Suppression of Constitutive but Not IL-1β-Inducible Expression of Monocyte Chemoattractant Protein-1 in Mesangial Cells by Retinoic Acids: Intervention in the Activator Protein-1 Pathway

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Abstract. Retinoic acid regulates a wide range of biologic processes, including inflammation. This study investigated the effect of all-trans-retinoic acid (t-RA) on the constitutive and cytokine-inducible expression of monocyte chemoattractant protein 1 (MCP-1) in rat mesangial cells. Serum-deprived mesangial cells exhibited substantial levels of MCP-1 mRNA, and the expression was markedly upregulated by interleukin-1β (IL-1β). Pretreatment with t-RA abrogated the constitutive mRNA expression but did not inhibit the IL-1β–inducible expression. The similar effects were observed by 9-cis-RA. The suppressive effect of t-RA required retinoic acid receptors. t-RA did not affect the stability of MCP-1 mRNA, indicating that its suppressive effect was at the transcriptional level. Experiments that used pharmacologic and genetic inhibitors showed that the IL-1β–inducible MCP-1 expression was dependent on nuclear factor-κB (NF-κB) and independent of activator protein 1 (AP-1). In contrast, the constitutive expression of MCP-1 was dependent on both NF-κB and AP-1. t-RA substantially inhibited the constitutive activity of AP-1 but did not inhibit NF-κB activity in mesangial cells. These data suggested that (*1*) constitutive and IL-1β–inducible expression of MCP-1 was differently regulated by AP-1 and NF-κB and (*2*) t-RA inhibited selectively the constitutive expression of MCP-1 via intervention in the AP-1 pathway.

Monocyte chemoattractant protein 1 (MCP-1) is a member of the chemokine family and specifically attracts monocytes (1). MCP-1 is produced ubiquitously by various cells, including resident glomerular cells (1–3). During glomerulonephritis, infiltration of monocytes/macrophages is a common pathologic feature (4). Expression of MCP-1 is observed in the mesangium of inflamed glomeruli (5–7), and MCP-1 synthesized by resident glomerular cells plays a role in macrophage attraction during glomerular inflammation (8). From this viewpoint, chemical inhibitors of MCP-1 may be useful for therapeutic intervention in glomerulonephritis.

The 5'-flanking region of the MCP-1 gene contains multiple 12-o-tetradecanoylphorbol-13-acetate response elements (TRE) and nuclear factor-κB (NF-κB) binding sites (9–11). It indicates potential roles of activator protein 1 (AP-1) and NF-κB in the regulation of MCP-1 expression. Indeed, activa-

tion of AP-1 is required for induction of MCP-1 by growth factors, lipopolysaccharide, and mechanical stress in osteoblastic cells, macrophages, and vascular endothelial cells, respectively (12–14). In tumor cells, fibroblasts, and mesangial cells, activation of NF-κB is essential for the induction of MCP-1 by interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and 12-o-tetradecanoylphorbol-13-acetate (9,15–17). In endothelial cells, NF-κB and AP-1 cooperatively upregulate expression of MCP-1 in response to IL-1β (18).

Retinoic acid (RA) is an active metabolite of vitamin A and regulates a wide range of biologic processes, including cell proliferation, differentiation, and morphogenesis (19). The action of RA is mediated by specific nuclear receptors, namely, retinoic acid receptors (RARα, β, γ) and retinoid X receptors (RXRα, β, γ). RA is known to function as a potent inhibitor of AP-1 (20). In glomerular cells, RA inhibits serum-induced proliferation and oxidant-initiated apoptosis via inhibition of AP-1 (21,22). Previous studies also showed that RA may function as an inhibitor of NF-κB (23). Based on these, RA might serve as an inhibitor of MCP-1 expression. In the present investigation, we examined the effect of all-trans-RA (t-RA) on the expression of MCP-1 in cultured rat mesangial cells. Our data suggested that t-RA inhibited selectively the constitutive expression of MCP-1 via intervention in the AP-1 pathway.

Materials and Methods
Mesangial Cells
Mesangial cells (SM43) were established from isolated glomeruli of a male Sprague-Dawley rat and identified as being of the mesangial cell phenotype as described previously (24). Cells were maintained in Dulbecco's modified Eagle's medium/Ham's F-12 (Life Technologies BRL, Gaithersburg, MD) supplemented with 100 U/ml penicillin G, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, and 10% fetal calf serum (FCS). Media containing 0.5% FCS were generally used for experiments.

Stable Transfectants
SM/JUNDN cells in which AP-1 is selectively inactivated were established by stable transfection of SM43 mesangial cells with a dominant-negative mutant of c-*jun*, TAM-67 (25). TAM-67 is a deletion mutant that lacks amino acids 3 to 122 of c-Jun (26). The protein encoded by this truncated c-*jun* gene retains the DNA binding and leucine zipper domains but lacks the transactivating domain. Overexpression of TAM-67, therefore, inhibits AP-1–mediated transactivation via blocking formation or binding of functional AP-1 complexes in a dominant-negative fashion (26). SM/JUNDN cells exhibit depressed activity of AP-1 under both constitutive and stimulated conditions (25).

SM/IκBαM cells in which NF-κB is selectively inactivated were established by overexpression of a super-repressor mutant of IκBα (IκBαM) (27), as described previously (17,22). IκBαM contains N- and C-terminal mutations and is resistant to both basal and stimulus-dependent degradation. When IκBαM is overexpressed, it functions as a dominant-negative mutant and blocks constitutive and inducible activation of NF-κB (27). SM/IκBαM cells exhibit blunted activation of NF-κB when stimulated by proinflammatory cytokines IL-1β and TNF-α (28).

As a control, mock-transfected mesangial cells SM/Neo that express *neo* alone were created, as described previously (29).

Pharmacologic Manipulations
Confluent mesangial cells cultured in the presence of 0.5% FCS for 24 h (serum-deprived cells) were treated with t-RA (0.5 to 5 μM; Sigma Immunochemicals, St. Louis, MO) or 9-cis-RA (1 to 5 μM; Sigma) for up to 24 h. Five μmol of t-RA was generally used for experiments. To examine effects of t-RA and 9-cis-RA on the inducible expression of MCP-1, we pretreated cells with RA for 2 h and stimulated them with human recombinant IL-1β (10 ng/ml; Otsuka Pharmaceutical Co. Ltd., Tokushima, Japan) for 6 and 24 h. To examine roles of RAR in the regulation of MCP-1 by t-RA, we treated mesangial cells with t-RA (2.5 μM) together with or without a selective pan-RAR antagonist, AGN193109 (5 μM; Allergan, Irvine, CA) (30) for 6 h.

The effect of t-RA on the stability of MCP-1 transcript was examined as follows. First, the effect of the RNA synthesis inhibition on the constitutive MCP-1 mRNA level was examined by exposing the serum-deprived cells to actinomycin D (500 ng/ml; Serva, Heidelberg, Germany) for 0 to 8 h. Next, serum-deprived cells were exposed to actinomycin D for 6 h in the presence or absence of t-RA during the last 1.5 to 6 h.

To examine the role of AP-1 in the expression of MCP-1, we treated serum-deprived cells with the c-Jun/AP-1 inhibitor curcumin (Sigma; 20 μM) (29) for 8 h. To examine the effect of curcumin on the inducible expression of MCP-1, we pretreated cells with curcumin for 2 h and stimulated them with IL-1β for 24 h.

Northern Blot Analysis
Total RNA was extracted by a single-step method (31) and subjected to Northern blot analysis, as described previously (13). In brief, RNA samples were electrophoresed on 1.2% agarose gels containing 10% formaldehyde and transferred onto nitrocellulose membranes. As probes, a mouse *JE*/MCP-1 cDNA (32) and a rat glyceraldehyde-3-phosphate dehydrogenase cDNA were labeled with ^{32}P-dCTP using the random priming method. The membranes were hybridized with probes at 65°C overnight in a solution containing 4$\times$ SSC (600 mM sodium chloride, 60 mM sodium citrate), 5$\times$ Denhardt's solution, 10% dextran sulfate, 50 μg/ml herring sperm DNA, and 50 μg/ml poly(A), washed at 50°C and exposed to x-ray films at −80°C.

Assessment of Cell Viability
Serum-deprived, confluent mesangial cells were treated with t-RA (5 μM) for 6 h and 24 h, and incidence of necrosis and apoptosis was examined quantitatively by trypan blue exclusion and Hoechst staining, respectively. For the latter, cells were fixed with 4% formaldehyde in phosphate-buffered saline (PBS) for 10 min and stained by Hoechst 33258 (10 μg/ml; Sigma) for 1 h. Apoptosis was identified by fluorescence microscopy using morphologic criteria including nuclear condensation and/or fragmentation. Both assays were performed in quadruplicate.

Transient Transfection
AP-1 binds to the particular *cis* element TRE and triggers transcription of target genes. To evaluate the activity of AP-1 in mesangial cells, we used a transient transfection assay (17,22,25,29). In brief, using the calcium phosphate coprecipitation method, mesangial cells that were cultured in 24-well plates (1.0 $\times$ 10^5/well) were transfected with a reporter plasmid pTRE-LacZ (a gift from Dr. A. Alberts, ICRF, UK) (33) or a control plasmid pCI-βgal (a gift from Promega, Madison, WI) at 0.3 to 0.6 μg/well. pTRE-LacZ introduces a β-galactosidase (βgal) gene (*lacZ*) under the control of TRE. pCI-βgal introduces *lacZ* under the control of the immediate-early enhancer/promoter of human cytomegalovirus. After incubation for 48 h in 0.5% FCS with or without t-RA (5 μM), cells were subjected to 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) assay, as described below. Activity of AP-1 was evaluated by counting X-gal–positive cells in each well. That is, the number of X-gal–positive cells transfected with pTRE-LacZ was normalized by the number of positive cells transfected with the control plasmid pCI-βgal. Assays were performed in quadruplicate.

Activity of NF-κB was similarly assessed by the transient transfection, as described previously (17,25,29). Mesangial cells were transfected with pCI-βgal, a κB reporter plasmid pHIVLTRβ-gal or its control construct pmuHIVLTRβ-gal (34) (gifts from Dr. A. Rattner, The Weizmann Institute of Science, Rehovot, Israel). pHIVLTRβ-gal introduces *lacZ* under the control of the HIV promoter that contains two κB motifs. The control plasmid pmuHIV-LTRβ-gal contains a κB-mutated HIV promoter. NF-κB activity was evaluated by the number of X-gal–positive cells in each group, which was normalized by the number of positive cells transfected with the control plasmid pCI-βgal. Each normalized value of the pHIVLTRβ-gal transfection was then subtracted by the normalized value of the pmuHIVLTRβ-gal transfection, and the resultant value was used as an indicator of NF-κB activity. Assays were performed in quadruplicate. The transfection efficiency achieved in these studies was approximately 0.1 to 0.4%.

X-gal Assay

X-gal assay was performed, as described previously (35). In brief, cells were fixed in 0.5% glutaraldehyde, 2 mM $MgCl_2$, and 1.25 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N'-tetraacetic acid in PBS at room temperature for 10 min and then incubated at 37°C for 2 to 4 h in a substrate solution containing 1 mg/ml X-gal, 20 mM $K_3Fe(CN)_6$, 20 mM $K_4Fe(CN)_63H_2O$, 2 mM $MgCl_2$, 0.01% sodium desoxycholate, and 0.02% NP-40 in PBS.

Statistical Analyses

Data were expressed as means $\pm$ SEM. Statistical analysis was performed using the nonparametric Mann-Whitney U test to compare data in different groups. $P < 0.05$ was used to indicate a statistically significant difference.

Results

Suppression of Constitutive but Not IL-1β–Inducible Expression of MCP-1 by t-RA

Cultured mesangial cells constitutively express low levels of MCP-1 mRNA in the absence of stimulation. We first examined the effect of t-RA on the constitutive expression of MCP-1 in mesangial cells. SM43 mesangial cells were serum deprived and treated with t-RA (5 μM) for 6 and 24 h. Northern blot analysis showed that the level of constitutive MCP-1 expression was reduced by the treatment with t-RA, modestly after 6 h and markedly after 24 h (Figure 1A).

The effect of t-RA on the level of MCP-1 mRNA was examined further using various concentrations. Mesangial cells were treated with t-RA at 0.5 to 5 μM for 24 h, and Northern blot analysis was performed. As shown in Figure 1B, substantial suppression of the steady-state level of MCP-1 was observed even at low concentrations, $e.g.$, 0.5 μM. The maximum effect was observed at concentrations higher than 2.5 μM.

Retinoic acids are known to induce apoptosis in various cell types, including tumor cells and embryonic cells. To exclude a possibility that the downregulation of MCP-1 was due to decrease in cell viability, we examined the incidence of apoptosis and necrosis in mesangial cells exposed to the highest concentration of t-RA (5 μM) for 6 and 24 h. As summarized in Table 1, no obvious induction of either apoptosis or necrosis was observed in the t-RA–treated cells.

IL-1β is known to be a prominent inducer of MCP-1 in mesangial cells (17). We next examined the effect of t-RA on the cytokine-inducible expression of MCP-1. Serum-deprived cells were pretreated with t-RA for 2 h and stimulated by IL-1β (10 ng/ml) for 24 h. Dramatic induction of MCP-1 was observed in IL-1β–stimulated cells, and the induction was not affected by the treatment with t-RA (Figure 1C).

Suppression of Constitutive but Not IL-1β–Inducible Expression of MCP-1 by 9-cis-RA

To examine whether the suppressive effect on MCP-1 was also observed by other retinoic acids, we examined the effect of 9-cis-RA on the constitutive expression of MCP-1 in mesangial cells. Serum-deprived cells were treated with 9-cis-RA (5 μM; nontoxic concentration) for 6 and 24 h and subjected to Northern blot analysis. Consistent with the effect of t-RA, the level of MCP-1 mRNA was dramatically reduced by the treatment with 9-cis-RA for 24 h (Figure 2A).

We examined further the effect of 9-cis-RA on the cytokine-inducible expression of MCP-1. Serum-deprived cells were pretreated with 9-cis-RA (5 μM) for 2 h and stimulated by IL-1β for 24 h. Marked induction of MCP-1 was observed in IL-1β–stimulated cells, and the induction was not affected by the treatment with 9-cis-RA (Figure 2B), which was consistent with the effect of t-RA.

Requirement of RAR for the Anti–MCP-1 Effect of t-RA

Both t-RA and 9-cis-RA induce target gene expression via RAR. To examine roles of RAR in the regulation of MCP-1 by RA, we treated mesangial cells with t-RA (2.5 μM) together with or without a selective pan-RAR antagonist, AGN193109 (5 μM), for 6 h, and Northern blot analysis was performed. As shown in Figure 3, AGN193109 completely abolished the suppressive effect of t-RA on MCP-1. AGN193109 itself did not obviously affect the basal level of MCP-1 (data not shown).

Effect of t-RA on the Stability of MCP-1 mRNA

Downregulation of the constitutive MCP-1 mRNA level by t-RA may be caused by transcriptional suppression or decreased stability of the transcript. To test the latter, we exam-
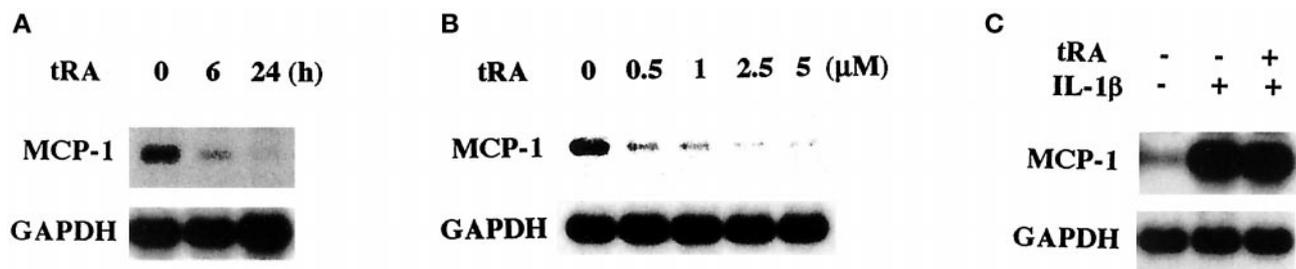

Figure 1. Suppression of constitutive but not interleukin-1β (IL-1β)-inducible expression of monocyte chemoattractant protein 1 (MCP-1) by all-trans-retinoic acid (t-RA). (A) Rat mesangial cells (SM43) were serum deprived (0.5% fetal calf serum [FCS] for 24 h), treated with t-RA (5 μM) for 6 and 24 h, and subjected to Northern blot analysis of MCP-1. Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is shown as a loading control. (B) Mesangial cells were treated with various concentrations of t-RA (0.5 to 5 μM) for 24 h, and Northern blot analysis was performed. (C) Mesangial cells were pretreated with (+) or without (−) t-RA for 2 h and stimulated with IL-1β (10 ng/ml) for 24 h, and Northern blot analysis was performed.

Table 1. Viability of mesangial cells treated with 5 μM t-RA (%)[a]

	t-RA Exposure Time (h)		
	0	6	24
Hoechst staining	98.0 ± 0.4	98.0 ± 0.4	98.9 ± 0.4
Trypan blue exclusion	97.9 ± 0.4	97.6 ± 0.3	97.9 ± 0.7

[a] t-RA, all-trans-retinoic acid. Serum-deprived, confluent mesangial cells were treated with t-RA (5 μM) for 6 h and 24 h, and incidence of apoptosis and necrosis was examined by Hoechst staining and trypan blue exclusion, respectively. Both assays were performed in quadruplicate. Data are means ± SEM.
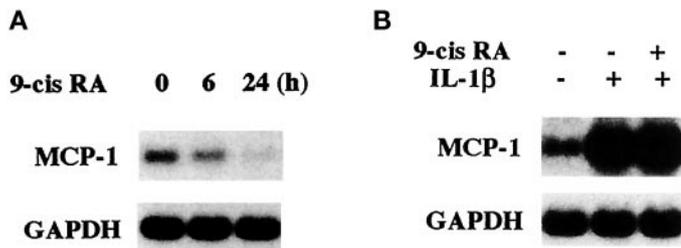

Figure 2. Suppression of constitutive but not IL-1β–inducible expression of MCP-1 by 9-cis-RA. (A) Mesangial cells were serum deprived, treated with 9-cis-RA (5 μM) for 6 and 24 h, and subjected to Northern blot analysis of MCP-1. (B) Mesangial cells were pretreated with (+) or without (−) 9-cis-RA for 2 h and stimulated with IL-1β (10 ng/ml) for 24 h, and Northern blot analysis was performed.
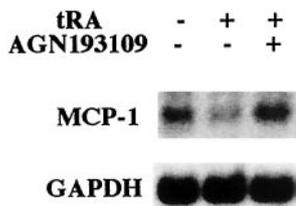

Figure 3. Requirement of retinoic acid receptors (RAR) for the anti–MCP-1 effect of t-RA. Mesangial cells were treated with t-RA (2.5 μM) together with or without a selective pan-RAR antagonist, AGN193109 (5 μM), for 6 h, and Northern blot analysis was performed.

ined whether t-RA affects the stability of MCP-1 mRNA. Serum-deprived mesangial cells were treated with actinomycin D (500 ng/ml) for 2 to 8 h, and the level of MCP-1 transcript was examined. As shown in Figure 4A, treatment with actinomycin D effectively reduced the level of MCP-1 mRNA after 6 h. Using this condition, we examined the effect of t-RA. Mesangial cells were exposed to actinomycin D for 6 h in the presence or absence of t-RA during the last 1.5 to 6 h. Northern blot analysis showed that treatment of t-RA did not affect the stability of MCP-1 mRNA at any time points (Figure 4B).

Roles of NF-κB and AP-1 in the Constitutive and IL-1β–Inducible Expression of MCP-1

The 5'-flanking region of the MCP-1 gene contains TRE and NF-κB binding sites. The roles of NF-κB and AP-1 in the

constitutive and IL-1β–inducible expression of MCP-1 were examined using mutant mesangial cells in which the function of NF-κB or AP-1 is selectively attenuated. SM/IκBαM cells stably express a super-repressor mutant of IκBα and exhibit blunted activation of NF-κB when stimulated by IL-1β and TNF-α (28). SM/JUNDN cells stably express a dominant-interfering form of c-Jun and show attenuated activity of AP-1 under both unstimulated and stimulated conditions (25). Mock-transfected SM/Neo cells, SM/IκBαM cells, and SM/JUNDN cells were treated with or without IL-1β, and levels of MCP-1 mRNA were evaluated by Northern blot analysis. Under the IL-1β–stimulated condition, SM/IκBαM cells exhibited blunted expression of MCP-1, when compared with SM43 and SM/Neo cells (Figure 5A, left). SM/JUNDN cells showed the same level of MCP-1 mRNA as that observed in control mesangial cells. However, under the unstimulated condition, the expression of MCP-1 was attenuated in both SM/IκBαM cells and SM/JUNDN cells (Figure 5A, right). These data suggested that AP-1 was involved in the constitutive expression but not in the inducible expression of MCP-1 in mesangial cells. This was confirmed further by using the pharmacologic inhibitor of c-Jun/AP-1, curcumin. Mesangial cells were pretreated with curcumin (20 μM) and stimulated with or without IL-1β. As shown in Figure 5B, the IL-1β–inducible expression of MCP-1 was not affected by curcumin. In contrast, the constitutive expression of MCP-1 was attenuated substantially by the treatment with curcumin.

Effects of t-RA on the Activity of AP-1 and NF-κB

As described above, the IL-1β–inducible expression of MCP-1 was dependent only on NF-κB, but its constitutive expression was dependent on both AP-1 and NF-κB. t-RA inhibited the constitutive MCP-1 expression without affecting the inducible expression. These data suggested a possibility that t-RA inhibited the constitutive expression of MCP-1 via selective intervention in the AP-1 pathway. To explore this possibility, we examined the effect of t-RA on the constitutive activity of AP-1 and NF-κB. Mesangial cells were transfected with reporter plasmids, serum-deprived in the presence or absence of t-RA for 48 h, and activity of AP-1 and NF-κB was examined. As reported previously (17,25), serum-depleted mesangial cells exhibited substantial levels of AP-1 and NF-κB activity. Treatment with t-RA significantly inhibited the constitutive activity of AP-1 (Figure 6A). In contrast, t-RA did not have any effects on the constitutive activity of NF-κB (Figure 6B).

Discussion

MCP-1 is induced in mammalian cells in response to pathologic stimuli, including cytokines/growth factors, bacterial components, and mechanical stress. Under various pathologic situations, the inducible expression of MCP-1 contributes to accumulation of monocytes/macrophages at inflammatory sites. Conversely, constitutive expression of MCP-1 is observed in certain normal tissues, including bronchial epithelium and renal glomeruli (36,37). Because resident macrophages are present in these tissues (38), the low levels of
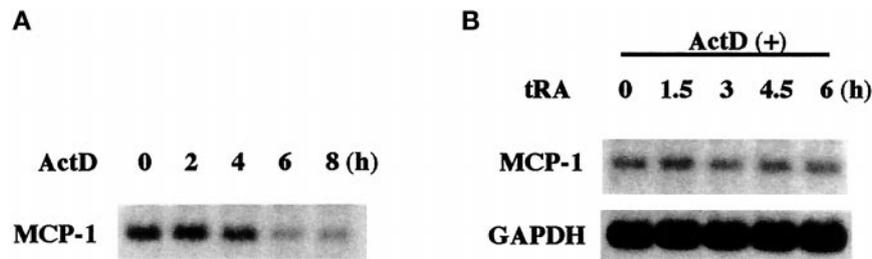
Figure 4. Effect of t-RA on the stability of MCP-1 mRNA. (A) Serum-deprived mesangial cells were treated with actinomycin D (ActD; 500 ng/ml) for 2 to 8 h, and the level of MCP-1 transcript was examined by Northern analysis. (B) Serum-deprived mesangial cells were exposed to actinomycin D for 6 h in the presence or absence of t-RA during the last 1.5 to 6 h and subjected to Northern blot analysis.
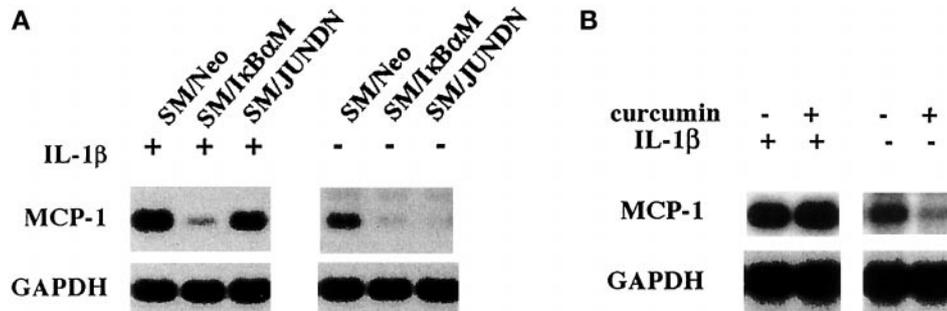

Figure 5. Roles of nuclear factor-κB (NF-κB) and activator protein 1 (AP-1) in the constitutive and cytokine-inducible expression of MCP-1. (A) Transfectants stably expressing a neomycin phosphotransferase gene (*neo*) alone (SM/Neo), *neo* and a dominant-negative mutant of c-*jun* (SM/JUNDN), and *neo* and a super-repressor mutant of IκBα (SM/IκBαM) were treated with (left) or without (right) IL-1β. Northern blot analysis was performed on the expression of MCP-1. (B) Mesangial cells were pretreated with (+) or without (−) the c-Jun/AP-1 inhibitor curcumin (20 μM) and treated with (left) or without (right) IL-1β for 8 h. Northern blot analysis was performed.

constitutive expression may contribute to continuous attraction of monocytes into these sites. Currently, little information is available regarding how the constitutive expression of MCP-1 is regulated in certain cell types. In the present study, we demonstrated that the constitutive and cytokine-inducible ex-
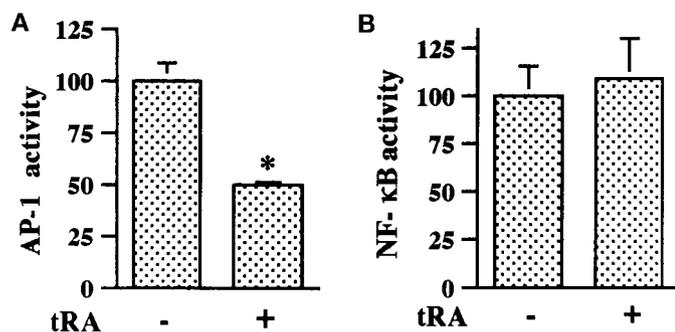

Figure 6. Effects of t-RA on the constitutive activity of AP-1 and NF-κB. Mesangial cells cultured in 24-well plates were transfected transiently with an AP-1 reporter plasmid pTRE-LacZ (A) or an NF-κB reporter plasmid pHIVLTRβ-gal (B). After the transfection, cells were incubated in 0.5% FCS in the presence (+) or absence (−) of t-RA (5 μM) for 48 h and subjected to 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) assay. The activity of AP-1 and NF-κB was evaluated as described in the Materials and Methods section. Assays were performed in quadruplicate. Data are shown as means ± SEM. *, statistically significant difference ($P < 0.05$).

pression of MCP-1 was regulated differently in mesangial cells, *i.e.*, the constitutive expression was dependent on but the cytokine-inducible expression was independent of AP-1.

RA generally has been regarded as an inhibitor of AP-1 (20). However, previous studies indicated that the anti–AP-1 activity of RA is somewhat controversial. For example, RA does not inhibit c-*jun* and c-*fos* expression and activity of AP-1 in activated myofibroblasts and monocytes (39,40). RA rather may upregulate expression of c-*fos*/c-*jun* and activity of AP-1 in tumor cells and embryonic stem cells (41–44). The effect of RA on the AP-1 pathway supposedly is different from cell type to cell type. In this investigation, we tested the effect of t-RA, an anti-inflammatory agent for glomerulonephritis (45), on the expression of MCP-1 in mesangial cells. Our results showed that t-RA inhibited the constitutive expression but not IL-1β–inducible expression. The suppressive effect of t-RA was via the inhibition of AP-1 because (*1*) the constitutive expression but not the cytokine-inducible expression was dependent on AP-1, (*2*) t-RA inhibited constitutive activity of AP-1 but not NF-κB, and (*3*) genetic and pharmacologic inhibitors of AP-1 suppressed only the constitutive expression of MCP-1.

The mechanisms involved in the suppressive effect of t-RA on the basal AP-1 activity are unknown. We previously showed that t-RA inhibited induction of c-*fos* and c-*jun* in H₂O₂-exposed mesangial cells. However, under the serum-deprived, unstimulated condition, t-RA did not suppress the constitutive expression of c-*fos* and c-*jun* but rather upregu-

lated both mRNA (Lucio-Cazana J, Kitamura M, unpublished data), which is consistent with previous reports (41–44). The anti–AP-1 effect of t-RA should, therefore, be ascribed to other mechanisms.

Biological actions of RA are mediated by RAR and RXR. The RAR family is known to be activated by t-RA and by 9-cis-RA, but the RXR family is activated only by 9-cis-RA (46). That both t-RA and 9-cis-RA inhibited MCP-1 expression suggested the importance of RAR in the transcriptional suppression by RA. Indeed, the experiments using the pan-RAR antagonist revealed that RAR were essential. A previous report showed that RA inhibited activation of AP-1 via physical interaction of RAR-RXR complexes with c-Jun (47). Sequestration of AP-1 proteins by RAR-RXR heterodimers (48) may be involved in the anti–AP-1 effect of t-RA. Another recent report also showed that disruption of homodimerization or herterodimerization of AP-1 components is a mechanism through which ligand-activated RAR suppress the AP-1 activity (49). Of note, RARα, RARβ, RARγ, and RXRα mRNA are constitutively expressed in serum-deprived mesangial cells (Xu Q, Kitamura M, unpublished observation).

In summary, these data elucidated the different mechanisms involved in the constitutive and cytokine-inducible expression of MCP-1. t-RA, a potential anti-inflammatory drug, has the ability to inhibit selectively the constitutive expression of MCP-1 via intervention in the AP-1 pathway.

Acknowledgments

References

1. Leonard EJ, Yoshimura T: Human monocyte chemoattractant protein-1 (MCP-1). *Immunol Today* 11: 97–101, 1990
2. Zoja C, Wang JM, Bettoni S, Sironi M, Renzi D, Chiaffarino F, Abboud HE, Van-Damme J, Mantovani A, Remuzzi G: Interleukin-1β and tumor necrosis factor-α induce gene expression and production of leukocyte chemotactic factors, colony-stimulating factors, and interleukin-6 in human mesangial cells. *Am J Pathol* 138: 991–1003, 1991
3. Rovin BH, Yoshiumura T, Tan L: Cytokine-induced production of monocyte chemoattractant protein-1 by cultured human mesangial cells. *J Immunol* 148: 2148–2153, 1992
4. Main IW, Nikolic-Paterson DJ, Atkins RC: T cells and macrophages and their role in renal injury. *Semin Nephrol* 12: 395–407, 1992
5. Stahl RAK, Thaiss F, Disser M, Helmchen U, Hora K, Schlondorff D: Increased expression of monocyte chemoattractant protein-1 in anti-thymocyte antibody-induced glomerulonephritis. *Kidney Int* 44: 1036–1047, 1993
6. Rovin BH, Rumancik M, Tan L, Dickerson J: Glomerular expression of monocyte chemoattractant protein-1 in experimental and human glomerulonephritis. *Lab Invest* 71: 536–542, 1994
7. Neugarten J, Feith GW, Assmann KJM, Shan Z, Stanley ER, Schlondorff D: Role of macrophages and colony-stimulating factor-1 in murine antiglomerular basement membrane glomerulonephritis. *J Am Soc Nephrol* 5: 1903–1909, 1995
8. Tang WW, Qi M, Warren JS: Monocyte chemoattractant protein 1 mediates glomerular macrophage infiltration in anti-GBM Ab GN. *Kidney Int* 50: 665–671, 1996
9. Ueda A, Okuda K, Ohno S, Shirai A, Igarashi T, Matsunaga K, Fukushima J, Kawamoto S, Ishigatsubo Y, Okubo T: NF-κB and Sp1 regulate transcription of the human monocyte chemoattractant protein-1 gene. *J Immunol* 153: 2052–2063, 1994
10. Rollins BJ, Stier P, Ernst T, Wong GG: The human homolog of the JE gene encodes a monocyte secretory protein. *Mol Cell Biol* 9: 4687–4695, 1989
11. Shyy Y-J, Li Y-S, Kolattukudy PE: Structure of human monocyte chemotactic protein gene and its regulation by TPA. *Biochem Biophys Res Commun* 169: 346–351, 1990
12. Takeshita A, Chen Y, Watanabe A, Kitano S, Hanazawa S: TGF-β induces expression of monocyte chemoattractant JE/monocyte chemoattractant protein 1 via transcriptional factor AP-1 induced by protein kinase in osteoblastic cells. *J Immunol* 155: 419–426, 1995
13. Kitamura M: Identification of an inhibitor targeting macrophage production of monocyte chemoattractant protein-1 as TGF-β1. *J Immunol* 159: 1404–1411, 1997
14. Shyy JYJ, Lin MC, Han J, Lu Y, Petrime M, Chien S: The *cis*-acting phorbol ester "12-o-tetradecanoylphorbol 13-acetate"-responsive element is involved in shear stress-induced monocyte chemotactic protein 1 gene expression. *Proc Natl Acad Sci USA* 92: 8069–8073, 1995
15. Murata M, Arata S, Nose K: Involvement of reactive oxygen species in the induction of chemokine JE/MCP-1 gene by phorbol-12-myristate-13-acetate in Balb 3T3 cells. *Cell Struct Funct* 22: 231–238, 1997
16. Rovin BH, Dickerson JA, Tan LC, Hebert CA: Activation of nuclear factor-κB correlates with MCP-1 expression by human mesangial cells. *Kidney Int* 48: 1263–1271, 1995
17. Ishikawa Y, Sugiyama H, Stylianou E, Kitamura M: Bioflavonoid quercetin inhibits interleukin-1-induced transcriptional expression of monocyte chemoattractant protein-1 in glomerular cells via suppression of nuclear factor-κB. *J Am Soc Nephrol* 10: 2290–2296, 1999
18. Martin T, Cardarelli PM, Parry GC, Felts KA, Cobb RR: Cytokine induction of monocyte chemoattractant protein-1 gene expression in human endothelial cells depends on the cooperative action of NF-κB and AP-1. *Eur J Immunol* 27: 1091–1097, 1997
19. De Luca LM: Retinoids and their receptors in differentiation, embryogenesis, and neoplasia. *FASEB J* 5: 2924–2933, 1991
20. Schüle R, Rangarajan P, Yang N, Kliewer S, Ransone LJ, Bolado J, Verma IM, Evans RM: Retinoic acid is a negative regulator of AP-1-responsive genes. *Proc Natl Acad Sci USA* 88: 6092–6096, 1991
21. Simonson MS: Anti-AP-1 activity of all-*trans* retinoic acid in glomerular mesangial cells. *Am J Physiol* 267: F805–F815, 1994
22. Moreno-Manzano V, Ishikawa Y, Lucio-Cazana J, Kitamura M: Suppression of apoptosis by all-trans-retinoic acid: Dual intervention in the c-Jun N-terminal kinase-AP-1 pathway. *J Biol Chem* 274: 20251–20258, 1999
23. Gille J, Paxton LL, Lawley TJ, Caughman SW, Swerlick RA: Retinoic acid inhibits the regulated expression of vascular cell adhesion molecule-1 by cultured dermal microvascular endothelial cells. *J Clin Invest* 99: 492–500, 1997
24. Kitamura M, Taylor S, Unwin R, Burton S, Shimizu F, Fine LG: Gene transfer into the rat renal glomerulus via a mesangial cell

689vector: Site-specific delivery, in situ amplification, and sustained expression of an exogenous gene in vivo. *J Clin Invest* 94: 497–505, 1994

25. Yokoo T, Kitamura M: Opposite, binary regulatory pathways involved in IL-1-mediated stromelysin gene expression in rat mesangial cells. *Kidney Int* 50: 894–901, 1996

26. Brown PH, Alani R, Preis LH, Szabo E, Birrer MJ: Suppression of oncogene-induced transformation by deletion mutant of c-*jun*. *Oncogene* 8: 877–886, 1993

27. Van Antwerp DJ, Martin SJ, Kafri T, Green DR, Verma IM: Suppression of TNF-α-induced apoptosis by NF-κB. *Science* 274: 787–789, 1996

28. Sugiyama H, Savill JS, Kitamura M, Zhao L, Stylianou E: Selective sensitization of tumor necrosis factor-α-induced apoptosis by blockade of NF-κB in primary glomerular mesangial cells. *J Biol Chem* 274: 19532–19537, 1999

29. Yokoo T, Kitamura M: Dual regulation of IL-1β-mediated matrix metalloproteinase-9 expression in mesangial cells by NF-κB and AP-1. *Am J Physiol* 270: F123–F130, 1996

30. Agarwal C, Chandraratna RA, Johnson AT, Rorke EA, Eckert RL: AGN193109 is a highly effective antagonist of retinoid action in human ectocervical epithelial cells. *J Biol Chem* 271: 12209–12212, 1996

31. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol chloroform extraction. *Anal Biochem* 162: 156–159, 1987

32. Rollins BJ, Morrison ED, Stiles CD: Cloning and expression of *JE*, a gene inducible by platelet-derived growth factor and whose product has cytokine-like properties. *Proc Natl Acad Sci USA* 85: 3738–3742, 1988

33. Meinkoth J, Alberts A, Feramisco J: Construction of mammalian cell lines with indicator genes driven by regulated promoters. *Ciba Found Symp* 150: 47–56, 1990

34. Rattner A, Korner M, Walker MD, Citri Y: NF-κB activates the HIV promoter in neurons. *EMBO J* 12: 4261–4267, 1993

35. Kitamura M, Kawachi H: Creation of an in vivo cytosensor using engineered mesangial cells: Automatic sensing of glomerular inflammation controls transgene activity. *J Clin Invest* 100: 1394–1399, 1997

36. Becker S, Quay J, Koren HS, Haskill JS: Constitutive and stimulated MCP-1, GRO α, β, and γ expression in human airway epithelium and bronchoalveolar macrophages. *Am J Physiol* 266: L278–L286, 1994

37. Wolf G, Schneider A, Helmchen U, Stahl RAK: AT$_1$-receptor antagonists abolish glomerular MCP-1 expression in a model of mesangial proliferative glomerulonephritis. *Exp Nephrol* 6: 112–120, 1998

38. Schreiner G, Kiely JM, Cotran RS, Unanue ER: Characterization of resident glomerular cells in the rat expressing Ia determinants and manifesting genetically restricted interactions with lymphocytes. *J Clin Invest* 68: 920–931, 1981

39. Davis BH, Coll D, Beno DW: Retinoic acid suppresses the response to platelet-derived growth factor in human hepatic Ito-cell-like myofibroblasts: A post-receptor mechanism independent of raf/fos/jun/egr activation. *Biochem J* 294: 785–791, 1993

40. Oeth P, Yao J, Fan ST, Mackman N: Retinoic acid selectively inhibits lipopolysaccharide induction of tissue factor gene expression in human monocytes. *Blood* 91: 2857–2865, 1998

41. Busam KJ, Geiser AG, Roberts AB, Sporn MB: Synergistic increase of phorbol ester-induced c-*fos* mRNA expression by retinoic acid through stabilization of the c-*fos* message. *Oncogene* 8: 2267–2273, 1993

42. de-Groot RP, Pals C, Kruijer W: Transcriptional control of c-*jun* by retinoic acid. *Nucleic Acids Res* 19: 1585–1591, 1991

43. Wan H, Dawson MI, Hong WK, Lotan R: Enhancement of Calu-1 human lung carcinoma cell growth in serum-free medium by retinoids: Dependence on AP-1 activation, but not on retinoid response element activation. *Oncogene* 15: 2109–2118, 1997

44. Desai SH, Niles RM: Characterization of retinoic acid-induced AP-1 activity in B16 mouse melanoma cells. *J Biol Chem* 272: 12809–12815, 1997

45. Wagner J, Dechow C, Morath C, Lehrke I, Amann K, Waldherr R, Floege J, Ritz E: Retinoic acid reduces glomerular injury in a rat model of glomerular damage. *J Am Soc Nephrol* 11: 1479–1487, 2000

46. Chambon P: A decade of molecular biology of retinoic acid receptors. *FASEB J* 10: 940–954, 1996

47. Schroen DJ, Brinckerhoff CE: Inhibition of rabbit collagenase (matrix metalloproteinase-1; MMP-1) transcription by retinoid receptors: Evidence for binding of RARs/RXRs to the -77 AP-1 site through interactions with c-Jun. *J Cell Physiol* 169: 320–332, 1996

48. Vincenti MP, White LA, Schroen DJ, Benbow U, Brinckerhoff CE: Regulating expression of the gene for matrix metalloproteinase-1 (collagenase): Mechanisms that control enzyme activity, transcription, and mRNA stability. *Crit Rev Eukaryot Gene Expr* 6: 391–411, 1996

49. Zhou XF, Shen XQ, Shemshedini L: Ligand-activated retinoic acid receptor inhibits AP-1 transactivation by disruption c-Jun/c-Fos dimerization. *Mol Endocrinol* 13: 276–285, 1999