

Suppression of Constitutive but Not IL-1 β -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-1 β (IL-1 β). Pretreatment with t-RA abrogated the constitutive mRNA expression but did not inhibit the IL-1 β -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-1 β -inducible MCP-1 expression was dependent on nuclear factor- κ B (NF- κ B) and independent of activator protein 1 (AP-1). In contrast, the constitutive expression of MCP-1 was dependent on both NF- κ B and AP-1. t-RA substantially inhibited the constitutive activity of AP-1 but did not inhibit NF- κ B activity in mesangial cells. These data suggested that (1) constitutive and IL-1 β -inducible expression of MCP-1 was differently regulated by AP-1 and NF- κ B 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- κ B (NF- κ B) binding sites (9–11). It indicates potential roles of activator protein 1 (AP-1) and NF- κ B 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- κ B is essential for the induction of MCP-1 by interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), and 12-o-tetradecanoylphorbol-13-acetate (9,15–17). In endothelial cells, NF- κ B and AP-1 cooperatively upregulate expression of MCP-1 in response to IL-1 β (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 (RAR α , β , γ) and retinoid X receptors (RXR α , 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- κ B (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.

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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 $\mu\text{g/ml}$ streptomycin, 0.25 $\mu\text{g/ml}$ 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/I κ B α M cells in which NF- κ B is selectively inactivated were established by overexpression of a super-repressor mutant of I κ B α (I κ B α M) (27), as described previously (17,22). I κ B α M contains N- and C-terminal mutations and is resistant to both basal and stimulus-dependent degradation. When I κ B α M is overexpressed, it functions as a dominant-negative mutant and blocks constitutive and inducible activation of NF- κ B (27). SM/I κ B α M cells exhibit blunted activation of NF- κ B when stimulated by proinflammatory cytokines IL-1 β 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-1 β (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-1 β 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 $\mu\text{g/ml}$ herring sperm DNA, and 50 $\mu\text{g/ml}$ 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 $\mu\text{g/ml}$; 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 (1.0×10^5 /well) were transfected with a reporter plasmid pTRE-LacZ (a gift from Dr. A. Alberts, ICRF, UK) (33) or a control plasmid pCI- β gal (a gift from Promega, Madison, WI) at 0.3 to 0.6 $\mu\text{g/well}$. pTRE-LacZ introduces a β -galactosidase (β gal) gene (*lacZ*) under the control of TRE. pCI- β gal 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- β gal. Assays were performed in quadruplicate.

Activity of NF- κ B was similarly assessed by the transient transfection, as described previously (17,25,29). Mesangial cells were transfected with pCI- β gal, a κ B reporter plasmid pHIVLTR β -gal or its control construct pmuHIVLTR β -gal (34) (gifts from Dr. A. Rattner, The Weizmann Institute of Science, Rehovot, Israel). pHIVLTR β -gal introduces *lacZ* under the control of the HIV promoter that contains two κ B motifs. The control plasmid pmuHIVLTR β -gal contains a κ B-mutated HIV promoter. NF- κ B 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- β gal. Each normalized value of the pHIVLTR β -gal transfection was then subtracted by the normalized value of the pmuHIVLTR β -gal transfection, and the resultant value was used as an indicator of NF- κ B 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 MgCl₂, 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 K₃Fe(CN)₆, 20 mM K₄Fe(CN)₆·3H₂O, 2 mM MgCl₂, 0.01% sodium desoxycholate, and 0.02% NP-40 in PBS.

Statistical Analyses

Data were expressed as means ± 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-

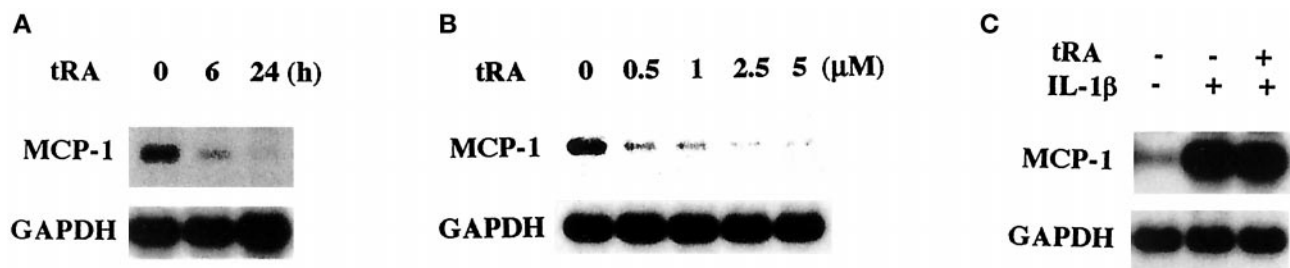


Figure 1. Suppression of constitutive but not interleukin-1β (IL-1β)-inducible expression of monocyte chemoattractant protein 1 (MCP-1) by all-trans-retinoic acid (t-RA). (A) Rat mesangial cells (SM43) were serum deprived (0.5% fetal calf serum [FCS] for 24 h), treated with t-RA (5 μM) for 6 and 24 h, 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.

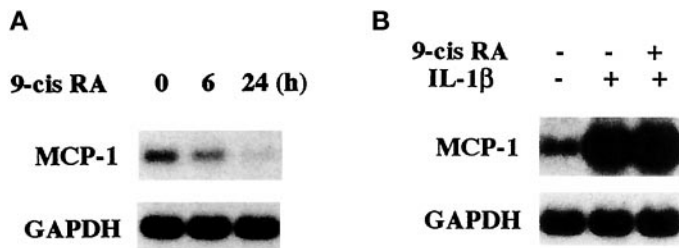


Figure 2. Suppression of constitutive but not IL-1β-inducible expression of MCP-1 by 9-cis-RA. (A) Mesangial cells were serum deprived, treated with 9-cis-RA (5 μM) for 6 and 24 h, and subjected to Northern blot analysis of MCP-1. (B) Mesangial cells were pretreated with (+) or without (-) 9-cis-RA for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

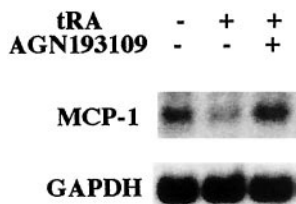


Figure 3. Requirement of retinoic acid receptors (RAR) for the anti-MCP-1 effect of t-RA. Mesangial cells were treated with t-RA (2.5 μM) together with or without a selective pan-RAR antagonist, AGN193109 (5 μM), for 6 h, and Northern blot analysis was performed.

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

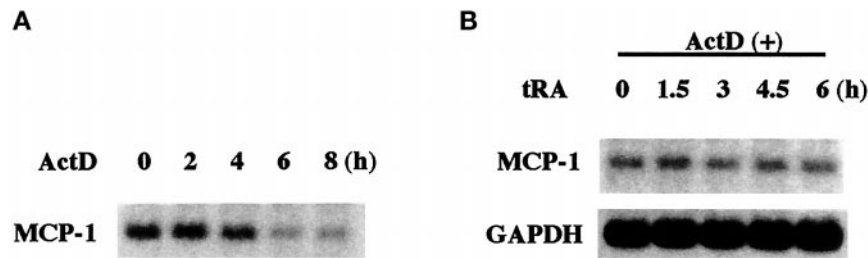


Figure 4. Effect of t-RA on the stability of MCP-1 mRNA. (A) Serum-deprived mesangial cells were treated with actinomycin D (ActD; 500 ng/ml) for 2 to 8 h, and the level of MCP-1 transcript was examined by Northern analysis. (B) Serum-deprived mesangial cells were exposed to actinomycin D for 6 h in the presence or absence of t-RA during the last 1.5 to 6 h and subjected to Northern blot analysis.

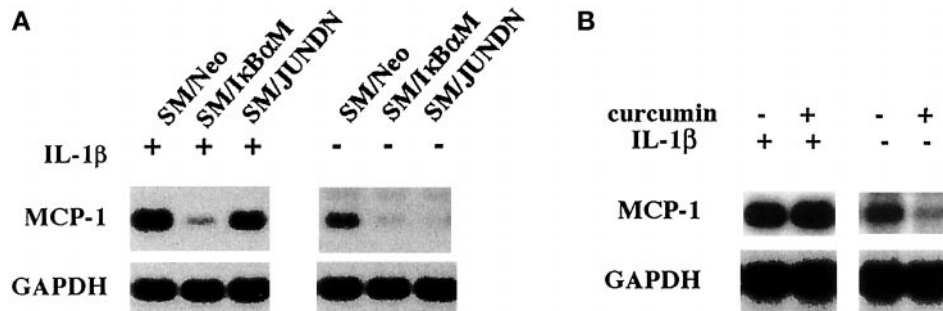


Figure 5. Roles of nuclear factor- κ B (NF- κ B) and activator protein 1 (AP-1) in the constitutive and cytokine-inducible expression of MCP-1. (A) Transfectants stably expressing a neomycin phosphotransferase gene (*neo*) alone (SM/Neo), *neo* and a dominant-negative mutant of *c-jun* (SM/JUNDN), and *neo* and a super-repressor mutant of $I\kappa B\alpha$ (SM/ $I\kappa B\alpha$ M) were treated with (left) or without (right) IL-1 β . 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 μ M) and treated with (left) or without (right) IL-1 β 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-

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-1 β -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- κ B, 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-

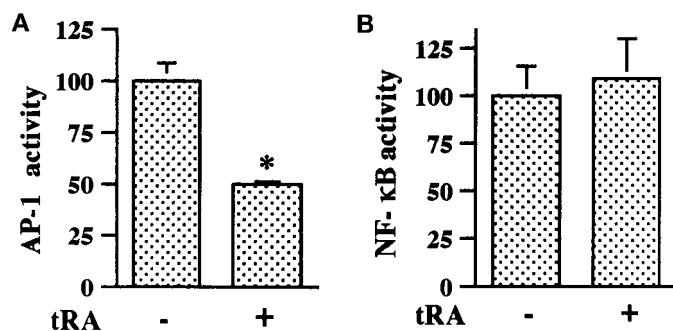


Figure 6. Effects of t-RA on the constitutive activity of AP-1 and NF- κ B. Mesangial cells cultured in 24-well plates were transfected transiently with an AP-1 reporter plasmid pTRE-LacZ (A) or an NF- κ B reporter plasmid pHIVLTR β -gal (B). After the transfection, cells were incubated in 0.5% FCS in the presence (+) or absence (-) of t-RA (5 μ M) 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- κ B was evaluated as described in the Materials and Methods section. Assays were performed in quadruplicate. Data are shown as means \pm SEM. *, statistically significant difference ($P < 0.05$).

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 heterodimerization of AP-1 components is a mechanism through which ligand-activated RAR suppress the AP-1 activity (49). Of note, RAR α , RAR β , RAR γ , and RXR α 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.

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