Research Article
Effect of retinoic acid in experimental diabetic nephropathy

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Summary Although the pathogenetic mechanism of diabetic nephropathy has not been elucidated, an inflammatory mechanism has been suggested to contribute to its progression. Monocyte chemoattractant peptide (MCP)-1 attracts macrophages and T cells, and ultimately injures renal tissue. In early diabetic nephropathy, urinary excretion of MCP-1 was elevated, and increased as renal damage became more severe. Podocytes are expected to have an inflammatory role in diabetic nephropathy, as the surface expression of chemokine receptors such as CCR and CXCR on these cells has been recently reported. Although retinoid (retinal), a known anti-inflammatory agent, has been reported to be beneficial in some experimental models of renal disease, it has not been determined to prevent disease progression in diabetic nephropathy. We investigated the effects of all-trans retinoic acid on the production of MCP-1 under high glucose conditions in cultured mouse podocytes. We also evaluated whether all-trans retinoic acid inhibits inflammatory changes and improves renal function during the early stages of diabetic nephropathy in streptozotocin-induced diabetic rats. In cultured podocytes, high glucose stimuli rapidly upregulated the MCP-1 mRNA transcript and protein release. Treatment with retinoic acid tended to suppress the MCP-1 gene transcript, and significantly inhibited MCP-1 protein synthesis induced by high glucose stimulation. Urinary protein excretion and the urinary albumin : creatinine ratio (ACR) were significantly higher in diabetic rats 4 weeks after the induction of diabetes mellitus compared with control rats, and retinoic acid treatment markedly decreased both proteinuria and urinary ACR (proteinuria: 1.25 ± 0.69 vs 0.78 ± 0.72 mg/mgCr, P = 0.056; urinary ACR: 0.47 ± 0.25 vs 0.21 ± 0.06 mg/mgCr, P = 0.088). Urinary excretion of MCP-1 was rapidly increased 2 days after induction of diabetes mellitus in diabetic rats, and further increased until rats were 4 weeks of age, compared with control rats. Retinoic acid treatment resulted in 30% reduction of the urinary level of MCP-1 compared with vehicle-treated diabetic rats (119.3 ± 74.2 vs 78.1 ± 62.7 pg/mgCr, P = 0.078). Immunohistochemistry revealed a significant increase in staining for MCP-1 and anti-monocyte/macrophage (ED-1) protein in the diabetic kidney, and retinoic acid treatment significantly suppressed intrarenal MCP-1 and ED-1 protein synthesis. In conclusion, podocytes are involved in the inflammatory reaction under diabetic circumstances, and these reactions were suppressed by retinoic acid. Retinoic acid also suppressed inflammatory changes in the diabetic rat kidney, and decreased proteinuria in diabetic rats. These results suggest that retinoic acid may have renoprotective effects in the early stages of diabetic nephropathy through an anti-inflammatory activity.

Key words: diabetic nephropathy, inflammation, monocyte chemoattractant peptide (MCP)-1, podocyte, retinoid, streptozotocin.

Introduction
The pathogenesis of diabetic nephropathy is complex. Hyperglycaemia, advanced glycosylation end products, abnormalities in the polyol pathway, oxidative stress and TGF-β are all involved in the progression of diabetic nephropathy. Recently, diabetic nephropathy, which has been categorized as a non-immunological renal disease, has been reported to be related to the inflammatory reaction. As with other immunological renal diseases, monocyte chemoattractant peptide (MCP)-1, IL-1β, adhesion molecule, and macrophage infiltration in the kidney have been reported in both human and animal diabetic models. 1,2 MCP-1, which is a representative chemokine, attracts inflammatory cells to renal tissue and activates inflammatory processes such as the stimulation of other cytokines and growth factors, and ultimately injures the kidney by induction of α1-type IV collagen synthesis. 3

Although the role of mesangial cells is well known in diabetic nephropathy, the role of podocytes, which support the glomerular basement membrane and maintain the integrity of the glomerular filtration barrier, has not been clearly defined. Since the development of an immortalized cell line, a...
body of experimental evidence has been collected on the role of podocytes in diabetic nephropathy. Podocyte loss presents even in early diabetic nephropathy, and this damage aggraves the functional loss for glomerular perme selectivity and proteinuria. However, podocyte involvement in the inflammatory response has not been previously reported. Considering that podocytes are the major synthesis site for IL-1α and IL-1β, and that they have receptors for various chemokines, the possibility exists that podocytes may also have a role in the inflammatory reaction.

Vitamin A (retinoid) is stored in the liver as retinol, and is transferred to tissue by transforming retinoid acid. Vitamin A is transformed to both trans and cis forms, and produces heterodimer, which binds to retinoic acid A receptor (RAR) and retinoic acid X receptor (RXR) to stimulate the retinoic acid response elements. These transcriptional factors contribute to the anti-inflammatory and antiproliferation effects of retinoic acid. Retinoid suppresses two important inflammatory transcriptional factors, nuclear factor (NF)-κB and activator protein (AP)-1, by activation of RXR. Retinoid can retard renal injury in the anti-Thy1.1-nephritis rat, and has an anti-inflammatory effect by blocking lipid peroxidation in streptozotocin (STZ)-induced diabetic rats. However, little is known about whether retinoid has a beneficial effect on diabetic nephropathy when comparing its effects in diabetes and in immunological renal diseases.

To evaluate whether podocytes are related to the inflammatory reaction, we determined MCP-1 expression in high-glucose stimulated podocytes, and the effect of retinoid on MCP-1 expression. We also evaluated whether retinoid acid inhibited inflammatory changes and improved renal function during the early stages of diabetic nephropathy in STZ-induced diabetic rats.

Materials and Methods

Cell culture and experimental design

A thermosensitive, SV40-transfected immortalized mouse podocyte cell line, obtained as a gift from Peter Mundel (Albert Einstein College of Medicine, New York, USA), was used for this experiment. All cells were grown in type I collagen coated dishes (Iwaki, Tokyo, Japan) in RPMI medium containing heat inactivated 10% FCS (Invitrogen, Carlsbad, CA, USA) and penicillin and streptomycin. Cells were incubated at 33°C with IFN-α (Invitrogen) until they reached confluency, and then cells were transferred to an incubator (37°C) to allow differentiation for 10–14 days. Studies were performed using a podocyte cell line at 20–24 passages. Identification of podocytes was performed using reverse transcription (RT)-PCR for podocyte specific markers such as Wilm’s Tumor protein (WT-1). Differentiation of podocytes was determined using RT–PCR on the basis of the expression of synaptotadpin, which is a differentiation marker. Differentiated podocytes were grown to subconfluence in the growth medium, and then cultured for 24 h in a medium containing 5 mmol/L D-glucose and 1% FCS before being exposed to experimental conditions. The normal glucose (NG) group used confluent cell monolayers cultured with 5 mmol/L D-glucose, while the high glucose (HG) group used 30 mmol/L D-glucose. For an osmotic control, we used 5 mmol/L D-glucose and 25 mmol/L mannitol. In the group receiving retinoic acid treatment, different concentrations of all-trans retinoic acid (Sigma-Aldrich, St Louis, MO, USA) dissolved in DMSO (Sigma-Aldrich) were added to the culture media at final concentrations of 0.5 µmol/L, 2.0 µmol/L and 5.0 µmol/L under high glucose conditions (30 mmol/L D-glucose). To elucidate the effect of DMSO in the all-trans retinoic acid treatment, some cells were treated with DMSO alone and used as a control. All experiments were performed under protection from light to protect the all-trans retinoic acid from degradation. All experimental groups were cultured in triplicate and harvested at 6 and 24 h for extraction of the total RNA and protein. To avoid any confounding effects by serum on MCP-1 expression, all experiments were performed in serum-free media.

Semi-quantitative PCR assay for MCP-1 mRNA expression in cultured podocytes

Total RNA extraction was performed from cultured podocytes with a Trizol reagent (monophasic solutions of phenol and guanidine isothiocyanate) and cDNA synthesized by an RT reaction using an RNA PCR kit (Applied Biosystems, Roche, Foster City, CA, USA) in a 20 µL mixture containing 1 µg RNA, 50 mmol/L KCl, 10 mmol/L Tris-HCl, 5 mmol/L MgCl2, 1 mmol/L of each dNTP, oligo-(dT) primers, 20 units of RNase inhibitor and 50 units of MuLV reverse transcriptase. The reaction mixture was incubated at 42°C for 60 min, then heated at 90°C for 7 min in a thermocycler (GeneAmp PCR system 9600; Perkin Elmer, Roche Molecular System, Branchburg, NJ, USA). Next, cDNA was amplified by 1.25 units of Taq DNA polymerase in a 25 µL reaction volume containing 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L MgCl2, 0.2 mmol/L deoxyxynucleoside triphosphate and 20 pmol of each primer. Sequence specific primers for mouse MCP-1 were used for the PCR reaction. The nucleotide sequences of each primer were: sense, 5’-AGT CAG TTG GTA TC-3′; antisense, 5’-TGA ACC ACC CTC AC-3′. As an internal control, β-actin was also amplified and the nucleotide sequences for the primers were: sense, 5’-GGT CAG GCA CCA GGG CGT GAT-3′; antisense, 5’-GTT CAT CTT CTC GGC GTT GCC CTT GTG GT-3′. The PCR conditions were 40 cycles (MCP-1) or 36 cycles (β-actin) of denaturation at 94°C for 1 min, annealing at 51°C (MCP-1) or 60°C (β-actin) for 1 min, and extension at 72°C for 3 min. The expected lengths of the PCR products were 415 bp for MCP-1 and 460 bp for β-actin. The number of PCR cycles selected represents a point before the plateau of amplification products, as described previously. To confirm the identity of each PCR product, each of the electrophoresed PCR bands was extracted with a DNA extraction kit (Qiagen, Valencia, CA, USA) and sequenced using an ABI automated DNA sequencing system (ABI Genetic Analyser 310; PRISM, Branchburg Park, NJ, USA). The RT–PCR products were separated by electrophoresis on a 2% agarose gel with ethidium bromide staining. After scanning at 300 p.i., densitometric analysis was performed for quantification using NIH image analysis software (version 1.61; National Institute of Health, Bethesda, MA, USA). The ratios of the concentration of β-actin to those of MCP-1 were evaluated.

Measurement of MCP-1 protein in culture supernatant

The amount of MCP-1 protein secreted by podocytes in culture medium was measured by a commercially available quantitative sandwich ELISA (Biosource, Camarillo, CA, USA). The assay method was designed to recognize mouse MCP-1 and showed no cross-reactivity with other cytokines or growth factors. The sensitivity of the ELISA for vascular endothelial growth factor (VEGF) was 9 pg/mL. The intra-assay coefficient of variation was 4.6% and the interassay coefficient of variation was 6.8%. For measurement of secreted MCP-1 in culture medium, conditioned media were collected at the end of the treatment period. All particulates were then
removed by centrifugation at 4000 g for 10 min and stored at −70°C before measurement of MCP-1 protein. Supernatants were diluted twofold with diluent according to the manufacturer’s instructions. At that point, 50 µL of sample with an equal volume of diluent was dispensed into a 96-microwell plate precoated with polyclonal antibody specific for mouse MCP-1. The plates were then incubated at room temperature for 2 h, washed four times, and developed with 100 µL of colour reagent per well. The intensity of the colour was measured in an ELISA reader at 450 nm. The assay was performed in duplicate and the results were corrected for the protein concentration under each condition.

**Animal study and experimental design**

Male Sprague–Dawley rats weighing 200–220 g were divided into four groups. Ten rats were used as a control for diabetic rats without induction of diabetes mellitus. Diabetes mellitus was induced by the intraperitoneal injection of STZ 65 mg/kg bodyweight (Sigma-Aldrich). Seven of the 24 diabetic rats were killed 48 h after STZ injection. The remaining diabetic rats (n = 17) were further randomized into treatment groups with daily intraperitoneal administration of 10 mg/kg bodyweight all-trans retinoic acid (Sigma-Aldrich) dissolved in corn oil and 5% DMSO (n = 7) for 4 weeks, or corn oil and DMSO as a vehicle control (n = 3) to exclude the effect of DMSO alone. The remaining seven rats were injected with saline, as untreated diabetic rats. Diabetic rats were confirmed when the tail blood sugar concentration was greater than 300 mg/dL. 48 h after STZ injection. Animals were given free access to rat chow. Animals were caged individually, and their weights and 24 h urine samples were collected in a metabolic cage. Blood samples were collected when the animals were killed, and plasma glucose levels were measured using a glucose oxidase based method; creatinine levels were determined by a modified Jaffe method. Twenty-four hour urine protein was measured by a nephelometric method using sulfosalicylic acid. The amount of urinary albumin excretion was also determined in 24 h urine samples. Urinary albumin concentrations were determined by competitive ELISA (Shibayagi, Shibukawa, Japan). Urinary albumin excretion values were normalized with respect to urine creatinine (urinary ACR). Two days or 4 weeks after STZ injection, rats were killed under anaesthesia by intraperitoneal injection of sodium pentobarbital 50 mg/kg bodyweight. All kidneys were perfused with normal saline before removal. Kidney was divided and stored at −70°C for morphological analysis. The study was performed in accordance with the institutional guidelines for animal research.

**Determination of MCP-1 level in urine**

To determine the urinary MCP-1 levels, 24 h urine specimens were collected, immediately centrifuged at 4000 g for 10 min, aliquoted, and stored at −70°C until required for testing. MCP-1 levels were measured by quantitative sandwich ELISA using a commercial kit (Biosource) according to the manufacturer’s instructions. This MCP-1 assay was specific for rat MCP-1. Before the study, the assay was validated for urine samples. Appropriate reductions in determined MCP-1 levels were observed by serially diluting urine samples. The assay was performed in duplicate, and the intensity of the colour was measured in an ELISA reader at 450 nm. The detection limit of the MCP-1 assay was 8 pg/mL, its coefficients of variation for intra-assay precision at 110.0 pg/mL was 4.0%, and interassay precision at 101.6 pg/mL was 9.7%. These ELISA assays showed no cross-reactivity with other cytokines or growth factors. To control for urine concentration differences, the urinary MCP-1 levels were expressed relative to the urinary creatinine content, and expressed as MCP-1 (pg/mg Cr).

**Immunohistochemical staining for MCP-1 and ED-1**

For immunohistochemical staining, renal tissue was immediately fixed in 10% neutral buffered formalin, cast in paraffin, sliced into 4 µm sections, and placed onto microscope slides. After removal of the paraffin in xylene and dehydration in graded alcohol, slides were immersed in distilled water. Kidney sections were transferred to a 10 mmol/L citrate buffer solution for antigen retrieval at a pH of 6.0, and then heated at 80°C for 30 min. After a wash in water, 3.0% peroxide/methanol was applied for 20 min to block endogenous peroxidase. To inhibit non-specific staining, slides were incubated at room temperature for 20 min under normal goat serum. The primary antibody, monoclonal rabbit antirat MCP-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a monoclonal rabbit antirat monococyte and macrophage (ED-1) antibody (RDI Research Diagnostic, Flanders, NJ, USA), was added at a 1:100 (MCP-1) or 1:1000 (ED-1) dilution for 1 h (MCP-1) or 2 h (ED-1) at room temperature. Negative control sections were stained under identical conditions by substituting the primary antibody with equivalent concentrations of normal rabbit IgG. Using an LASB2 kit/HRP (DAKO, Carpinteria, CA, USA), kidney sections were sequentially treated with normal goat serum, primary antibody, link antibody, streptavidin–biotin horseradish peroxidase and diaminobenzidine. Sections were then counterstained with Mayer’s haematoxylin.

To evaluate MCP-1 and ED-1 staining, each glomerular or tubulointerstitial grid field was graded semiquantitatively. Each score reflects changes in the extent rather than in the intensity of staining, and depends on the percentage of positive grid field or glomeruli. Cases in which more than 5% of resident cells showed positive staining were regarded as positive. Three scores were awarded: 0, absent or less than 25% staining; 1, 25–50% positive staining; 2, 50–75% positive staining; and 3, more than 75% positive staining. For each sample, 50–60 glomeruli were evaluated and the average score was calculated. Each slide was scored by an observer who was unaware of the experimental details.

**Statistical analysis**

We used non-parametric analysis because most of the variables, particularly urinary MCP-1, were not normally distributed, even after logarithmic transformation. The Mann–Whitney U-test was used to compare two groups. A significance level of 5% was chosen for all tests (P < 0.05). All statistical analyses were performed using SPSS for Windows 10.0 (SPSS, Chicago, IL, USA). Data are expressed as mean ± SE.

**Results**

**Effect of high glucose and retinoic acid on MCP-1 mRNA expression in cultured podocytes**

Monocyte chemoattractant protein (MCP)-1 mRNA expression rapidly increased under high glucose conditions, with the maximal effect observed at the 24 h treatment (0.18 ± 0.04 vs. 2.17 ± 0.71, P < 0.05). Cells exposed to 25 mmol/L D-mannitol as an osmotic control showed no difference in the expression of MCP-1 compared with MCP-1 expression found in the normal glucose group. Treatment with all-trans retinoic acid under high glucose conditions showed a tendency to decrease MCP-1 gene expression, but the decrease was not statistically significant. The DMSO group also did not show any significant difference to the control group (Fig. 1).
Effect of high glucose and all-trans retinoic acid (RA) on the expression of monocyte chemoattractant protein (MCP)-1 mRNA in cultured mouse podocytes. (A) Representative reverse transcription (RT)-PCR result showing a 415 bp product. In this figure, the RT–PCR result for 6 h incubation samples is shown. Podocytes were exposed to different concentrations of RA for 6 and 24 h under HG conditions (30 mmol/L). MM, 100 bp DNA ladder; NG, normal glucose (5 mmol/L). (B) Densitometric analysis of RT–PCR data. Results are expressed as an optical density ratio of MCP-1 : β-actin. Data are shown as mean ± SE. *P < 0.05 versus normal glucose group. □, NG; ■, HG (30 mmol/L); *, HG + RA (0.5 µmol/L); [ ], HG + RA (2 µmol/L); [ ], HG + RA (5 µmol/L); □, HG + DMSO.

Effect of high glucose and retinoic acid on MCP-1 protein synthesis in cultured podocytes

The level of MCP-1 protein released after exposure to high glucose was significantly increased after 6 h of incubation (NG: 2.96 ± 0.57 pg/mg protein; HG: 7.77 ± 1.27 pg/mg protein; P < 0.05) (Fig. 2). High glucose-induced MCP-1 overexpression was persistently observed at 24 h of incubation (NG: 11.59 ± 1.17 pg/mg protein; HG: 22.45 ± 2.03 pg/mg protein; P < 0.05). Treatment with all-trans retinoic acid under high glucose conditions significantly suppressed the synthesis of MCP-1 protein at 6 h of incubation, even at a concentration of 0.5 µmol/L (HG: 7.77 ± 1.27 pg/mg protein; HG + retinoic acid: 3.50 ± 1.40 pg/mg protein; P < 0.05), although we could not find any dose-dependent effect. However, high glucose-induced MCP-1 protein synthesis was abolished by treatment with all-trans retinoic acid, and MCP-1 protein synthesis was dose dependently suppressed at 24 h of incubation (HG: 22.45 ± 2.03 pg/mg protein; RA 0.5 µmol/L: 12.8 ± 1.89 pg/mg protein; RA 2.0 µmol/L: 8.26 ± 1.12 pg/mg protein; RA 5.0 µmol/L: 9.13 ± 0.18 pg/mg protein; P < 0.05).

Baseline characteristics of diabetic rats

Streptozotocin-induced diabetic rats showed a higher blood glucose concentration and 24 h urine amount than controls at 2 days after induction of diabetes mellitus. At 4 weeks, blood glucose concentration of diabetic rats was persistently higher compared with control rats, and blood glucose level was not different with or without retinoic acid treatment. In the DMSO group, blood glucose and daily urine amount were not different from the diabetic rat group. Diabetic rats showed a higher ratio of kidney : bodyweight compared with controls (P = 0.017), but the ratio was not different among diabetic rats, irrespective of the treatment with retinoic acid or DMSO (Table 1).

Proteinuria, urinary ACR and urinary MCP-1 excretion

At 2 days, there was no significant difference in daily proteinuria and microalbumin excretion between control and diabetic rats. However, proteinuria and urinary ACR were significantly increased in diabetic rats compared with control rats at 4 weeks (proteinuria: 0.59 ± 0.04 vs 1.25 ± 0.21 mg/mgCr, P = 0.009; urinary ACR: 0.07 ± 0.01 vs 0.47 ± 0.25 mg/mgCr, P = 0.035) (Fig. 3). Interestingly, urinary concentration
of MCP-1 was higher in diabetic rats than in control rats at 2 days, without significant difference in the proteinuria (31.5 ± 12.1 vs 75.5 ± 58.4 pg/mgCr, P = 0.109), and the level of urinary MCP-1 further increased to threefold in diabetic rats compared with control rats at 4 weeks (39.5 ± 13.3 vs 119.3 ± 74.2 pg/mgCr, P = 0.019) (Fig. 4). Increased proteinuria and urinary ACR in diabetic rats tended to be decreased in diabetic rats with retinoic acid treatment (proteinuria: 1.25 ± 0.69 vs 0.78 ± 0.72 mg/mgCr, P = 0.056; urinary ACR: 0.47 ± 0.25 vs 0.21 ± 0.06 mg/mgCr, P = 0.088). However, there was no change in the proteinuria in diabetic rats with DMSO treatment compared with control rats. In addition, diabetic rats with retinoic acid treatment also showed lower urinary MCP-1 levels than the diabetic rats (119.3 ± 74.2 vs 78.1 ± 62.7 pg/mgCr, P = 0.078), and urinary level of MCP-1 in diabetic rats with DMSO treatment was not changed (Fig. 4).

Table 1  Basic characteristics of control and diabetic rats

<table>
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<tr>
<th>Time</th>
<th>Experimental group</th>
<th>Bodyweight (g)</th>
<th>Ratio†</th>
<th>Blood glucose (mmol/L)</th>
<th>Urine amount (mL/day)</th>
</tr>
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<tr>
<td>2 days</td>
<td>Control rats</td>
<td>236.6 ± 5.77</td>
<td>0.39 ± 0.02</td>
<td>6.18 ± 0.52</td>
<td>8.5 ± 2.12</td>
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<td>Diabetic rats</td>
<td>210.7 ± 9.32</td>
<td>0.61 ± 0.04*</td>
<td>30.76 ± 4.39**</td>
<td>51.5 ± 4.43**</td>
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<tr>
<td>4 weeks</td>
<td>Control rats</td>
<td>340 ± 17.3</td>
<td>0.46 ± 0.09</td>
<td>6.24 ± 0.89</td>
<td>14.5 ± 3.53</td>
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<td></td>
<td>Diabetic rats (untreated)</td>
<td>234 ± 55.4</td>
<td>0.67 ± 0.11*</td>
<td>32.83 ± 0.61**</td>
<td>53.6 ± 5.50**</td>
</tr>
<tr>
<td></td>
<td>Diabetic rats (retinoic acid)</td>
<td>263.3 ± 32.1</td>
<td>0.54 ± 0.10*</td>
<td>31.17 ± 4.6**</td>
<td>48.5 ± 10.1**</td>
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<td>Diabetic rats (DMSO)</td>
<td>270 ± 14.1</td>
<td>0.61 ± 0.02*</td>
<td>33.27 ± 0.67**</td>
<td>53.0 ± 4.24**</td>
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</table>

†Ratio kidney weight : bodyweight. Values are expressed as mean ± SE. Statistical difference was performed among groups with the same duration of experimental period. *P < 0.05 versus control rats, **P < 0.001 versus control rats.
synthesis was rapidly increased in the diabetic kidney, and further increased after 4 weeks of induction of diabetes mellitus (1.54 ± 0.15 vs 4.28 ± 1.41, \( P < 0.01 \)). In comparison with MCP-1 synthesis, ED-1 was mainly detected in the glomeruli (Fig. 5b,7). Interestingly, retinoic acid treatment significantly suppressed tubulointerstitial MCP-1 protein synthesis (1.4 ± 0.6 vs 0.25 ± 0.27, \( P < 0.01 \)) and intraglomerular ED-1 protein synthesis (4.28 ± 1.41 vs 2.45 ± 0.86, \( P = 0.048 \)).

Discussion

In this study, we demonstrated that cultured podocytes synthesized MCP-1 in response to high glucose stimuli, and that retinoic acid treatment suppressed MCP-1 synthesis induced by high glucose stimulation. This result indicates that podocytes, as well as mesangial cells, have a role in the inflammatory process under diabetic circumstances. In addition, an increased urinary level of MCP-1 was observed during the early stage of diabetic nephropathy in STZ-induced diabetic rats. It is of interest that retinoic acid treatment suppressed proteinuria and inflammatory reactions, which was shown by significant inhibition of renal MCP-1 and ED-1 staining in the renal tissue of diabetic rats. To the best of our knowledge, these results are the first showing that podocytes can actively take part in the inflammatory process under diabetic circumstances and that retinoic acid may have a beneficial role in early diabetic nephropathy because of its anti-inflammatory action.

Podocyte damage occurs in early diabetic nephropathy without morphological changes. Podocyte loss was a predictive factor for the development of proteinuria, and the number of podocytes can be related to the severity of proteinuria in diabetic nephropathy.\(^4,12\) Podocytes are known to produce major proteins related to the progression of renal disease such as TGF-\(\beta\) and VEGF.\(^13\) Thus, podocytes are anticipated to synthesize inflammatory mediators such as mesangial cells.

In our experiment, we showed that urinary excretion of MCP-1 was rapidly increased after induction of diabetes mellitus, and urinary levels of MCP-1 were significantly increased during the early stage of diabetic nephropathy in diabetic rats. These findings agree with the previous report that urinary MCP-1 excretion increased in patients with early diabetic nephropathy, and that its amount increases as albuminuria becomes severe.\(^14\)

It has been suggested that the origin of increased urinary MCP-1 excretion is mainly from mesangial cells and proximal tubular cells stimulated by high glucose and protein overload. In the present study, MCP-1 was also synthesized by glomerular podocytes, and MCP-1 was mainly detected in the tubulointerstitial tissues, which corresponds with earlier results.\(^15\) The reason for this discrepancy between the in vitro and in vivo results for MCP-1 expression is unknown, but may be related to the complexity of the in vivo state in the regulation of MCP-1 synthesis. Wada et al.\(^15\) reported that MCP-1 expression was mainly in the tubulointerstitial area rather than in the glomeruli in diabetic nephropathy. In contrast, other researchers showed glomerular MCP-1 to be increased in diabetic nephropathy.\(^2\) Disagreement among the studies may be ascribed to the difference in the experimental animals used or to the different models of diabetic nephropathy.
In diabetes mellitus, retinoid enhanced wound healing in the db/db mouse. In addition, RXR, which forms a heterodimer binding to peroxisome proliferative-activated receptors (PPAR-α), prevents hyperglycaemia and hyperlipidaemia in obese mice. Retinoid also has an antioxidant effect on the heart in the diabetic rat, and reduces the risk for atherosclerosis in patients with insulin-dependent diabetes mellitus, which suggests that retinoid may have an anti-inflammatory effect in diabetes. However, its role in diabetic nephropathy is not known. Retinoid has been reported to suppress NF-κB and activator protein (AP)-1 in non-diabetic nephropathy. Retinoid also retards renal disease progression by suppressing important mediators such as angiotensin II, endothelin and TGF-β in the anti-Thy1.1 nephritis rat model. Utimura et al. reported that mycophenolate mofetil, which is known for its anti-inflammatory and antiproliferative properties, prevents renal disease progression by suppressing expression of lymphocytes, macrophages and adhesion molecules. These reports suggest that anti-inflammatory agents can prevent renal disease progression in diabetic nephropathy.

Although changes in proteinuria and urinary MCP-1 excretion in response to retinoic acid treatment were not statistically significant in this study, the overall beneficial response to retinoid and the significant inhibitory effects on both intraglomerular ED-1 expression and tubulointerstitial MCP-1 expression suggest that retinoid may be beneficial in the early stage of diabetic nephropathy, because of its anti-inflammatory effects. Considering that retinoic acid treatment did not affect metabolic alterations induced by diabetes mellitus such as hyperglycaemia and renal hypertrophy, the beneficial effect of retinoic acid in this study implies the importance of the anti-inflammatory action.

The beneficial effects of retinoic acid in this study might be due to the antihypertensive effect of retinoids as well as to the anti-inflammatory action. The antihypertensive mechanism of retinoids has not yet been clarified, and has been considered to be alleviation of renal damage by retinoic acid and blockade of angiotensin II action. Although we did not measure blood pressure in experimental rats, it has been reported that RARα agonist is less effective in reduction of blood pressure, and most of the antihypertensive effect of retinoids was observed in the acute and chronic experimental nephritic rat models.

In conclusion, podocytes are involved in the inflammatory process under diabetic circumstances, and MCP-1 synthesis was upregulated by high glucose stimulation, which was significantly suppressed by retinoic acid treatment. In the diabetic kidney, upregulation of MCP-1 and ED-1 synthesis occurred in the early stages of diabetic nephropathy, and retinoic acid treatment produced a beneficial effect through

**Figure 6** Representative immunohistochemical staining for monocyte chemoattractant protein (MCP)-1 in experimental groups at 4 weeks of age after induction of diabetes mellitus. (a) Control rats, (b) untreated diabetic rats, (c) all-trans retinoic acid-treated diabetic rats and (d) vehicle control (DMSO-treated diabetic rats). Positive staining for MCP-1 was detected mainly in the tubulointerstitial tissues, and particularly in proximal tubules (short arrow).
reduction of the renal inflammatory process. These results suggest that retinoic acid may be a new therapeutic agent in the treatment of diabetic nephropathy.

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