Therapeutic effects and mechanism of conditioned media from human mesenchymal stem cells on anti-GBM glomerulonephritis in WKY rats

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Recent studies have demonstrated that conditioned media derived from mesenchymal stem cells (MSC-CM) have therapeutic effects in various experimental diseases. However, the therapeutic mechanism is not fully understood. In the present study, we investigated the therapeutic effects and mechanism of MSC-CM in experimental anti-glomerular basement membrane glomerulonephritis. We administered either MSC-CM or vehicle from day 0 to day 10 after the induction of nephrotoxic serum nephritis in Wistar-Kyoto rats. In vitro, we analyzed the effects of MSC-CM on TNF-α-mediated cytokine production in cultured normal human mesangial cells, proximal tubular (HK-2) cells, human umbilical vein endothelial cells, and monocytes (THP-1 and peripheral blood mononuclear cells). Compared with vehicle treatment, MSC-CM treatment improved proteinuria and renal dysfunction. Histologically, MSC-CM-treated rats had reduced crescent formation and glomerular ED1+ macrophage infiltration and increased glomerular ED2+ macrophage infiltration. Increased serum monocyte chemotactic protein (MCP)-1 levels were observed in MSC-CM-treated rats. Renal cortical mRNA expression levels of proinflammatory cytokines, such as TNF-α and IL-6, and of the T helper cell 1 cytokine interferon-γ were greatly decreased by MSC-CM treatment. In vitro, pretreatment with MSC-CM blocked TNF-α-mediated IL-8 release in normal human mesangial cells and HK-2 cells. TNF-α-mediated MCP-1 release was enhanced by pretreatment with MSC-CM in human umbilical vein endothelial cells and HK-2 cells and was strikingly enhanced in THP-1 cells. Stimulation of peripheral blood mononuclear cells with a combination of MCP-1 and IL-4 enhanced the expression of M2-associated genes compared with IL-4 alone. We demonstrated that MSC-CM had therapeutic effects in experimental antiglomerular basement membrane glomerulonephritis that were mediated through anti-inflammatory effects that were partly due to acceleration of M2 macrophage polarization, which might be mediated by MCP-1 enhancement.

conditioned media; stem cells; macrophages; monocyte chemotactrant protein-1; glomerulonephritis; glomerular basement membrane; Wistar-Kyoto

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cytokines depending on each microenvironment and the inappropriate timing of MSC-CM injection (46).

We recently reported therapeutic effects of hMSCs in Wistar-Kyoto (WKY) rats with anti-glomerular basement membrane (GBM) GN, a model of rapidly progressive GN that resembles human crescentic GN (39). Treatment with hMSCs significantly attenuated anti-GBM GN and the subsequent development of glomerular fibrosis through anti-inflammatory and immunomodulatory effects, especially a marked increase of serum IL-10 levels indicating paracrine mechanisms of hMSCs in repairing GN. In the present study, we examined whether administration of MSC-CM could also attenuate this model of GN and explored its mechanism of action. Here, we show that MSC-CM attenuates GN through anti-inflammatory effects. In addition, we found that M2-type macrophage polarization could be mediated by monocyte chemotactrant protein (MCP)-1 that was induced by MSC-CM.

MATERIALS AND METHODS

Preparation of MSC-CM. hMSCs were obtained from the Center for the Preparation and Distribution of Adult Stem Cells (http://medicine.tamhsc.edu/irm/msc-distribution.html) and cultured as previously described (39). The supernatant was harvested as MSC-CM, filtered through a 0.22-μm membrane filter (Millex-GS 33 mm, Millipore), and stored at −80°C until later use.

Ethics statement. All animals were given humane care in compliance with institutional guidelines using protocols approved by the Animal Care Committee of Showa University (Tokyo, Japan).

Experimental protocol. Seven-week-old female WKY rats weighing 135–150 g were purchased from Charles River Japan (Kanagawa, Japan) and used in all of the experiments. Animals were housed in the animal care facility of Showa University (25°C, 50% humidity, 12:12-h dark-light cycle) with free access to food and water. A total of 39 female WKY rats were injected intravenously with 20 μl nephrotoxic serum, which was prepared as previously described (22), on day 0. Groups of animals were given 0.5 ml of either MSC-CM or vehicle by daily intraperitoneal administration from day 0 after nephrotoxic serum injection to day 10; all rats were killed on either day 4 or 10. Five female WKY rats at the age of 7 wk were used as normal controls without nephritis. At the end of the study, rats were anesthetized with pentobarbital (100 mg/kg), their blood was collected by cardiac puncture, and their kidneys were collected. The right kidney was snap frozen in liquid nitrogen for biochemical and gene analyses, and the left kidney was fixed in 2% paraformaldehyde-PBS for histological analysis.

Proteinuria and creatinine determination. For the analysis of proteinuria, rats were housed individually in metabolic cages for 24-h urine collection. Urine samples were collected on the day before death. Urinary protein was determined using the Biuret method. Serum and urinary creatinine (Cr) levels were measured using an automated analyzer (Hitachi, Tokyo, Japan) according to the manufacturer’s instructions.

Measurement of circulating anti-rabbit IgG antibody. The level of circulating anti-rabbit IgG antibody in rats with nephritis was measured by ELISA as previously described (21).

Light microscopy. Tissues fixed in 2% paraformaldehyde-PBS were embedded in paraffin using routine protocols. Paraffin-embedded materials were sectioned at 2 μm thickness for routine staining with periodic acid-Schiff. One-micrometer-thick sections were used for periodic acid-methenamine silver staining (silver). The number of crescentic glomeruli per 100 glomeruli of each rat was calculated and expressed as a percentage. The percentage of area occupied by crescents in each glomeruli was estimated and assigned one of the following scores: 0, absent; 1, <1/4; 2, between 1/4 and 1/2; 3, between 1/2 and 3/4; and 4, >3/4 of the whole glomerulus (21). The mean score of 50 glomeruli was then calculated as the crescent score. All histological analyses were performed without knowledge of the origin of the slides.

Immunohistochemistry. The antibodies used in this study were as follows: mouse anti-rabbit ED1 antibody (BMA, Augst, Switzerland), mouse anti-rabbit ED2 (CD163) antibody (BMA), and mouse anti-rat CD8 antibody (clone number: X8, Antigenix America, Huntington Station, NY). Biotinylated rabbit anti-mouse IgG and peroxidase-conjugated streptavidin (LSAB 2 kit/HRP) were purchased from Dako (Glostrup, Denmark). Immunohistochemical staining for ED1 (1:50 dilution), ED2 (1:100 dilution), and CD8 (1:50 dilution) were performed as previously described (21). CD8+ cells were estimated by counting the numbers of these cells within 50 glomeruli and dividing the total number by 50. The extent of ED1 or ED2 staining of each glomerulus was graded for 50 glomeruli on a four-point scale: 0, absent; 1, weak; 2, moderate; and 3, severe (21). The mean score was then calculated as the ED1 or ED2 score. All histological analyses were performed without knowledge of the origin of the slides.

Inmunofluorescence. Tissues were snap frozen in liquid nitrogen and cut into 4-μm-thick sections. The deposits of rabbit IgG and rat IgG in the kidney sections were evaluated using previously described methods (21).

Real-time RT-PCR. In vivo gene expression levels of rat TNF-α, IL-1β, IL-6, MCP-1, interferon (IFN)-γ, IL-4, IL-10, IFN-γ, and GAPDH in kidney tissues (cortex) and in vitro gene expression levels of human chemokine (C-X-C motif) ligand (CXCL)11, indole (INDO), MRC11, chemokine (C-C motif) ligand (CCL)13, and GAPDH in macrophages were analyzed using real-time RT-PCR as previously described (20). mRNA expression was normalized using GAPDH as an endogenous control to correct for the differences in the amount of total RNA added to each reaction.

Quantification of serum cytokine levels. Serum rat IL-17 (eBioscience, San Diego, CA), IL-6, IL-10, IFN-γ, TNF-α, IL-1β (R&D Systems, Minneapolis, MN), and MCP-1 (Thermo, Waltham, MA) protein levels in vivo and human MCP-1 and IL-8 (R&D Systems) protein levels in vitro were determined using commercially available ELISA kits according to the instructions of the respective manufacturers.

Cell cultures. Human proximal tubular cells (HK-2 cells) were obtained from the American Type Culture Collection (Manassas, VA), and normal human mesangial cells (NHMCs) were purchased from Lonza (Basel, Switzerland). These cells were cultured in DMEM-F-12 (1:1) (supplemented with 10% FBS and antibiotics). Human umbilical vein endothelial cells (HUVECs) were purchased from PromoCell (Heidelberg, Germany) and cultured in endothelial cell growth medium-2 (supplemented with 10% FBS and antibiotics). The human monocytic leukemia cell line (THP-1 cells) was obtained from the Human Science Research Resources Bank (Osaka, Japan), and cells were cultured in RPMI 1640 (supplemented with 10% FBS and antibiotics). All of these cells were cultured in an atmosphere of 5% CO2–95% air at 37°C in a humidified incubator.

Isolation of human peripheral blood mononuclear cells and differentiation into macrophages. To obtain resting macrophages (M0 macrophages), peripheral blood mononuclear cells (PBMCs) provided from healthy donors were isolated with Lymphoprep (Axis-Shield, Oslo, Norway), cultured for 2 h under standard cell culture conditions in RPMI 1640 (supplemented with 10% FBS and antibiotics), and then seeded at a density of 5 × 10⁶ cells/well in six-well plates. Nonadherent cells were discarded, and resting macrophages were obtained by maintaining adherent monocytes in RPMI 1640 for 7 days. M1 or M2 macrophages were obtained by stimulating PBMCs with IFN-γ (20 ng/ml) or IL-4 (30 ng/ml) for 7 days, respectively.

Reagents. Recombinant human TNF-α, IL-4, IFN-γ, and MCP-1 were obtained from R&D Systems.
RESULTS

Effects of MSC-CM on biochemical parameters in rats with nephritis. WKY rats with nephrotoxic serum nephritis were given either MSC-CM or vehicle daily and euthanized at either day 4 or 10. There were no significant changes in body weight between vehicle-treated control WKY rats (WKY-vehicle) and MSC-CM-treated WKY (WKY-CM) rats throughout the experimental period (data not shown). Figure 1, A–C, shows the results of urinary total protein levels, serum Cr levels, and total kidney weight for each group. These three parameters were significantly higher in WKY-vehicle rats than in control WKY rats without nephritis [WKY-NTS(−)]. Urinary protein in WKY-Vehicle rats reached 53.09 ± 10.84 mg/day on day 4 and 108.45 ± 6.63 mg/day on day 10. MSC-CM treatment significantly improved urinary protein levels on day 10 (108.45 ± 6.63 vs. 77.94 ± 6.68 mg/day, \( P < 0.01 \); Fig. 1A). Whereas serum Cr levels on day 4 were not different between WKY-vehicle and WKY-CM groups, levels were decreased significantly in WKY-CM rats compared with WKY-vehicle rats on day 10 (day 10: 0.33 ± 0.01 vs. 0.31 ± 0.01 mg/dl, \( P < 0.05 \); Fig. 1B). MSC-CM treatment also suppressed kidney hypertrophy of rats with nephritis on day 10 (1.59 ± 0.04 vs. 1.53 ± 0.02 g, \( P < 0.05 \); Fig. 1C).

Effects of MSC-CM on the process of heterologous antibody deposition or autologous antibody production. Rabbit IgG was detected in an intense linear pattern along the glomerular capillaries in WKY-CM rats and WKY-vehicle rats. Rat IgG was also detected along the glomerular capillaries. There was no significant difference in rabbit IgG and rat IgG glomerular staining between WKY-CM rats and WKY-vehicle rats (data not shown). Furthermore, there was no significant difference in the levels of serum anti-rabbit IgG antibody between WKY-CM rats and WKY-vehicle rats (data not shown). These findings suggest that MSC-CM does not significantly affect the process of heterologous antibody deposition or autologous antibody production.

Effects of MSC-CM on renal histological findings in rats with nephritis. We next analyzed the potential of MSC-CM to improve renal histological findings. Figure 2 shows representative silver-stained sections of each treatment group on days 4 and 10. The glomeruli on day 4 showed severe fibrinoid necrosis and cellular crescent formation (Fig. 2A). The crescentic glomeruli started to transform from cellular to fibrocellular on day 10 (Fig. 2C). MSC-CM treatment significantly reduced the percentage of crescent formation (\( P < 0.05 \)) and crescent score (\( P < 0.05 \)) on day 10 compared with vehicle treatment (Fig. 2, C and D, and Table 1).

Effects of MSC-CM on CD8+ cell influx and ED1+/ED2+ macrophage accumulation in rats with nephritis. Since CD8+ cells and macrophages play a pivotal role in the pathogenesis of this experimental model of anti-GBM GN, the glomerular infiltration of these cells was examined using immunohistochemistry (Fig. 3). The number of CD8+ cells in glomeruli on days 4 and 10 did not differ significantly between the two groups (Table 1). To identify the phenotype of macrophages affected by MSC-CM, glomeruli were immunostained for the expression of ED1, which is a general macrophage marker, and for ED2, which is an anti-inflammatory M2 macrophage marker. Figure 3, A–H, shows typical immunostaining for ED1+ or ED2+ macrophages in glomeruli in each group on days 4 and 10. There were fewer ED1+ macrophages in the glomeruli of WKY-CM rats on day 10 than in the glomeruli of WKY-vehicle rats (Fig. 3, C and D, and Table 1). In contrast, the number of ED2+ macrophages in glomeruli was significantly reduced by MSC-CM treatment compared with vehicle treatment on day 10 (\( P < 0.01 \); Fig. 3, G and H, and Table 1). The number of ED1+ or ED2+ macrophages on day 4 did not differ significantly between the two groups.

Effects of MSC-CM on renal cortical proinflammatory cytokine expression in rats with nephritis. Since MSC-CM significantly reduced inflammatory cell infiltration into glomeruli on day 10, the levels of renal cortical proinflammatory cytokines, which are fundamental to the pathogenesis of crescentic GN, were examined using real-time RT-PCR. Gene expression levels of the proinflammatory cytokines TNF-α, IL-1β, MCP-1, and IL-6 were much higher in WKY-vehicle rats than in WKY-NTS(−) rats. MSC-CM significantly decreased gene expression of these cytokines. MSC-CM treatment decreased the levels of TNF-α, IL-1β, and MCP-1, and IL-6 was decreased by 10.22 (\( P < 0.05 \)) and 0.32 (\( P < 0.01 \)) on day 4, and 10.22 (\( P < 0.05 \)) and 0.32 (\( P < 0.01 \)) on day 10, respectively (Fig. 4A–D, Table 1).
expression levels of TNF-α (P < 0.05), MCP-1 (P < 0.01), and IL-6 (P < 0.01) but not IL-1β on day 4 compared with WKY-vehicle rats (Table 2). The levels of these proinflammatory cytokines did not differ significantly between the two groups on day 10, when the peak of inflammation had already ceased (Table 2).

To determine the systemic effect of MSC-CM on proinflammatory cytokines, serum levels of TNF-α, MCP-1, and IL-6 were measured using ELISA kits. Serum TNF-α and IL-6 levels on days 4 and 10 in the majority of the samples of the study groups were below the detection level (data not shown). However, as shown in Fig. 4, MSC-CM treatment was associated with a significant increase in the serum MCP-1 level in rats with nephritis on day 10 compared with vehicle treatment (P < 0.05).

Effects of MSC-CM on polarization of T helper 1/T helper 2 and T helper 17/regulatory T cells in rats with nephritis. As shown in Table 2, mRNA expression of renal cortical IFN-γ, IL-4, IL-10, IL-17, and Foxp3 was significantly higher in WKY-vehicle rats than in WKY-NTS(−) rats, as assessed using real-time RT-PCR. On day 4, IFN-γ (P < 0.01) and Foxp3 (P < 0.05) gene expression were significantly downregulated by MSC-CM treatment compared with vehicle treatment. MSC-CM treatment did not affect IL-4, IL-10, or IL-17 gene expression. To examine the systemic effect of MSC-CM on T helper (Th)1, Th2, and Th17, serum levels of IFN-γ, IL-10, and IL-17, respectively, were measured on days 4 and 10 using ELISA kits. Levels of serum IFN-γ, IL-10, and IL-17 on days 4 and 10 did not differ significantly between the two groups (data not shown).

Results of multiplex suspension array for 42 cytokines in control media and MSC-CM. To analyze the difference in soluble factors present in MSC-CM and vehicle, a comprehensive evaluation of 42 cytokines was performed using a multiplex suspension array. Table 3 shows the differences in the levels of soluble factors between MSC-CM and the medium of vehicle control. Comprehensive analysis of these cytokines showed that the levels of IL-6, IL-8, MCP-1, PDGF-AA, and VEGF were markedly higher, whereas only the level of PDGF-AB/BB was significantly lower in MSC-CM compared with vehicle control media.

Table 1. Assessment of histological and immunohistochemical findings

<table>
<thead>
<tr>
<th></th>
<th>WKY-vehicle</th>
<th>WKY-CM</th>
<th>WKY-vehicle</th>
<th>WKY-CM</th>
</tr>
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<tbody>
<tr>
<td>% Crescent</td>
<td>50.40 ± 6.99</td>
<td>47.67 ± 9.07</td>
<td>75.08 ± 3.54</td>
<td>62.27 ± 2.72*</td>
</tr>
<tr>
<td>Crescent score (0–4)</td>
<td>0.72 ± 0.09</td>
<td>0.71 ± 0.14</td>
<td>1.74 ± 0.16</td>
<td>1.24 ± 0.17*</td>
</tr>
<tr>
<td>CD8+ cells/glomerular cross section</td>
<td>5.84 ± 0.39</td>
<td>5.83 ± 0.41</td>
<td>3.20 ± 0.18</td>
<td>3.22 ± 0.21</td>
</tr>
<tr>
<td>ED1 score (0–3)</td>
<td>1.79 ± 0.15</td>
<td>1.92 ± 0.15</td>
<td>1.59 ± 0.07</td>
<td>1.33 ± 0.05†</td>
</tr>
<tr>
<td>ED2 score (0–3)</td>
<td>1.03 ± 0.28</td>
<td>1.31 ± 0.08</td>
<td>1.60 ± 0.49</td>
<td>2.05 ± 1.01†</td>
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</table>

Data are means ± SE. Wistar-Kyoto (WKY) rats were treated with either vehicle (WKY-vehicle group) or conditioned media from mesenchymal stem cells (MSC-CM); WKY-CM group) on day 0 and euthanized on days 4 or 10. Semiquantitative assessment of the crescent score, ED1 score, and ED2 score as well as quantitative assessment of crescent formation and CD8+ cells number in each group are shown. Each group contained 10–14 rats, and 50 glomeruli/rat were evaluated in a blind fashion. *P < 0.05 and †P < 0.01 vs. WKY rats with the same duration of vehicle treatment (by Mann-Whitney test).
Effects of MSC-CM on TNF-α-induced MCP-1 production in endothelial cells, mesangial cells, proximal tubular cells, and monocytes in vitro. Unexpectedly, the serum MCP-1 level on day 10 was significantly higher in WKY-CM rats than in WKY-vehicle rats (Fig. 4). To clarify the cellular source of the MCP-1 induced by MSC-CM treatment, we next investigated the effects of MSC-CM on TNF-α-induced MCP-1 production in HUVECs, NHMCs, HK-2 cells, and THP-1 cells in vitro. These cells were pretreated with MSC-CM or vehicle and then exposed to TNF-α (10 ng/ml), and MCP-1 protein levels in the supernatant were then measured. Stimulation with TNF-α alone increased MCP-1 production in HUVECs, NHMCs, and HK-2 cells but not in THP-1 cells compared with cells treated with medium alone. Pretreatment with MSC-CM further increased TNF-α-induced MCP-1 production in HUVECs (P < 0.05) and HK-2 cells (P < 0.01). In THP-1 cells, pretreatment with MSC-CM dramatically enhanced TNF-α-mediated MCP-1 production compared with vehicle pretreatment (12.10 ± 1.06 vs. 330.20 ± 8.84 pg/ml, P < 0.0001, vehicle vs. MSC-CM; Fig. 5). These findings suggest that MSC-CM-induced MCP-1 secretion from monocytes may contribute to the increased level of serum MCP-1 that was observed in WKY-CM rats in vivo.

Effects of MSC-CM on TNF-α-induced IL-8 production in endothelial cells, mesangial cells, proximal tubular cells, and monocytes in vitro. Since MSC-CM strongly enhanced TNF-α-mediated MCP-1 production, we further analyzed the effects of MSC-CM on TNF-α-induced production of the neutrophil chemotactic factor IL-8 in HUVECs, NHMCs, HK-2 cells, and THP-1 cells in vitro. HUVECs, NHMCs, HK-2 cells, and THP-1 cells were exposed to TNF-α (10 ng/ml) after pretreatment with MSC-CM, and IL-8 protein levels in the supernatant were measured. Although TNF-α stimulation of HUVECs, NHMCs, and HK-2 cells led to an increase in IL-8 release, pretreatment with MSC-CM significantly blocked this TNF-α-mediated IL-8 release in NHMCs (P < 0.05) and HK-2 cells (P < 0.001) but not in HUVECs (Fig. 6). In THP-1 cells, neither stimulation with TNF-α alone nor pretreatment with MSC-CM induced IL-8 production (Fig. 6D).

Effects of a combination of MCP-1 and IL-4 on the expression of M2-associated genes in PBMCs. As described above, immunohistochemical staining for ED1 and ED2 showed that MSC-CM decreased infiltrating macrophages and promoted M2 macrophage polarization in this model of nephritis (Table 1). Furthermore, as shown in Figs. 4 and 5, MCP-1 levels were significantly enhanced by MSC-CM treatment both in vivo and in vitro. We therefore next investigated the effects of a combination of MCP-1 and IL-4 on the expression of M2-associated genes in PBMCs.

### Table 2. Results of real-time RT-PCR for cytokine genes in the renal cortex

<table>
<thead>
<tr>
<th>Gene</th>
<th>Day 4</th>
<th>Day 10</th>
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<tr>
<td></td>
<td>WKY-vehicle</td>
<td>WKY-CM</td>
</tr>
<tr>
<td>TNF-α</td>
<td>40.80 ± 4.61</td>
<td>18.57 ± 4.21*</td>
</tr>
<tr>
<td>IL-1β</td>
<td>27.18 ± 3.70</td>
<td>15.63 ± 3.92</td>
</tr>
<tr>
<td>MCP-1</td>
<td>397.42 ± 46.12</td>
<td>163.99 ± 28.12*</td>
</tr>
<tr>
<td>IL-6</td>
<td>91.21 ± 8.37</td>
<td>34.81 ± 8.78†</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>68.62 ± 8.23</td>
<td>28.78 ± 8.81†</td>
</tr>
<tr>
<td>IL-4</td>
<td>2.63 ± 1.84</td>
<td>2.10 ± 0.75</td>
</tr>
<tr>
<td>IL-10</td>
<td>7.78 ± 1.28</td>
<td>6.00 ± 3.17</td>
</tr>
<tr>
<td>IL-17</td>
<td>7.99 ± 4.57</td>
<td>7.33 ± 3.46</td>
</tr>
<tr>
<td>Foxp3</td>
<td>13.52 ± 6.63</td>
<td>6.51 ± 4.09*</td>
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</table>

Data are means ± SE. Real-time RT-PCR for cytokines and profibrogenic genes in each group are shown. Rats were treated with either vehicle or MSC-CM at day 0 and euthanized on days 4 and 10. Values were normalized to the GAPDH values and then expressed as relative quantification. MCP, Monocyte chemotactic protein; IFN, interferon. *P < 0.05 and †P < 0.01 vs. WKY rats with same duration of vehicle treatment (by Mann-Whitney test).
examined the effect of MCP-1 on macrophage phenotype using PBMCs.

PBMCs were coincubated with either the Th1 cytokine IFN-γ or the Th2 cytokine IL-4 in the presence or absence of MCP-1, and the expression of M1- and M2-associated genes was assessed using real-time RT-PCR. This RT-PCR analysis showed that IFN-γ alone caused an upregulation of M1-associated genes CXCL11 and INDO and that the M2-associated genes MRC11 and CCL13 were upregulated by IL-4 alone. Figure 7 shows that the expression of M1-associated genes did not differ significantly between treatment with IFN-γ alone or with a combination of IFN-γ and MCP-1. On the other hand, stimulation with a combination of IL-4 and MCP-1 significantly enhanced the expression of the M2-associated genes MRC11 and CCL13 compared with that induced by IL-4 alone (MRC11: \( P < 0.05 \) and CCL13: \( P < 0.05 \)). However, stimulation with MCP-1 alone did not affect the expression of either M1 or M2 macrophage genes (data not shown). These results indicate that MCP-1 might promote M2 macrophage polarization in some situations.

**DISCUSSION**

In the present study, the major findings were as follows. First, daily intraperitoneal administration of MSC-CM demonstrated both a functional and an histological benefit in rats with anti-GBM GN, as evaluated by a significant reduction in urinary protein level, serum Cr level, kidney weight, and the degree of glomerular crescent formation. Second, in MSC-CM-treated rats, there was a significant reduction in glomerular infiltration of ED1+ macrophages, which are largely involved in the pathogenesis of this model of GN, in addition to upregulation of M2-type macrophages in glomeruli, concomitant with a significant reduction in the mRNA expression of M1-associated proinflammatory cytokines including TNF-α, IL-6, and IFN-γ in the renal cortex. Third, serum MCP-1 levels were significantly increased in MSC-CM treated rats versus vehicle-treated rats, whereas serum levels of other cytokines were not significantly changed by MSC-CM. Fourth, comprehensive analysis of cytokines in MSC-CM showed that levels of IL-6, MCP-1, VEGF, IL-8, and PDGF-AA were markedly higher compared with control media. Fifth, stimulation with TNF-α increased MCP-1 production in cultured endothelial cells (HUVECs), mesangial cells (NHMCs), and proximal tubular cells (HK-2 cells), and MCP-1 production by HUVECs and HK-2 cells was further increased by pretreatment with MSC-CM. Of note, pretreatment with MSC-CM dramatically enhanced TNF-α-mediated MCP-1 production in cultured monocytes (THP-1 cells). Sixth, pretreatment with MSC-CM significantly reduced TNF-α-induced IL-8 production in NHMCs and HK-2 cells. Finally, stimulation of PBMCs with a combination of MCP-1 and IL-4 significantly enhanced the expression of M2-associated genes compared with IL-4 alone, whereas MCP-1 addition did not affect the expression of M1-associated genes stimulated with IFN-γ.

Many studies have shown that MSCs themselves have beneficial therapeutic effects on various experimental disease models via paracrine/endocrine mechanisms. We previously
reported that hMSCs have anti-inflammatory and immuno-modulatory effects on anti-GBM GN. There were some differences in the mechanisms by which hMSCs and MSC-CM repair GN. In the previous study using hMSCs, immunomodulatory effects such as modification of the Th1/Th2 and/or Th17/T regulatory (Treg) balance, especially a large increase in serum Th2 cytokine IL-10 levels, contributed to repair of the early phase of GN (39). MSC treatment has been shown to suppress the Th1 and Th17 response and to induce the Th2 (30) and Treg response (7, 14). Interestingly, such a clear involvement of immunomodulatory effects in the GN repair mechanisms of MSC-CM was not observed in the present study. Thus, although the expression of Th1 cytokine IFN-γ mRNA was inhibited by MSC-CM compared with vehicle treatment, contrary to our expectation, the mRNA expression of Foxp3, which is a transcription factor that is specifically expressed by Treg cells, was also inhibited. Moreover, no changes in the levels of serum cytokines such as IL-10 and IL-17 that were seen in the previous study were observed in the present study with MSC-CM treatment. Instead, serum MCP-1 levels were significantly enhanced after the administration of MSC-CM. However, it is possible that the reparative mechanisms of MSCs and MSC-CM were influenced by differences in the experimental protocol, such as differences in the administration method (intravenously or intraperitoneally), dose, and timing of administration.

Fig. 5. Effects of MSC-CM on TNF-α-induced MCP-1 production in endothelial cells [human umbilical vein endothelial cells (HUVECs)], mesangial cells [normal human mesangial cells (NHMCs)], proximal tubular cells (HK-2 cells), and monocytes (THP-1 cells) in vitro. The indicated cells were preincubated with MSC-CM or control medium for 1 h followed by stimulation with TNF-α (10 ng/ml) or medium. After 24 h of culture, MCP-1 levels in the supernatant of HUVECs (A), NHMCs (B), HK-2 cells (C), and THP-1 cells (D) were measured using ELISA. The horizontal dotted lines show the results of cultures incubated with medium alone. Data are presented as means ± SE; n = 6. ###P < 0.001 compared with cultures with medium alone; *P < 0.05, **P < 0.01, and ***P < 0.001 compared with cultures with the addition of TNF-α (by t-test).

Fig. 6. Effects of MSC-CM on TNF-α-induced IL-8 production in endothelial cells (HUVECs), mesangial cells (NHMCs), proximal tubular cells (HK-2 cells), and monocytes (THP-1 cells) in vitro. The indicated cells were preincubated with MSC-CM or control medium for 1 h followed by stimulation with TNF-α (10 ng/ml) or medium. After 24 h of culture, IL-8 levels in the supernatant of HUVECs (A), NHMCs (B), HK-2 cells (C), and THP-1 cells (D) were measured using ELISA. The horizontal dotted lines show the results of cultures with medium alone. Data are presented as means ± SE; n = 6. ###P < 0.001 compared with cultures with medium alone; *P < 0.05 and ***P < 0.001 compared with cultures with the addition of TNF-α (by t-test).
On the other hand, an anti-inflammatory effect of MSC-CM, which was characterized as decreased ED1+ macrophage infiltration and inversely enhanced M2 macrophage recruitment, with diminished M1-associated proinflammatory cytokines in glomeruli, clearly contributed to the MSC-CM-mediated repair of GN in the present study. Macrophages can be divided into either classical macrophages (M1) or alternatively activated macrophages (M2). Classical macrophages (M1) are associated with inflammation and tissue injury by producing proinflammatory cytokines such as TNF-α, IL-6, IFN-γ, and IL-12 (4, 16). In contrast, alternatively activated macrophages (M2) are associated with wound healing and tissue repair by producing anti-inflammatory cytokines such as IL-10, TGF-β, and IL-1 receptor antagonist (4, 16). Wang et al. (44) showed that M1 macrophages acted as effectors but M2 macrophages can be induced to provide protection against renal injury when infused ex vivo and programmed these macrophages into immunodeficient mice with adriamycin nephropathy. Previous reports have shown that macrophages are crucial for the initiation and subsequent progression of anti-GBM nephritis in WKY rats (10, 19, 40). Consistent with these findings, a variety of strategies used to inactivate glomerular macrophage infiltration have proven effective in inhibiting crescentic formation in GN (6, 17, 21). Although statin and ANG II receptor blockade attenuated anti-GBM GN in WKY rats with augmentation of M2 macrophages and upregulation of anti-inflammatory cytokines similar to our results, the underlying mechanisms of switching from an M1 to M2 phenotype were not elucidated (2, 11). In vitro, we found that MSC-CM significantly enhanced TNF-α-mediated MCP-1 production in cultured endothelial cells, proximal tubular cells, and monocytes. Furthermore, we found that a combination of MCP-1 and IL-4 significantly enhanced the expression of M2-associated genes in PBMCs compared with IL-4 alone, whereas the expression of M1-associated genes was not affected by a combination of MCP-1 and IFN-γ compared with IFN-γ alone. Consistent with these in vitro results, serum MCP-1 levels were significantly increased by MSC-CM administration in vivo. The combined findings indicate that the increase in MCP-1 that was induced by MSC-CM predominantly from monocytes altered macrophage polarization toward the M2 phenotype in the present study. We consider that the decreased expression levels of renal cortical MCP-1 mRNA resulting from MSC-CM treatment was the result of histological improvement due to a reduction in M1 macrophages, which are the main source of MCP-1 in this GN (19).

Although MCP-1, a member of the chemokine (C-C motif) family, is known as an inflammatory chemokine, previous experimental studies have suggested that MCP-1 has versatile functions in kidney diseases depending on disease models or disease phases (10, 37). Deficiency of MCP-1 has been shown to aggravate AKI after renal ischemia-reperfusion injury, corroborating the importance of MCP-1 in repairing AKI (37). In the same experimental model as the present study, administration of anti-MCP-1 antibody led to a decrease in glomerular macrophage accumulation on day 4, but this inhibitory effect was not observed on day 8 (10). The ability of MCP-1 to switch the macrophage phenotype towards M2 has been reported (35, 36), and it is speculated that this activity of MCP-1 is an important mechanism in the repair of renal injury after MSC-CM administration in the present study. Although several reports have demonstrated the ability of MSCs to polarize macrophages toward the M2 phenotype in vivo (13, 45) and in vitro (1, 25, 27), there has been no report suggesting that MCP-1 is associated with the underlying mechanism. Interestingly, Adullter-Lieber et al. (1) reported that IL-6 mediated the M2 polarizing effect of human cardiac adipose tissue-derived MSCs on macrophages by increasing the levels of the M2-associated cytokines IL-10 and IL-13. Thus, we speculate that the mechanisms underlying M2 macrophage polarization by MSCs or MSC-CM may be diverse depending on the regional microenvironment.

In vitro experiments, we found that MSC-CM significantly reduced TNF-α-induced IL-8 production in mesangial cells and proximal tubular cells. These results indicate that MSC-CM have an anti-inflammatory effect on renal intrinsic cells without macrophage interaction. MSC-CM contained higher levels of cytokines such as IL-6, IL-8, MCP-1, PDGF-

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<td>IL-4 + MCP-1</td>
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* *P < 0.05 compared with cultures with the addition of IFN-γ or IL-4 (by t-test).
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K. I., K. Honda and T. Shibata approved final version of manuscript. H.O. conception and design of research; M.I. and H.O. edited and revised results of experiments; K.I. prepared figures; K.I. drafted manuscript; M.I. and performed experiments; K.I. and M.I. analyzed data; K.I. and M.I. interpreted mechanism by which anti-GBM GN might be repaired by MSC-CM therapy.

In conclusion, our data suggest that MSC-CM has anti-inflammatory effects that are partly induced by augmentation of MCP-1 and by subsequent switching of macrophages toward the M2 phenotype and that this mechanism might be a potential mechanism by which anti-GBM GN might be repaired by MSC-CM therapy.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

K.I., M.I., K.M., N.W., T. Suzuki, Y.Y., T. Saito, K. Hihara, and S.T. performed experiments; K.I. and M.I. analyzed data; K.I. and M.I. interpreted results of experiments; K.I. prepared figures; K.I. drafted manuscript; M.I. and H.O. conception and design of research; M.I. and H.O. edited and revised manuscript; K. Honda and T. Shibata approved final version of manuscript.

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