroscopic findings of the bowels at day 49. Note the bowel adhesions in the PBS-MGO and GFP-MGO rats mimicking a cocoon-like mass (arrow heads). GFP, green fluorescent protein; IL, interleukin; MGO, methylglyoxal; PBS, phosphate-buffered saline.

reports showing that IL-10 signaling in immune cells mediates the inhibitory effects through the inhibition of nuclear factor- κ B, a transcription factor which controls the expression of numerous inflammatory regulators.³⁸ Recent studies have also revealed that IL-10 has a pivotal role in the regulation of tissue scarring by both modulating recruitment and inducing the differentiation of inflammatory cells, thereby reducing their release of proinflammatory cytokines, decreasing extracellular matrix (ECM) synthesis, and increasing ECM breakdown by regulating proteolysis, and thus causing the downregulation of TGF- β expression.^{10,39} The current study did not allow us to independently assess the impact of IL-10 on the chronic inflammatory milieu and the pathogenic process for the PF. We also failed to precisely evaluate the individual role of IL-10 in macrophage infiltration in the peritoneum because the magnitude of such phenomena in the PBS-MGO, GFP-MGO, and IL-10-MGO groups only became comparable at the end point of this study. Nevertheless, the fact that the attenuated fibrogenic events in the presence of IL-10 and the numerous previous findings described above led us to consider immunomodulation with IL-10 to be a potentially beneficial strategy for the management of peritoneal injury resulting in PF.

In clinical settings, some patients with PD treatment occasionally manifest encapsulating peritoneal sclerosis (EPS), an aggressive phenotype of PF that has been described to

constitute a spectrum of peritoneal fibrosing syndromes.^{1,3,40} Some cases of EPS develop under conditions of severe inflammatory insult, while no apparent causes of ongoing inflammation are confirmed in other cases, and this discrepancy may be caused, at least in part, by some pathophysiological predisposition.³ Indeed, several previous studies have shown the residual renal function to be significantly lower in patients with EPS,^{41,42} thus implying that an abnormal fibrogenic response may be amplified in proportion to the decline in the residual renal function.¹ The histological alterations observed in EPS include the loss of mesothelial cells and the development of a thick, acellular collagen rind that encloses the loops of the bowel with extensive intra-abdominal adhesions.³ Previously, we demonstrated that an abdominal cocoon mimicking human EPS may be inducible in rats treated with the peritoneal injection of MGO.⁶ We believe that some of the MGO-treated subjects in the current study may thus have a similar pathological status despite the lack of equivalence verification by a histological analysis. As a result, the macroscopic appearance of the abdomen with no adhesive change in the bowel that was observed in the IL-10-MGO group may therefore suggest the therapeutic potential of IL-10 for EPS. Interestingly, even the postoperative intraperitoneal adhesion formation has been proposed as a promising therapeutic target of IL-10.⁴³

Although the present study provides information regarding the effects of IL-10 on the peritoneal characteristics of MGO-treated rats, our results should be interpreted within the context of the study's limitations. The number of animals included in each group was small, thus implying that the study may be underpowered to evaluate the pathophysiological parameters, and some selection bias may also be present, and thus the validity of our findings should be evaluated in an increased number of rats in the future. In addition, precisely how the IL-10 signaling pathway results in the inhibition of a broad spectrum of mediators associated with the development of PF needs to be better characterized. Otherwise, it may be interesting to determine whether IL-10 works, as shown in the current study, under conditions of a deteriorated renal function. Indeed, our findings demonstrated that the various adverse effects of MGO, including the induction of PF, became more prominent under conditions associated with a uremic milieu.¹ Alternatively or additionally, the careful assessment for the biological activities of MMP-2 and TGF- β , which may not necessarily be evaluated by the amounts of their gene transcripts,^{29,44} should be addressed. Finally, PF is a multistep process orchestrated by a complex mixture of numerous growth factors, cytokines, proteolytic enzymes, and matrix molecules. Therefore, the optimal treatment for inducing an acceptable peritoneal response to various types of stimuli is expected to require a combination of appropriate factors. At present, we feel that the procedure applied in the current experimental model for the sustained expression of IL-10 may not be a practical approach in an actual clinical setting. Nevertheless, our observation highlights the pragmatic and therapeutic context, thereby suggesting that the regulation of the inflammatory cascade with IL-10 may have great potential in treating the development of PF. The determination of whether or not the magnitude of PF modulation by such a strategy is parallel to that of peritoneal function, which was not analyzed in the current study, remains an urgent priority.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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