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Tumor Necrosis Factor-α Induces Fractalkine Expression Preferentially in Arterial Endothelial Cells and Mithramycin A Suppresses TNF-α-Induced Fractalkine Expression

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Fractalkine is an unusual tumor necrosis factor (TNF)-α-induced chemokine. The molecule is tethered to cells that express it and produces strong and direct adhesion to leukocytes expressing fractalkine receptor. However, the potential mechanism and significance of TNF-α-induced fractalkine expression in vascular endothelial cells are poorly understood. Here we show that in primary cultured endothelial cells TNF-α-induced fractalkine mRNA expression is mediated mainly through phosphatidylinositol 3′-kinase activation and nuclear factor (NF)-κB mediated transcriptional activation, along with GC-rich DNA-binding protein-mediated transcription. Interestingly, GC-rich DNA-binding protein inhibitors, mithramycin A and chromomycin A3, strongly suppressed TNF-α-induced fractalkine mRNA expression, possibly through inhibition of transcriptional activities by NF-κB and Sp1. In fact, direct inhibition of NF-κB and Sp1 bindings by decoy oligonucleotides suppressed TNF-α-induced fractalkine expression. Histologically, TNF-α-induced fractalkine expression was observed markedly in arterial and capillary endothelial cells, endocardium, and endothelium of intestinal villi, and slightly in venous endothelial cells, but not at all in lymphatic endothelial cells of intestine. Mithramycin A markedly suppressed TNF-α-induced fractalkine expression *in vivo*. These results indicate that TNF-α-stimulated fractalkine expression could act as part of arterial endothelial adhesion to leukocytes and monocytes during inflammation and atherosclerosis. NF-κB and Sp1 inhibitors such as mithramycin A may provide a pharmacological approach to suppressing these processes. *(Am J Pathol 2004, 164:1663–1672)*

The vascular endothelium plays a central role in the recruitment and migration of circulating effector cells to sites of inflammation and immune response.[1–3] The migration of leukocytes into extravascular tissues involves a cascade of molecular events, including the elaboration of chemotactic factors, response to these factors, interaction of leukocytes with endothelial cells, and leukocyte transmigration through the wall of the blood vessel.[1–3] Chemokines were first described as chemoattractant cytokines synthesized at sites of inflammation, and are major regulatory proteins for the recruitment and trafficking of leukocytes.[4,5]

Among more than 50 known chemokines, fractalkine is the sole member of the CX3C family, and has unique structural and functional attributes.[6,7] It is a large protein of 373 amino acids containing multiple domains.[6,7] In its extracellular domain, the first 76 amino acids of fractalkine comprise a chemokine domain with a novel arrangement of cysteine residues (CysXaaXaaXaaCys), followed by an extended mucin-like stalk, a transmembrane domain, and an intracellular domain of 37 amino acids.[6,7] Thus, it is expressed as a transmembrane molecule on the cell surface. Fractalkine binding to its seven transmembrane domain G protein-coupled receptor, CX3CR1, triggers signaling, but also directly mediates cell adhesion.[8] In fact, fractalkine binds CX3CR1 rapidly and firmly, which leads to tethering and arrest of leukocytes under conditions of normal blood flow even in the absence of CX3CR1 signaling.[8,9] CX3CR1 is also expressed on subsets of peripheral $CD4^+$ and $CD8^+$ T cells, natural killer cells, and $CD16^+$ monocytes.[8,10,11] Furthermore, CX3CR1 defines circulating effector-killer lymphocytes and modulates trafficking.[12] These observations suggest that fractalkine and CX3CR1 fulfill special roles in tethering and rolling, arrest, stable adhesion, and transendothelial migration of CX3CR1-leukocytes at sites of fractalkine-expressing endothelium.

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Similar to other cytokines, fractalkine expression is markedly induced by inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin-1, and interferon-γ in primary cultured endothelial cells.[13,14] Activation of nuclear factor (NF)-κB could play a central role in inflammatory cytokine-induced fractalkine expression at the transcriptional level.[14] However, the signaling mechanisms by which inflammatory cytokines induce fractalkine mRNA expression are not well known. Given that different endothelia show heterogeneity in structural and functional characteristics,[15] inflammatory reactions at different sites could be heterogeneous.[16] However, the localization and regulation of fractalkine expression by different endothelia during inflammation is not well known. Therefore, the goals of the present study were to determine the second messenger/transcriptional mechanisms leading to fractalkine expression by TNF-α, and the localization and regulation of fractalkine expression by TNF-α.

Materials and Methods

Materials and Cell Culture

Recombinant human TNF-α, soluble fractalkine, and anti-fractalkine antibodies were purchased from R&D Systems (Minneapolis, MN). Phosphatidylinositol 3′-kinase (PI 3′-kinase) inhibitor wortmannin was purchased from RBI, Inc. ADP ribosylation factor inhibitor brefeldin-A and MEK 1/2 inhibitor PD98059 were purchased from Calbiochem (San Diego, CA). Phospholipase C (PLC) inhibitor U73122 was purchased from Biomol Research Laboratory (Plymouth Meeting, PA). Sphingosine kinase inhibitor *N*,*N*-dimethylsphingosine was purchased from ICN Pharmaceuticals (Costa Mesa, CA). NF-κB inhibitor pyrrolidine dithiocarbamate and protein kinase C inhibitor chelerythrine chloride were purchased from Sigma-Aldrich (St. Louis, MO). Media, sera, and most other biochemical reagents were purchased from Sigma-Aldrich, unless otherwise specified. Human umbilical vein endothelial cells (HUVECs) were prepared from human umbilical cords by collagenase digestion as previously described.[17] The endothelial identity of the cultures was confirmed by the presence of factor VIII detected by immunofluorescence. HUVECs were maintained in M-199 medium supplemented with 20% (v/v) fetal bovine serum at 37°C in a 5% CO_2 atmosphere. The primary cultured cells used in this study were between passages 2 and 4.

RNase Protection Assay (RPA)

The partial cDNA of human fractalkine (nucleotides 482 to 893, GenBank accession NM002996) was amplified by polymerase chain reaction and subcloned into pBluescript II KS+ (Stratagene, La Jolla, CA). After linearizing with *Eco*RI, ^{32}P-labeled anti-sense RNA probes were synthesized by *in vitro* transcription using T7 polymerase (Ambion Maxiscript kit; Ambion, Austin, TX) and gel purified. RPA was performed on total RNAs using the Ambion RPA kit. An anti-sense RNA probe of human cyclophilin (nucleotides 135 to 239, GenBank accession X52856) was used as an internal control for RNA quantification.

Western Blot Analysis

For Western blot analysis, samples were mixed with sample buffer, boiled for 10 minutes, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under denaturing conditions, and electroblotted to nitrocellulose membranes. The nitrocellulose membranes were blocked by incubation in blocking buffer, incubated with anti-fractalkine monoclonal antibody, washed, and incubated with horseradish peroxidase-conjugated secondary antibody. Signals were visualized by chemiluminescent detection according to the manufacturer's protocol (Amersham, Buckinghamshire, UK). The membrane was reblotted with anti-actin antibody to verify equal loading of protein in each lane.

Electrophoretic Mobility Shift Assay (EMSA)

EMSA for NF-κB proteins was performed as previously described.[18] Briefly, the cells were lysed in a hypotonic buffer (10 mmol/L HEPES, pH 7.9, 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 0.5 mmol/L dithiothreitol, 0.5 mmol/L phenylmethyl sulfonyl fluoride) containing 0.6% NP-40 and microfuged at 4000 rpm for 15 minutes. The pellet was lysed in 15 μl of a high-salt buffer (20 mmol/L HEPES, pH 7.9, 420 mmol/L NaCl, 25% glycerol, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L ethylenediaminetetraacetic acid, 0.5 mmol/L phenylmethyl sulfonyl fluoride, 0.5 mmol/L dithiothreitol) for 20 minutes on ice. Seventy-five μl of storage buffer (20 mmol/L HEPES, pH 7.9, 100 mmol/L NaCl, 20% glycerol, 0.2 mmol/L ethylenediaminetetraacetic acid, 0.5 mmol/L phenylmethyl sulfonyl fluoride, 0.5 mmol/L dithiothreitol) was added, agitated for 10 seconds by vortexing, and then microfuged at 14,000 rpm for 20 minutes. Nuclear extracts (10 μg) were incubated with ~20,000 cpm of ^{32}P-labeled NF-κB binding site oligomer 5′-AGTTGAGGGGACTTTCCCAGGC-3′ (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 minutes at 20°C. EMSA for SP1 protein was performed with biotin-labeled SP1 binding site oligomer 5′-GATCCGGTCCCCCACCATCCCCCGCCATTTCCA and signals were detected by chemiluminescence imaging according to the manufacturer's protocol (EMSA Gel-Shift kit; Panomics, Redwood City, CA).

Construction of Decoy Oligodeoxynucleotide (ODN) for Sp1 and NF-κB

The sequences of dumbbell-shaped, decoy ODN against the NF-κB binding site (NF-DO), its mismatched NF-κB decoy ODN (MNF-DO), against the Sp-1 binding site (SP-DO) and mismatched SP-1 decoy ODN (MSP-DO) are constructed as previously described[19] as follows (with consensus sequences underlined): NF-DO, 5′-GGATCCC*GGGGACTTTCCC*GCACAAAAGTGC*GGGAAAGTCCCC*G-

3′; MNF-DO, 5′GGATCCC*TATAACTTTCCC*GCACAA-AAGTGC*GGGAAAGTTATA*G-3′; SP-DO, 5′-*GGGG*CTA-GTTTTCTAG*CCCCGCCCC*GCTCGTTTTCGAGC*GGGG-C*-3′; MSP-DO, 5′-*ATGGC*TAGTTTTCTAG*CCATGCCA-T*GCTCGTTTTCGAGC*ATGGC*-3′. Decoy ODNs were annealed for 2 hours with a steady temperature descent from 80°C to 25°C. T4 DNA ligase (1 U) was added to the mixture, followed by incubation for 24 hours at 16°C to generate a covalently ligated dumbbell-shaped decoy ODN molecule. Each decoy ODN comprises two loops and one stem containing two NF-κB or one Sp1 consensus sequence in tandem. The stability and cellular uptake of the ODNs were similar to those previously described.[19]

In Vitro *Decoy ODN Transfer*

HUVECs were fed with fresh culture medium the day before the decoy ODN was added and washed twice with Opti-MEM (Gibco Invitrogen Corp., Carlsbad, CA) immediately before the experiment. HUVECs were transfected with decoy ODN (100 nmol/L) combined with LipofectAMINE 2000 (molar ratio; DNA:lipid = 1:3) (Gibco Invitrogen Corp.). The decoy ODN:lipid mixture was added dropwise to cells, according to the manufacturer's instructions. HUVECs were incubated at 37°C for 5 hours. After the addition of fresh medium containing 10% fetal bovine serum, cells were maintained in a CO_2 incubator until use. Transfection efficiency was ~72 to 83% as measured by fluorescence-labeled decoy ODN. Addition of NF-DO (100 nmol/L) or SP-DO (100 nmol/L) produced ~60 to 70% inhibition in TNF-α (10 ng/ml)-induced NF-κB or Sp1 activity, whereas MNF-DO (100 nmol/L) or MSP-DO (100 nmol/L) did not produce any changes in the TNF-α-induced NF-κB or Sp1 activity. At 100 nmol/L, none of the four ODNs alone produced any changes in basal NF-κB or Sp1 activity.

Animal Experiments

Inbred male Sprague-Dawley rats (150 to 200 g) were obtained from Daehan Experimental Animal Center (Daejeon, Korea) and were maintained on standard laboratory chow and water *ad libitum*. The rats (180 to 220 g) were divided into three groups: vehicle (0.1% dimethyl sulfoxide) (n = 6), TNF-α (10 μg/kg) (n = 6), and TNF-α (10 μg/kg) plus mithramycin A (50 μg/kg/day) (n = 6). Control buffer and TNF-α were injected intravenously through the tail vein. The vehicle (total volume, 100 μl) or mithramycin A was injected intravenously through the tail vein once per day for 3 days before TNF-α administration. At 12 hours after injection of vehicle or TNF-α, rats were anesthetized with pentobarbital (30 mg/kg), and subsequently sacrificed by cervical dislocation. All animal studies were reviewed and approved by the animal care and use committee of the Korean Advanced Institute of Science and Technology.

Immunohistochemical Analysis of Fractalkine Expression

After sacrifice, the hearts and small intestines (jejunums) were quickly excised, rinsed with PBS, and frozen in OCT in methyl-butane on dry ice. Frozen tissue blocks were sectioned at 10 μm and 8 to 12 sections of heart or jejunum from each mouse were incubated with anti-fractalkine antibody at 4°C overnight. Signals were visualized with the Cell and Tissue Staining Kit (R&D Systems, Minneapolis, MN). The sections were counterstained with Meyer's hematoxylin and were viewed and photographed with an Axioskope2 plus microscope (Carl Zeiss, Göttingen, Germany) equipped with color charge-coupled device camera (ProgResC14; Jenoptik, Jena, Germany) and monitor. Fractalkine expression was semiquantitated by grading the degree of immunostaining (very strong = 5, strong = 4, moderate = 3, weak = 2, none = 1). Three to five endothelial portions of each section were graded. Tissues were examined from several tissues of the heart (artery, vein, endocardium, and cardiac valves) and jejunum (artery, vein, and villous endothelium). Two independent, blinded investigators graded the expression by the charge-coupled device camera. Interinvestigator variation was <5%.

Densitometric Analyses and Statistics

All signals were visualized and analyzed by densitometric scanning (LAS-1000; Fuji Film, Tokyo, Japan). Data are expressed as mean ± SD. Statistical significance was tested using one-way analysis of variance followed by the Student-Newman-Keuls test. Statistical significance was set at $P < 0.05$.

Results

TNF-α Increased Expression of Fractalkine mRNA and Protein in HUVECs

Similar to previous reports,[13,14] addition of TNF-α (10 ng/ml) markedly increased the expression of fractalkine mRNA in a time-dependent manner (Figure 1A). The higher expression levels continued to 24 hours. Addition of TNF-α increased the fractalkine mRNA at 4 hours in a dose-dependent manner (Figure 1B). Addition of TNF-α (10 ng/ml) maximally increased expression of fractalkine protein at 12 hours, and the level continued to be higher than control for up to 24 hours (Figure 1C). The maximum mean increase in fractalkine was 3.7-fold.

Inhibitors Changed TNF-α-Stimulated Expression of Fractalkine mRNA

To examine the second messenger and transcriptional mechanisms leading to induction of fractalkine by TNF-α, various kinase inhibitors and transcription factor inhibitors were added to TNF-α (10 ng/ml)-treated HUVECs.

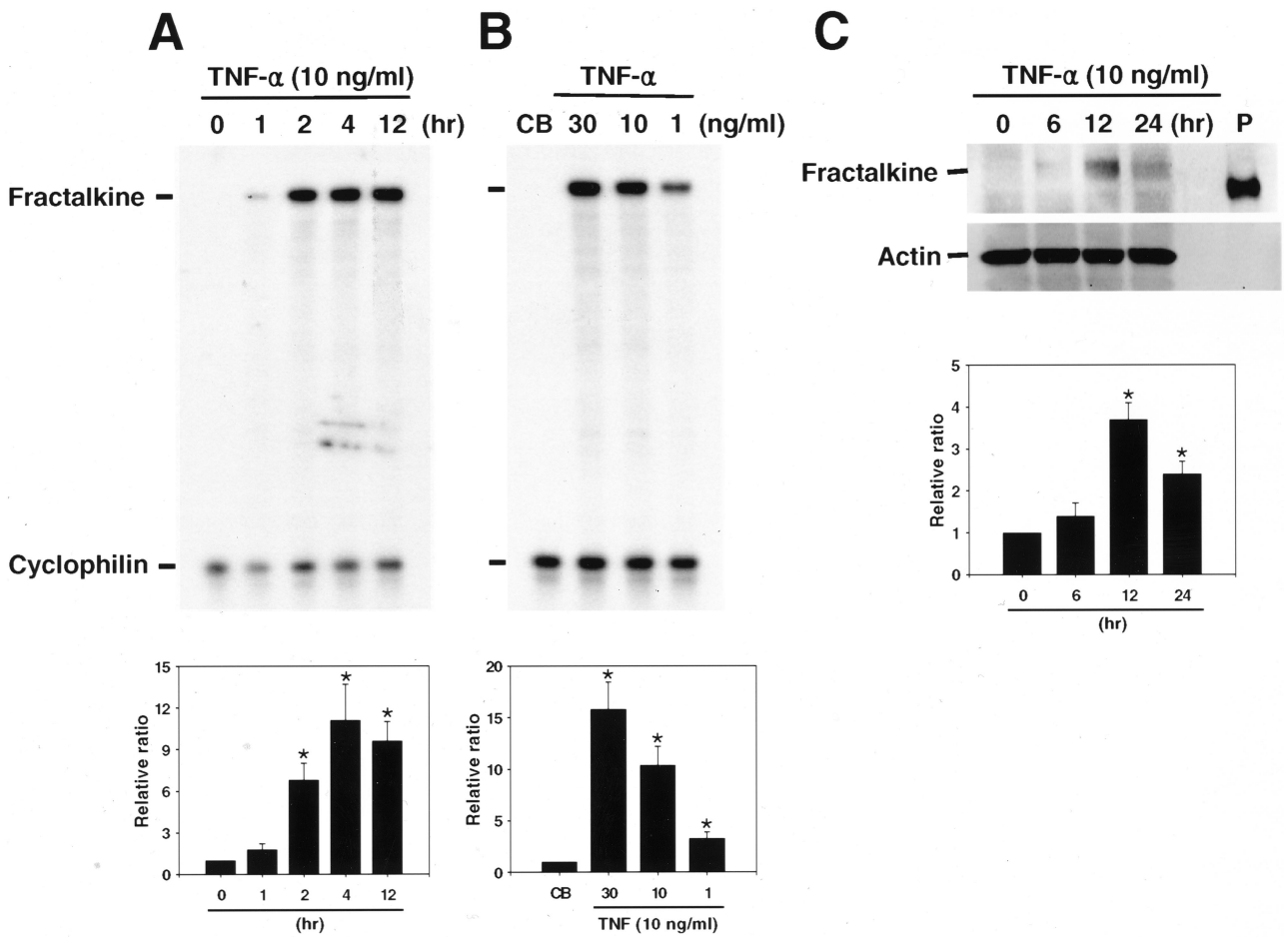

Figure 1. Induction of fractalkine by TNF-α. **A** and **B:** RPAs of fractalkine mRNA in TNF-α-stimulated HUVECs. HUVECs were incubated with TNF-α for the indicated times and indicated amounts for 4 hours. Total RNAs (10 μg) were subjected to multiplex RPA probed with anti-sense fractalkine RNA probe (426 bp). Equivalent loading was confirmed by probing the same reactions with an anti-sense cyclophilin RNA probe (105 bp). **C:** Western blot analyses of fractalkine protein in TNF-α-stimulated HUVECs. HUVECs were incubated for the indicated times with TNF-α (10 ng/ml). Each lane contains 50 μg of cellular protein. The Western blot was probed with an anti-fractalkine antibody (**top**) and reprobed with an anti-actin antibody (**middle**) to verify equal loading of protein in each lane. P, soluble fractalkine without transmembrane anchor domain (10 ng) was loaded as a positive control. **Bottom**: Densitometric analyses are presented as the relative ratio of fractalkine to cyclophilin or actin. The relative ratio measured at time 0 or the ratio relative to control buffer (CB) is arbitrarily presented as 1. Bars represent the mean ± SD from three experiments. *, $P < 0.05$ *versus* time 0 or CB.

NF-κB inhibitor pyrrolidine dithiocarbamate (50 μg/ml) suppressed TNF-α-induced expression of fractalkine mRNA. Co-treatment with PI 3′-kinase inhibitor wortmannin (30 nmol/L) also suppressed TNF-α-induced fractalkine mRNA expression. However, ADP ribosylation factor inhibitor brefeldin-A (50 μg/ml), MEK 1/2 inhibitor PD98059 (50 μmol/L), PLC inhibitor U73122 (1 μmol/L), sphingosine kinase inhibitor *N*,*N*-dimethylsphingosine (5 μmol/L), and protein kinase C inhibitor chelerythrine chloride (5 μmol/L) did not produce any changes (Figure 2). Interestingly, GC-rich DNA-binding protein inhibitor mithramycin A (500 nmol/L) completely suppressed TNF-α-induced expression of fractalkine mRNA (Figure 2). These results suggested that TNF-α-stimulated expression of fractalkine might be mediated mainly through activation of NF-κB and PI 3′-kinase pathways, along with GC-rich DNA binding protein-mediated transcription. The process seems to be independent of the MEK/ERK, PLC, and protein kinase C pathways.

Mithramycin A and Chromomycin A3 Suppressed TNF-α-Induced Expression of Fractalkine mRNA

We further characterized the effect of mithramycin A on TNF-α-induced fractalkine mRNA expression. Mithramycin A suppressed TNF-α-induced expression of fractalkine mRNA in a dose-dependent manner (Figure 3A). Addition of mithramycin A (10 nmol/L) suppressed ~30 to 40% of TNF-α-induced expression of fractalkine mRNA, whereas addition of mithramycin A (100, 500, and 1000 nmol/L) almost completely suppressed TNF-α-induced fractalkine mRNA expression. Chromomycin A3 (10 nmol/L), a structural analog of mithramycin A, suppressed TNF-α-induced expression of fractalkine mRNA in a dose-dependent manner (Figure 3B). Addition of chromomycin A3 (10 nmol/L) suppressed ~20 to 30% of TNF-α-induced fractalkine mRNA expression, whereas

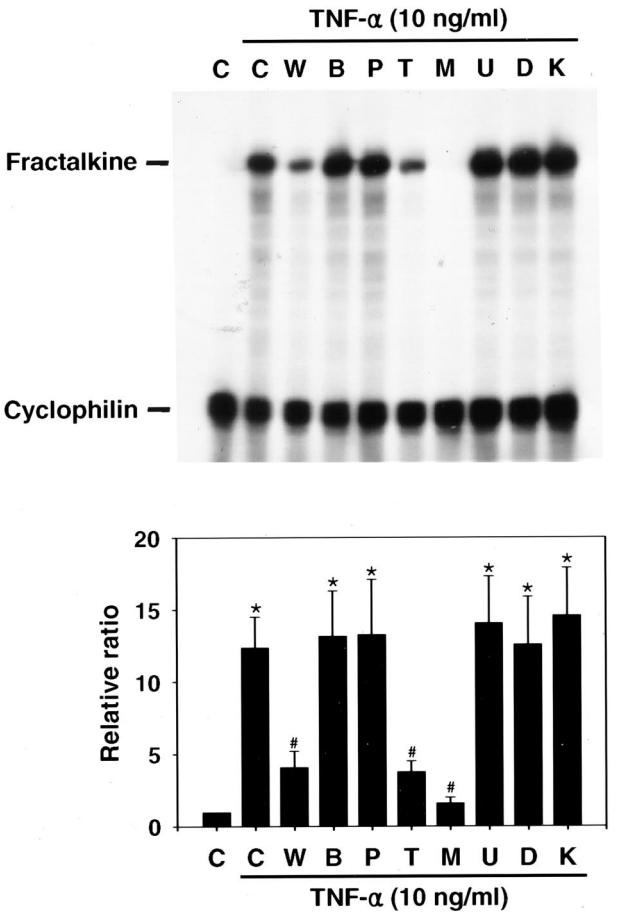

Figure 2. RPA of fractalkine mRNA in TNF-α-stimulated HUVECs co-treated with inhibitors. **Top:** HUVECs were incubated with TNF-α (10 ng/ml) for 4 hours in the presence of control buffer (C), wortmannin (W, 30 nmol/L), brefeldin-A (B, 50 μg/ml), PD98059 (P, 50 μmol/L), pyrrolidine dithiocarbamate (T, 50 μg/ml), mithramycin A (M, 500 nmol/L), U73122 (U, 1 μmol/L), *N,N*-dimethylsphingosine (D, 5 μmol/L), or chelerythrine chloride (K, 5 μmol/L). Total RNAs (10 μg) isolated from the cells were subjected to RPA. Note that wortmannin and pyrrolidine dithiocarbamate partially suppressed TNF-α-induced fractalkine mRNA expression, whereas mithramycin A completely suppressed TNF-α-induced fractalkine mRNA expression. Results were similar in three independent experiments. **Bottom**: Densitometric analyses are presented as the relative ratio of fractalkine to cyclophilin. The relative ratio to control buffer (C) is arbitrarily presented as 1. Bars represent the mean ± SD from three experiments. *, $P < 0.05$ *versus* C; #, $P < 0.05$ *versus* TNF-α (10 ng/ml) only.

addition of chromomycin A3 (100, 500, and 1000 nmol/L) almost completely suppressed TNF-α-induced fractalkine mRNA expression. These data suggest that mithramycin A and chromomycin A3 are potential inhibitors of TNF-α-induced fractalkine mRNA expression, possibly by inhibiting the binding of GC-rich DNA-binding proteins to the promoter of the fractalkine gene. To assess any possible toxicity of mithramycin A to HUVECs, we performed careful microscopic observation of the cells, an apoptosis assay by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling, and a DNA synthesis assay by [^{3}H]-thymidine incorporation. In the range from 0.1 to 30 nmol/L, mithramycin A exposure for 24 hours did not produce any notable effects on HUVECs. However, at concentrations of 100, 300, and 1000 nmol/L, mithramycin A for 24 hours induced apoptosis in ~2.2%, 3.1%, and 4.7% of cells and reduced

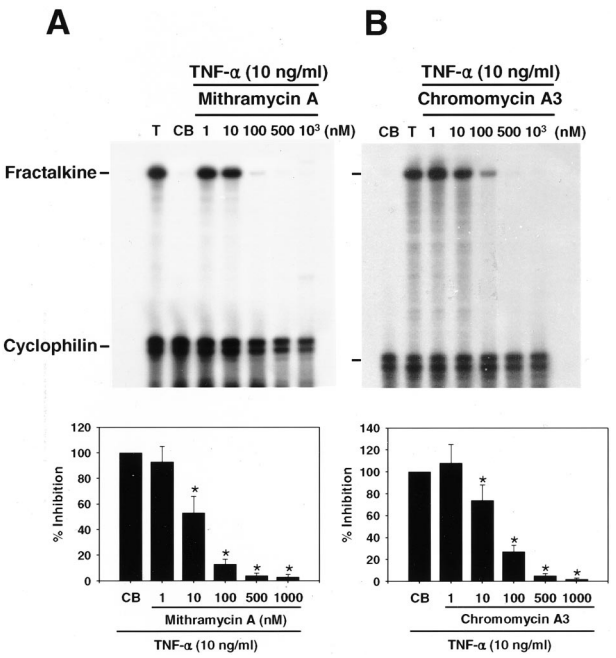

Figure 3. RPA of fractalkine mRNA in TNF-α-stimulated HUVECs co-treated with mithramycin A and chromomycin A3. **A** and **B:** HUVECs were incubated with control buffer (CB), TNF-α (T, 10 ng/ml), or TNF-α plus the indicated amount of mithramycin A and chromomycin A3 for 4 hours. Total RNAs (10 μg) isolated from the cells were subjected to RPA. Densitometric analyses are presented as the relative ratio of fractalkine mRNA to cyclophilin mRNA. The relative percentage measured with TNF-α (10 ng/ml) only is arbitrarily presented as 100%. Results were similar in three independent experiments. Bars represent the mean ± SD from three experiments. *, $P < 0.05$ *versus* T.

DNA synthesis ~18%, 37%, and 48%, respectively. Thus, mithramycin A at less than 30 nmol/L is not toxic, whereas concentrations of mithramycin A greater than 100 nmol/L may have toxic effects with significant reduction of DNA synthesis and induction of apoptosis.

Mithramycin A Suppressed TNF-α-Induced Sp1 and NF-κB Activities

Because Sp1 is a representative GC-rich DNA-binding protein and NF-κB is involved in TNF-α-induced fractalkine mRNA expression, we examined the effect of mithramycin A on Sp1 and NF-κB activities using EMSA. EMSA analyses revealed increased Sp1 and NF-κB (p65/p50) binding to the nuclear extracts of TNF-α (10 ng/ml)-stimulated HUVECs, whereas mithramycin A (100 nmol/L) alone only slightly suppressed the Sp1 and NF-κB (p65/p50) binding (Figure 4, A and B). Co-treatment with TNF-α and mithramycin A (10 nmol/L or100 nmol/L) suppressed the Sp1 and NF-κB (p65/p50) binding in a dose-dependent manner (Figure 4, A and B). Co-treatment with TNF-α and chromomycin A3 (10 nmol/L or 100 nmol/L) produced similar results (data not shown). These data suggest that mithramycin A and chromomycin A3 suppressed the TNF-α-induced fractalkine mRNA expression mainly through suppression of Sp1 and NF-κB activities in endothelial cells. To examine whether fractalkine mRNA expression is directly regulated by NF-κB and Sp1, double-stranded *cis*-element decoy ODNs for NF-

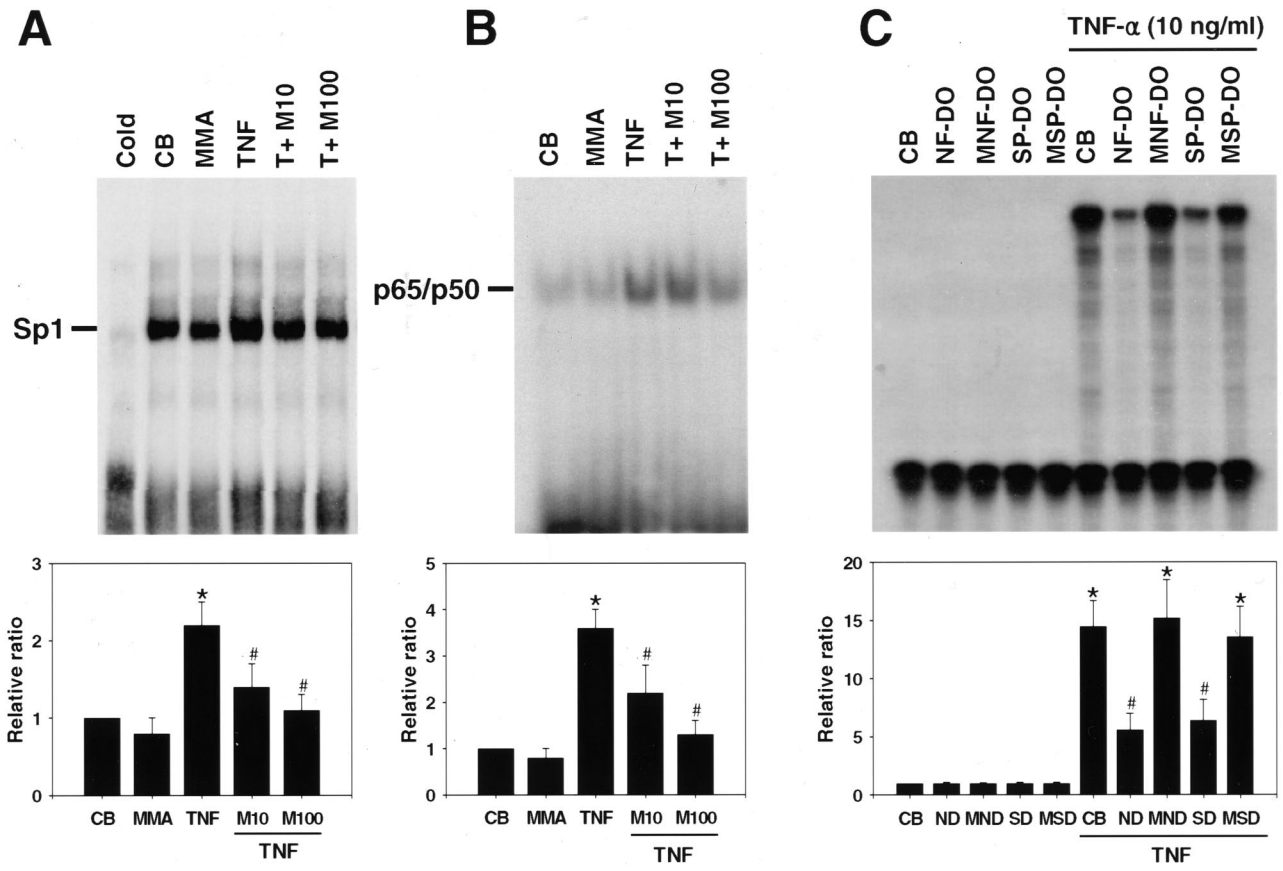

Figure 4. EMSA of Sp1 and NF-κB in TNF-α-stimulated HUVECs co-treated with mithramycin. **A** and **B:** HUVECs were incubated with control buffer (CB), mithramycin A (MMA, 100 nmol/L), TNF-α (TNF, 10 ng/ml), TNF plus mithramycin A (10 nmol/L) (T+M10), or TNF plus mithramycin A (100 nmol/L) (T+M100) for 2 hours. The binding activities of Sp1 (**A**) and NF-κB (**B**) in nuclear extracts were assayed by EMSA assay. **Bottom**: Densitometric analyses are presented as the relative ratio of Sp1 or NF-κB activity. The relative ratio to control buffer is arbitrarily presented as 1. Bars represent the mean ± SD from three experiments. *, $P < 0.05$ *versus* CB; #, $P < 0.05$ *versus* TNF. **C:** RPA of fractalkine mRNA in TNF-α-stimulated HUVECs co-treated with decoy ODNs of NF-κB or Sp1 binding site. HUVECs were transfected with NF-DO (100 nmol/L, ND), MNF-DO (100 nmol/L, MND), SP-DO (100 nmol/L, SD), or MSP-DO (100 nmol/L, MSD), incubated for 36 hours, and treated with control buffer (CB) or TNF-α (10 ng/ml) for 6 hours. Results were similar in three independent experiments. **Bottom**: Densitometric analyses are presented as the relative ratio of fractalkine to cyclophilin. The relative ratio to CB is arbitrarily presented as 1. Bars represent the mean ± SD from three experiments. *, $P < 0.05$ *versus* CB; #, $P < 0.05$ *versus* TNF-α (10 ng/ml).

κB- or Sp1-binding site was introduced into HUVECs. Matched decoy ODNs of NF-κB or Sp1 (NF-DO or SP-DO), significantly suppressed TNF-α (10 ng/ml)-induced fractalkine mRNA expression, whereas mismatched decoy ODNs (MNF-DO or MSP-DO) did not produce any changes in the TNF-α-induced fractalkine mRNA expression (Figure 4C). At the concentration used (100 nmol/L), none of the ODNs alone produced any changes in basal fractalkine mRNA expression (Figure 4C). Thus, fractalkine mRNA expression may be directly regulated by NF-κB and Sp1 in endothelial cells.

Mithramycin A Suppressed TNF-α-Induced Fractalkine Expression in Cardiac Endothelial Cells

We also examined the effect of TNF-α on fractalkine expression in rat heart using immunohistochemistry. We observed endogenous expression of fractalkine mainly in arterial endothelial cells, but almost no expression of fractalkine in capillary endothelial cells, venous endothelial cells, endocardium, myocardium, pericardium, or cardiac valves in normal adult rat (Figure 5). Intravenous injection of TNF-α (10 μg/kg) increased fractalkine expression markedly at 12 hours in arterial endothelial cells, capillary endothelial cell, endocardium, endocardial surface of cardiac valves, but not in venous endothelial cells, myocardial cells, or pericardium (Figures 5 and 7). These data suggest that TNF-α-induced fractalkine expression is endothelial cell-specific. Pretreatment with mithramycin A (50 μg/kg/day for 3 days) dramatically suppressed TNF-α-induced fractalkine expression in arterial and capillary endothelial cells, endocardium, and cardiac valves (Figures 5 and 7).

Mithramycin A Suppressed TNF-α-Induced Fractalkine Expression in Small Intestinal Endothelial Cells

We further examined the effect of TNF-α on fractalkine expression in rat small intestine using immunohistochemistry. We observed endogenous expression of fractalkine mainly in arterial endothelial cells, but only slight or almost no expression of fractalkine in villous endothelial,

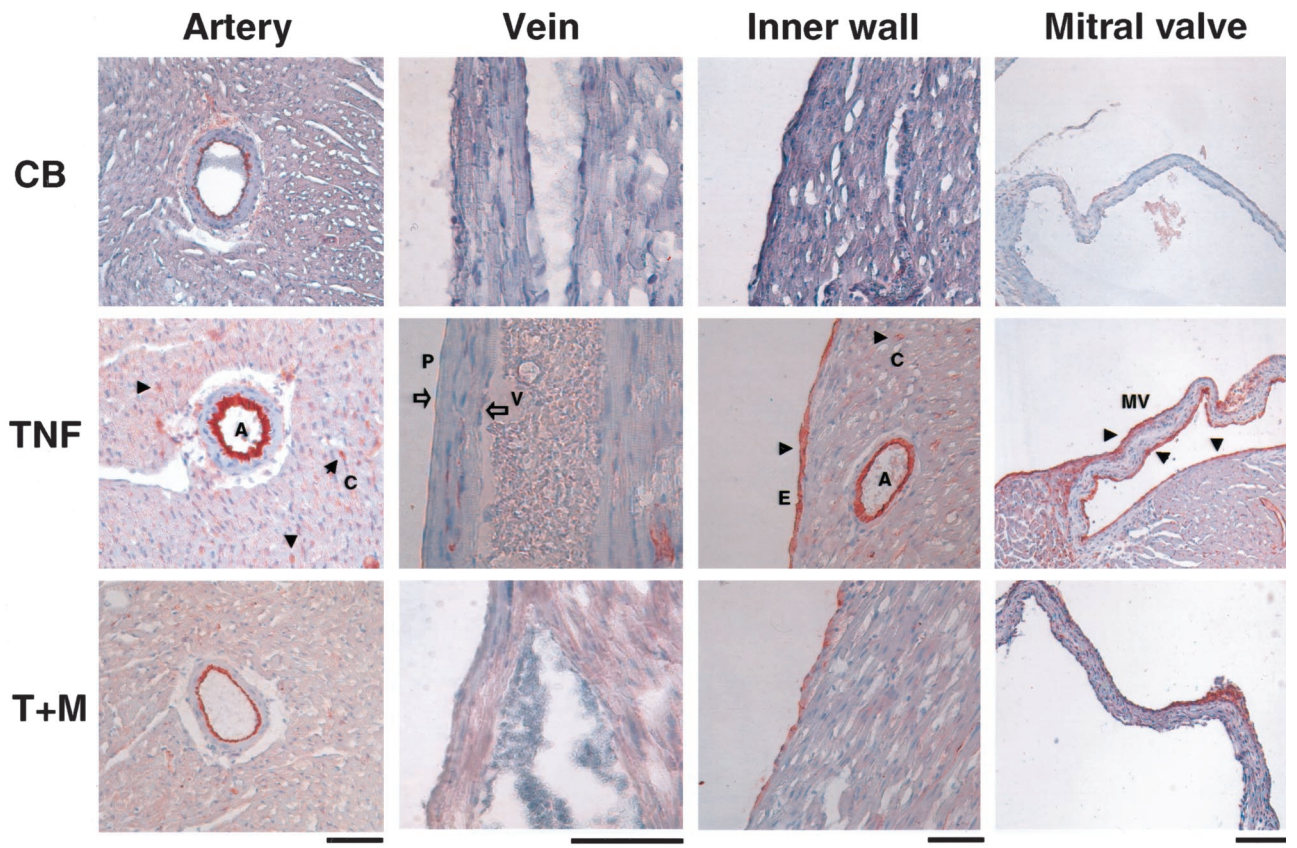

Figure 5. Immunohistochemical analyses of fractalkine expression in rat heart. Rats were given control buffer (CB), TNF-α (TNF, 10 μg/kg), or TNF-α plus mithramycin A (50 μg/kg/day for 3 days) (T+M). Note that there is endogenous expression of fractalkine in arterial endothelial cells. TNF-α increased fractalkine expression markedly in arterial and capillary endothelial cells, endocardium, endocardial surface of cardiac valves (**filled arrowheads**), but not in venous endothelial cells, myocardial cells, and pericardium (**arrows**). A, artery; V, vein; C, capillary; P, pericardium; E, endocardium; MV, mitral valve. Pretreatment with mithramycin A dramatically suppressed TNF-α-induced fractalkine expression. Results were similar in three independent experiments. Scale bars, 100 μm.

venous, and lymphatic endothelial cells, and epithelial cells (Figure 6). Intravenous injection of TNF-α (10 μg/kg) increased fractalkine expression markedly at 12 hours in arterial arteriolar endothelial cells and villous endothelium, slightly in venous endothelial cells, but not in lymphatic endothelial cells and epithelial cells (Figures 6 and 7). These data suggest that TNF-α-induced fractalkine expression is endothelial cell-specific. Pretreatment with mithramycin A (50 μg/kg/day for 3 days) dramatically suppressed TNF-α-induced fractalkine expression in arterial endothelial cells and villous endothelium (Figures 6 and 7).

Discussion

Fractalkine is an unusual chemokine because, in addition to a classic chemokine domain, it has a mucin-like stalk, a transmembrane domain, and a cytoplasmic tail allowing the molecule to be tethered to cells that express it, which include inflamed endothelial cells.[6,7] Even under conditions of physiological flow, fractalkine can mediate strong, direct, and rapid adherence of leukocytes expressing CX3CR1, which include most natural killer cells and $CD16^+$ monocytes as well as subpopulations of both $CD4^+$ and $CD8^+$ cytotoxic T lymphocytes.[8–11] Importantly, fractalkine-dependent adhesion of NK cells to endothelium has been shown to promote endothelial cell damage.[20] Thus, the important roles of fractalkine in endothelial inflammation and injury have been recently documented.[21] However, the signaling mechanisms by which inflammatory cytokines induce fractalkine mRNA expression are not well known. Our pharmacological assays revealed that TNF-α-stimulated expression of fractalkine occurs mainly through activation of NF-κB and PI 3′-kinase pathways, along with GC-rich DNA-binding protein-mediated transcription. The process seems to be independent of the MEK/ERK, PLC, and protein kinase C pathways. Therefore, inhibitors that suppress the activities in PI 3′-kinase, NF-κB, and GC-rich DNA-binding protein-mediated transcription could have the potential to suppress fractalkine expression and fractalkine-mediated vascular inflammation.

Notably, our results indicate that inhibitors of GC-rich DNA-binding protein-mediated transcription, mithramycin A and chromomycin A3, are strong inhibitors of TNF-α-induced fractalkine mRNA expression in the nanomolar range. Our EMSA suggests that this inhibition may possibly occur through suppression of the binding of GC-rich DNA binding proteins and NF-κB on the fractalkine gene promoter. Mithramycin A and chromomycin A3 are isolated from *Streptomyces griseus* and *Streptomyces plicatus*, respectively.[22] Structurally, they have the same chromophore with differences in the nature of the sugar

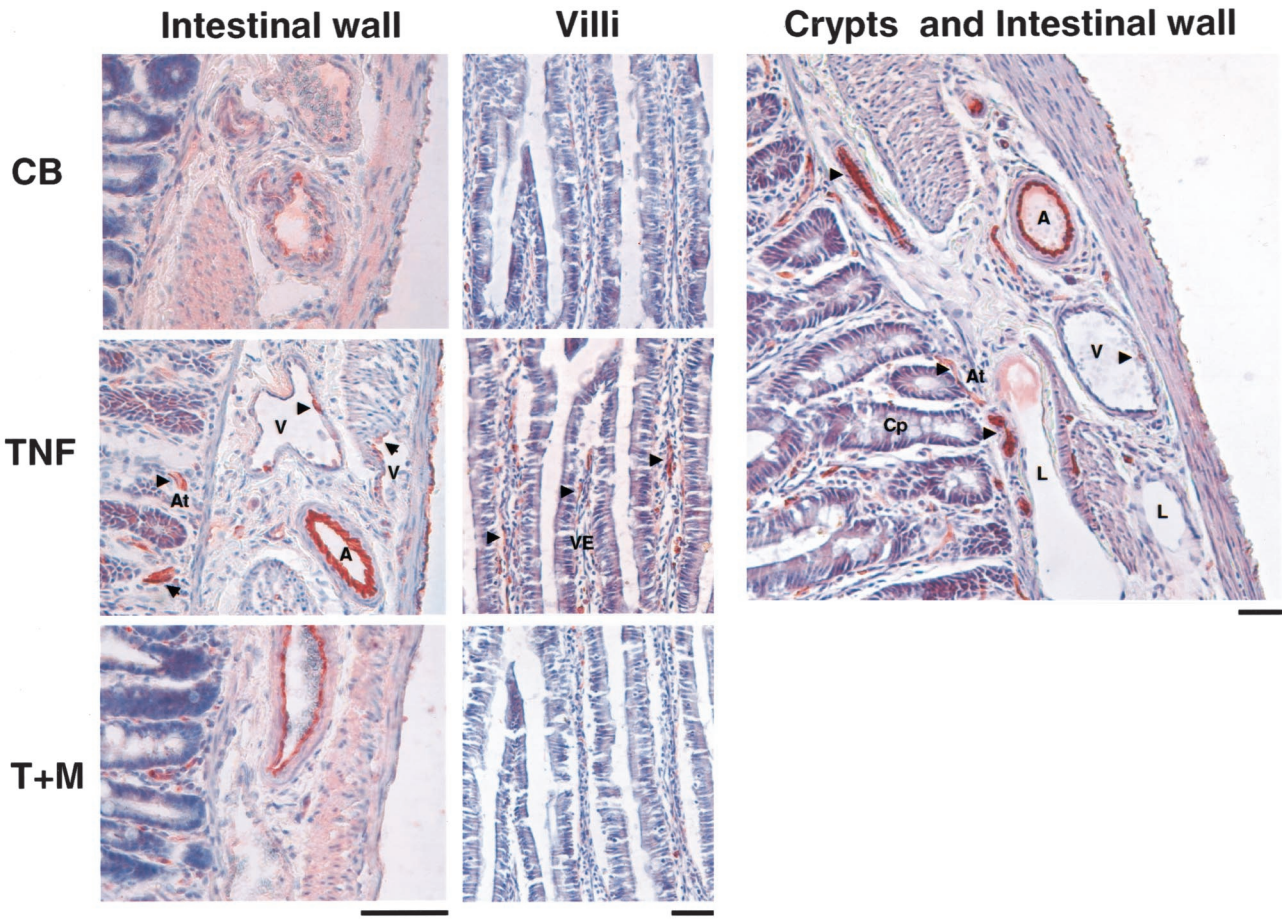

Figure 6. Immunohistochemical analyses of fractalkine expression in rat small intestine. Rats were given control buffer (CB), TNF-α (TNF, 10 μg/kg), or TNF-α plus mithramycin A (50 μg/kg/day for 3 days) (T+M). Note that there is endogenous expression of fractalkine in arterial endothelial cells. TNF-α increased fractalkine expression markedly in arterial, arteriolar, and villous endothelial cells, slightly in venous endothelial cells (**filled arrowheads**), but not in lymphatic endothelial cells or in cryptic or villous epithelial cells. Crypts and intestinal wall, TNF-α (TNF, 10 μg/kg)-treated sample. Note that red blood cells are present in artery and vein but not in lymphatics. A, artery; At, arteriole; V, vein; VE, villous endothelium, L, lymphatics; Cp, crypts. Pretreatment with mithramycin A dramatically suppressed TNF-α-induced fractalkine expression. Results were similar in three independent experiments. Scale bars, 100 μm.

residues connected on either side of the aglycone ring via *O*-glycosidic linkages.[23] They bind to double-stranded DNA at the minor groove with specificity for GC bp, thereby blocking the template activity of DNA during transcription by RNA polymerases.[24] Thus, mithramycin A and chromomycin A3 are known to bind GC-rich promoters, such as Sp1- and Egr-1-binding sites, and thus

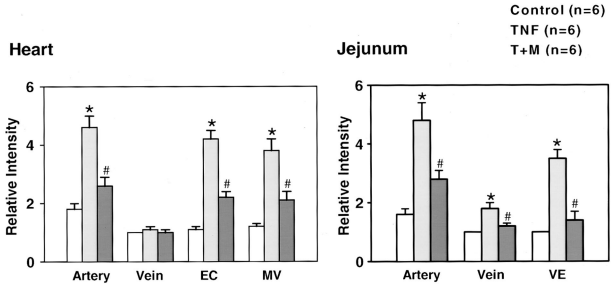

Figure 7. Semiquantitative analysis of fractalkine expression in substructures of heart and jejunum. The indicated portions of heart [artery, vein, endocardium (EC), and mitral valve (MV)] and jejunum [artery, vein, and villous endothelium (VE)] were immunostained. For each section, three to five endothelial portions of the tissue were graded on a scale from 1 (no staining) to 5 (very strong). Bars represent the mean ± SD from each of the group. *, $P < 0.05$ *versus* CB; #, $P < 0.05$ *versus* TNF-α (10 ng/ml) only.

inhibit their activation of gene transcription. Our EMSA indicated that mithramycin A suppressed not only Sp1 binding but also NF-κB binding to the DNA of TNF-α-stimulated endothelial cells. Therefore, it is possible that mithramycin A suppresses TNF-α-induced fractalkine mRNA expression through suppression of NF-κB and GC-rich DNA-binding proteins such as Sp1 and Egr-1. Indeed, direct inhibition of NF-κB and Sp1 through introduction of specific decoys suppressed TNF-α-induced fractalkine mRNA expression. Thus, fractalkine mRNA expression may be directly regulated by NF-κB and Sp1 in endothelial cells. Given that both Sp1 and Egr1 nuclear activator proteins are involved in vascular injury and inflammation,[25] mithramycin A could be useful for preventing vascular injury and inflammation. In the future, it will be important to determine how mithramycin A and chromomycin A3 suppress NF-κB activity in endothelial cells. Moreover, identification and characterization of the fractalkine promoter is warranted for clarifying the regulation of fractalkine expression.

Recruitment of circulating leukocytes to the arterial intima contributes to the formation of atherosclerotic lesions.[26] Leukocyte emigration from blood into tissues is

mediated by multiple adhesion molecules and chemokines that orchestrate specific steps of emigration and regulate preferential recruitment of different leukocytes, depending on the expression patterns of the corresponding chemokine receptors. A number of adhesion molecules (ie, VCAM-1, P-selectin, and ICAM-1), chemokines (ie, MCP-1/CCL2 and interleukin-8/CXCL8), and related chemokine receptors (ie, CCR2 and CXCR2), have been functionally implicated in atherosclerosis.[26] Our immunohistochemical analyses in heart and intestine revealed that fractalkine was expressed predominantly in arterial endothelial cells under normal conditions. However, TNF-α increased fractalkine expression predominantly in arterial and capillary endothelial cells, whereas little or no induction of fractalkine expression in venous and lymphatic endothelial cells was observed. Furthermore, TNF-α increased fractalkine expression markedly in endocardium of cardiac walls, the endocardial surfaces of cardiac valves, and the endothelium of intestinal villi. These data suggest that TNF-α-induced fractalkine expression is endothelial cell-specific. Considering the interaction between fractalkine-expressing endothelial cells and CX3CR1-expressing leukocytes *in vivo*, fractalkine must be involved in arterial inflammation rather than venous inflammation. In fact, recent reports indicated that fractalkine/CX3CR1 is closely involved in formation of arterial atherosclerosis.[27–29] Prominent expression of fractalkine in smooth muscle cells located in macrophage-rich areas of atherosclerotic plaques was observed in *apoE*$^{-/-}$ mice on a high-fat Western diet.[27] Moreover, CX3CR1−/− mice have less artheroma formation.[27] Polymorphisms of CX3CR1 at I249 and M280 are associated with decreased risk of coronary artery disease and atherosclerosis.[28,29] Atherosclerosis is an inflammatory disease.[27] Thus, fractalkine/CX3CR1 could be an adhesion molecule and receptor involved in arterial inflammation and formation of atherosclerosis.

Mithramycin A is known to inhibit tumors and bone resorption by cross-linking GC-rich DNA, thus blocking binding of Sp-family transcription factors to gene regulatory elements.[22–24] Thus, mithramycin A has been proposed as a therapeutic agent in severe neoplastic diseases such as chronic myelogenous leukemia, testicular cancer, and Paget's disease.[30–32] Our results revealed that pretreatment with mithramycin A dramatically suppressed TNF-α-induced fractalkine expression in endothelial cells. In addition, our preliminary results indicate that mithramycin A is also a strong inhibitor of inflammatory cytokine-induced VCAM-1 and ICAM-1 in endothelial cells. Thus, mithramycin A has the potential to be used as an anti-inflammatory drug by decreasing adhesiveness between cytokine-induced leukocytes and endothelial cells through suppression of adhesion molecule expression. Therefore, mithramycin A and its analogs warrant further evaluation as potential anti-inflammatory drugs.

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