Selective Regulation of ICAM-1 and RANTES Gene Expression after ICAM-1 Ligation on Human Renal Fibroblasts

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Abstract. Leukocyte infiltration of the cortico-interstitium is characteristic of many forms of progressive renal disease. The principal adhesion molecule expressed on resident interstitial cells and recognized by leukocytes is intercellular adhesion molecule–1 (ICAM-1). ICAM-1 is an inducible transmembrane receptor, which forms the counter-receptor for the leukocyte β2 integrins. ICAM-1–dependent binding induces the synthesis of the chemokine RANTES and of ICAM-1 itself. This study examines some of the signaling pathways involved in this induction. After ICAM-1 cross-linking on fibroblasts, the mRNA and protein for both RANTES and ICAM-1 were induced. This induction was calcium-dependent and inhibited by BAPTA-AM. The p38, ERK1, and ERK2 MAP kinases were activated in a [Ca2+]i-dependent manner, with a maximum phosphorylation at approximately 3 min after cross-linking. Through the use of selective inhibitors of p38 MAP kinase (SB203580) or MEKK (PD98059), p38 but not ERK activation was shown to be essential for the induction of ICAM-1. Neither was involved in RANTES activation, however. These mechanisms differed from those initiated by TNF-α, which were not [Ca2+]i-dependent. Electrophoretic mobility shift analysis demonstrated a time-dependent induction of both AP-1 and NF-κB binding activity in nuclear extracts, maximal at approximately 15 min after ICAM-1 cross-linking. Only AP-1 activation, however, was calcium-dependent, suggesting the central involvement of this transcription factor in ICAM-1 and RANTES induction after the ligation of ICAM-1. This study suggests an independent mechanism of inflammatory amplification, which may be characteristic of a persistent leukocytic involvement in areas of chronic inflammation rather than in cytokine-induced acute inflammation. steadmanr@cf.ac.uk

An influx of leukocytes, particularly macrophages and lymphocytes, into the glomerulus and subsequently into the cortical interstitium is characteristic of most forms of progressive renal disease (see reference 1 for review and references 2–11). The initial interaction of the infiltrating cells is with the endothelial lining of the vessels. After migration through the endothelium, however, the principal interaction of leukocytes is with cortical fibroblasts, which, under inflammatory conditions, have the phenotypic appearance of myofibroblasts (8,12–16). This interaction is mediated through the increased expression of intercellular adhesion molecule-1 (ICAM-1) (17–22) on the surface of the myofibroblasts and is central to the progression of inflammatory renal disease (2–11).

ICAM-1 is a heavily glycosylated, single-chain transmembrane receptor induced by inflammatory cytokines at sites of inflammation, where it forms the counter-receptor for the leukocyte β2 integrins (23–25). We have recently demonstrated, for the first time, that leukocyte adherence to primary cultures of human renal fibroblasts was a stimulus for ICAM-1 induction (26). This induction was initiated as a result of the ICAM-1/β2 integrin interaction and could be mimicked by cross-linking ICAM-1 with specific antibodies. Taken together with the observations that ICAM-1 cross-linking initiated the synthesis of vascular cell adhesion molecule-1 (26,27) and the chemokines IL-8 and RANTES (28), these observations suggest that cellular activation, through ICAM-1, may trigger a potent positive feedback mechanism for mediating the accumulation of leukocytes at sites of inflammation.

An immediate response to the cross-linking of ICAM-1 on fibroblasts or endothelial cells was the triggering of a rise in intracellular free calcium ([Ca2+]i) (26). This [Ca2+]i flux was an essential prerequisite for the de novo synthesis of the mRNA for ICAM-1 and for the increased expression of ICAM-1 protein on the cell surface. Chirathaworn et al. (29) described...
tyrosine phosphorylation of the cell cycle regulator cdc2, leading to transient inhibition of cdc2 kinase activity after ICAM-1 ligation. Holland and Owens (30) reported that cross-linking ICAM-1 on a B lymphoma line induced the activation of members of the src family of kinases along with an increase in the activity of Raf-1 kinase and the Mitogen-Activated Protein (MAP) kinases, ERK-1 and ERK-2. ERK activation has also been reported after ICAM-1 cross-linking on human umbilical vein endothelial cells (27). Others, however, have observed, in microvascular endothelial cells, activation of the small GTP-binding protein, rho associated with the phosphorylation of elements of the actin cytoskeleton but in the absence of MAP kinase activation (31,32). Furthermore, a separate study implicated p38 MAP kinase activation in the cytoskeletal rearrangement of microvascular endothelial cells after ICAM-1 cross-linking (33). There thus appears to be some cell-specific selectivity in the signaling pathways activated in response to ICAM-1 ligation, which may be indicative of different responses or levels of response to leukocyte adherence in different tissues.

Previous studies have identified the activation of the AP-1 transcription factor after ICAM-1 cross-linking (31,32). In contrast, although there are two potential sites for NF-κB binding in the promoter regions of both ICAM-1 and RANTES (34–36), activation of this transcription factor after ICAM-1 ligation has not been reported. Indeed, whereas AP-1 was activated by an ERK 1-dependent mechanism in endothelial cells, there was no concurrent activation of NF-κB (27). NF-κB is a transcription factor ubiquitously associated with the induction of a range of pro-inflammatory cytokines and adhesion molecules (37); therefore, this is an interesting finding that we have now examined in primary cultures of human renal fibroblasts. The present report demonstrates that binding activity for both NF-κB and AP-1 was induced by ICAM-1 cross-linking in these cells. Only AP-1 induction, however, appeared linked to the calcium-dependent activation pathways.

Cell Culture

The isolation and characterization of human renal cortical fibroblasts have been described previously (8,26). The cells were used between passages 2 and 7 after selection. Human lung fibroblasts (AG02262) at passages between two and four were purchased from N.I.A. Ageing Cell Repository, Corriel Institute, Camden, NJ. All cells were cultured in Dulbecco Modified Eagles Medium (DMEM) with 10% FCS, penicillin/streptomycin, 0.5 mg/ml insulin, 0.5 mg/ml transferrin, and supplemented with 10% FCS at 37°C in a humidified atmosphere of 5% CO2.

ICAM-1 Cross-Linking

Cells were grown to near confluence. The culture medium was then removed, and the cells incubated in serum-free medium for 72 h. This has been shown in previous studies to place the cells in synchronous, quiescent culture in which ICAM-1 is not expressed (8,26). To induce a reproducible, low level of ICAM-1 expression on the surface of the cells, they were then incubated in the presence of 1 × 10−12 M TNF-α for 24 h. This has been shown to induce the mRNA for ICAM-1 within 1 to 3 h. By 12 to 24 h, the mRNA for ICAM-1 was again undetectable. The protein levels for ICAM-1 on the cell surface, however, only became detectable at 4 h after cross-linking and reached a peak around 24 h. Using a picomolar concentration of TNF-α ensured that this protein expression was maximal, allowing the newly expressed receptor to be cross-linked and any subsequent effect on ICAM-1 mRNA to be examined. After TNF-α treatment, the cells were washed in warm PBS and then incubated with anti-CD54 monoclonal IgG (10 μg/ml) in serum-free culture medium for 1 h. After washing the cells with warm PBS, cross-linking was carried out using goat anti-mouse IgG (10 μg/ml; Sigma-Aldrich Co Ltd.) for the times indicated. Control conditions included cells incubated for 24 h in serum-free medium without TNF-α before the addition of anti-ICAM-1 antibody; cells incubated with irrelevant IgG of the same isotype in place of the anti-ICAM-1 antibody; and cells in which there was only the addition of secondary cross-linking antibody, without the addition of primary IgG. These controls were always negative for gene induction, as reported previously (26). The cells were then analyzed for the surface expression of ICAM-1 protein, lysed for RNA extraction, or extracted for the isolation and analysis of cytoplasmic and nuclear proteins. In certain experiments, to inhibit specific signal transduction pathways, SB203580 (p38 MAP kinase inhibitor) (38–41), PD98059 (MEK inhibitor) (42,43), and BAPTA-AM (a chelator of [Ca2+]i) (44,45) were added at the appropriate concentrations for 1 h before initiating cross-linking. All inhibitors were obtained from CN Biosciences, Nottingham, UK.

Surface Expression of ICAM-1

ICAM-1 expression on fibroblasts after ICAM-1 cross-linking or cytokine stimulation was assessed using a modified ELISA previously described (8,46). Growth-arrested fibroblasts were cultured in 96-well plates and cross-linked for defined times as described in the presence or absence of specific inhibitors at the appropriate concentrations. Cells were subsequently fixed in 1% glutaraldehyde for 5 min, washed in three changes of PBS, pH 7.3, and incubated with monoclonal anti-ICAM-1 antibodies (0.5 μg/ml) for 60 min at 37°C. After three washes, horseradish peroxidase-conjugated goat anti-mouse antibody (1:200, manufacturers recommended dilution) was added for 60 min at 37°C. The wells were again washed in three changes of PBS, pH 7.3, and substrate (2.2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid, 1 mg/ml in 0.15 M citrate-phosphate buffer, pH 4.0) was added.

Materials and Methods

Materials

All reagents were purchased from Sigma-Aldrich Co Ltd, Poole, Dorset, UK, unless otherwise stated. All cell culture reagents were purchased from Life Technologies/BRL Life Technologies Ltd, Paisley, UK, except fetal calf serum (FCS; Perbio Science NV/SA, Northumberland, UK).

Antibodies

Anti–ICAM-1 monoclonal RR1.1 was a kind gift of Dr. Robert Rothlein, Boehringer Ingelheim, NJ. Rabbit polyclonal anti-p38 (phosphorylation-state-independent) and rabbit anti-phospho-p38 (detects dual phosphorylation on Thr180 and Tyr 182) were from New England Biolabs, Hitchin, UK. Rabbit anti-phospho-ERK 1 and 2 (detects dual phosphorylation on Thr 183 and Tyr 185) was from Promega, Southampton, UK. Rabbit polyclonal anti-MAP kinase (ERK 1 and 2) (phosphorylation-state-independent) was from Sigma-Aldrich Co Ltd.
After 30 min, the optical density at 410 nm was determined in a Dynatech MR 5000/340 plate reader (Dynatech Laboratories Ltd.).

**Secretion of RANTES**

The concentration of RANTES in the medium of cells rested in serum-free medium, incubated with TNF-α or after ICAM-1 cross-linking for times up to 48 h, was measured by ELISA following the manufacturer’s protocol (R&D Systems).

**Chemotactic Analysis of Conditioned Medium**

Chemotaxis was performed in a 30-well plate apparatus (purchased from Guy Duremberg, Pasadena, CA) as described previously (47). Conditioned medium or recombinant RANTES in serum-free medium were added to the lower well of the plate and a Nucleopore, 12-mm diameter polycarbonate filter (3.0-μm pore size; Costar Corporation, Cambridge, MA) that had previously been treated for 1 h at 37°C in serum-free medium was lowered over the well. The upper section of the plate was then assembled, and a suspension of mononuclear cells prepared as described previously from freshly obtained whole blood (48) was added to the upper well. The plate was incubated at 37°C for 2 h, and the filters removed and fixed in 3.5% paraformaldehyde. Cells that had passed through the filters were aspirated from the lower wells and also fixed in 3.5% paraformaldehyde either in suspension or after Cytospin centrifugation onto glass slides. The morphology of cells that had traversed the filters was examined in five high power fields (hpf) (>400) for each of duplicate well. The expression of CD11a (measured by FACS analysis or immunohistochemistry) was used to exclude granulocytes. Chemotactic migration specifically due to RANTES was confirmed by addition of recombinant RANTES to the medium in the upper wells to abolish the chemotactic gradient. Each sample was assayed in quadruplicate, and the numbers of mononuclear cells migrating were calculated as the mean ± SD for each treatment.

**Preparation of Cell Lysates for SDS-PAGE and Western Blotting**

At the appropriate times following cross-linking, ice cold PBS was added to the cells. The cell monolayer was removed by scraping, pelleted, (500 x g for 10 min) and the pellet lysed by incubation in lysis buffer (50 mM Tris/HCl, pH 7.5, containing 0.1% SDS 150 mM NaCl, 1.0% Triton X-100, 1% NP40, 1mg/ml leupeptin, 1mg/ml pepstatin, 1mg/ml aprotinin, 0.5% deoxycholate, 1 mM PMSF, 1 mM Na3VO4, 50 mM NaF, and 2 mM EDTA), on ice for 1 hr. Cellular debris was removed by centrifugation (15 min at 10,000 x g) and the protein content of the cell lysate quantified using the Detergent Compatible protein assay (BioRad Laboratories Ltd, Hemel Hempstead, UK).

**Preparation of Nuclear Extracts**

Nuclear extracts were prepared by previously published methods (49). Briefly, at the appropriate times following cross-linking, cells were washed in 20 ml of ice cold PBS and pelleted for 5 min at 500 x g. The pellet was re-suspended and washed in 1 ml of hypotonic buffer (Buffer A) pH 7.9, containing 10 mM HEPES, 1.5 mM MgCl2, 10 mM KCl, 50 μM dithiothreitol (DTT), 100 μM phenanthroline, 1 μg/ml pepstatin, 100 μM EDTA, 10 mM NaF, 100 μM sodium orthovanadate, and 25 mM β-glycerophosphate. Cells were then lysed in 100 μl of Buffer A, containing 0.2% (vol/vol) NP40, and incubated at 4°C for 10 min. Lysates were cleared by centrifugation at 10,000 x g for 10 min and the supernatants decanted, snap frozen, and stored at −70°C. The pellet obtained contained a crude nuclear fraction and was resuspended in 100 μl of nuclear lysis buffer (20mM Hepes pH 7.9, 420 mM NaCl, 1.5 mM MgCl2, 0.2mM EDTA, 25% vol/vol glycerol, and 100 μM DTT). Lysed nuclei were incubated at 4°C for 15 min, vortexed and pelleted at 10,000 x g for 10 min. The supernatants containing soluble nuclear proteins were collected as nuclear extracts, snap frozen and stored at −70°C.

**RNA Isolation**

Total cellular RNA was extracted from fibroblast monolayers grown in single wells of 6 well plates (Falcon, Becton Dickinson Ltd, Oxford, UK) (26). Initially cells were washed with serum free medium, and then lysed with 1 ml of RNA Isolator (Sigma-Genosys, Cambridge, UK). One tenth volume of chloroform:isoamyl alcohol (24:1) was added and the mixture shaken for 30 s. Samples were incubated for 5 min at 4°C to allow the aqueous and phenol phases to separate. Following centrifugation at 11,000 x g the aqueous phase was mixed with an equal volume of isopropanol and RNA was precipitated at −20°C for 24 h before reverse transcription.

**Reverse Transcription**

Precipitated RNA was pelleted at 11000 x g and washed twice with 500 μl of 70% ethanol. The RNA pellet was dried and then resuspended in 10 μl of sterile DNase/Rnase-free water. The concentration of RNA in each sample was calculated from the absorbance at 260 nm.

The purity of RNA was assessed by flat bed electrophoresis at 70 V through a 3% agarose gel (BRL, Ultrapure, electrophoresis grade Life Technologies/BRL Life Technologies Ltd). Reverse transcription used one microgram of RNA added to 1 μl of random hexamers (hexadeoxyribonucleotides, pd[N]6, 100 μmol, Pharmacia Biosystems Ltd, Milton Keynes, UK), 5 μl of NTP (mixed nucleotides: dATP, dCTP, dGTP, dTTP, 2500 nmol: Life Technologies/BRL (Life Technologies Ltd)), 2 μl 10X PCR buffer (containing 1 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM MgCl2 and 0.001% w/v gelatin) and 2 μl Dithiothreitol (0.1 M DTT). This solution was heated to 95°C for 5 min, followed by cooling to 4°C for 2 min. One microliter of RNAsin (ribonuclease inhibitor: Promega Ltd, Southampton, UK) and 1 μl of M-MLV Superscript (Rnase H- reverse transcriptase: Life Technologies/BRL (Life Technologies Ltd)) were added to each reaction tube and vortexed. After vortexing the samples were incubated for 10 min at room temperature (20°C), 42°C for 40 min and then 95°C for 5 min. Negative controls for reverse transcriptase (-RT) were included. The cDNA library transcribed was then stored at −20°C until analysis by PCR.

**PCR**

PCR was carried out in 50 μl final volume of 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 200 μM dNTP, 0.001% gelatin, 0.25 μmol of each primer forward and reverse and 1.25 U AmpliTaq DNA polymerase (Applied Biosystems Ltd, Warrington, UK). Mineral oil (50 μl) was added to each tube and thermal cycling was carried out in a Perkin Elmer Euteron DNA thermal cycler 480. Primers were designed from the published cDNA sequences and were synthesized by Sigma-Genosys Ltd, Cambridge, UK (Table 1). Two microliters of cDNA product was PCR- amplified in 50 μl final volume of 10 mM Tris, pH 8.3, 50 mM KCL, 1.5 mM MgCl2, 200 μM dNTP, 0.001% gelatin, 0.5 μM of the primer pairs and 0.125 U of DNA polymerase (PE Applied Biosystems, Warrington, UK) for 26 cycles in a Perkin Elmer 480 thermal cycler. Negative control comprised of -RT and water substituted -PCR also underwent the
reaction. Amplification of cDNA was performed using the following thermal cycles:

Heating to 72°C for ten minutes was followed by 94°C for 3 min, 55°C for 1 min and 72°C for 1 min. The amplification cycles were carried out at 94°C for 40 s, 55°C for 1 min, 72°C for 1 min, 26 cycles (for β-actin), 30 cycles (for ICAM-1 and RANTES) and finally 1 cycle of 94°C for 40 s followed by 60°C for 10 min. PCR products were separated by electrophoresis in 3% agarose gels. Controls with water substituting for either mRNA in the RT reaction or cDNA in the PCR reaction were also included. cDNA was visualized under ultraviolet light and each of the bands was compared by densitometry (BioRad GS 670) to that of the corresponding β-actin (used as the housekeeping gene). The results were expressed as ratios normalized to those of β-actin.

**Northern Blot Analysis**

Total RNA (up to 10 μg) was run on a denaturing agarose gel and transferred by vacuum blotting onto a Hybond nylon membrane (Amersham, U.K.). mRNA for ICAM-1, RANTES or the actin housekeeping gene was detected by hybridization with a 32P internally-labeled cDNA probe synthesized by DNA random hexanucleotide priming. Signal intensity was detected on high performance autoradiography film (Hyperfilm, Amersham) before stripping the probe from the blot in boiling 0.1% (w/v) SDS for rehybridisation.

**Western Blot Analysis**

Cell extracts (each containing 100 μg protein) were analyzed by SDS-polyacrylamide gel electrophoresis under non-reducing conditions, followed by Western blotting. Proteins were transferred to nitrocellulose and membranes were blocked for 1 hr in 5% powdered milk, 0.5% Tween-20 in PBS (blocking buffer). After washing (0.5% Tween-20 in PBS), membranes were incubated in primary antibody overnight at 4°C. Proteins to which antibody bound were visualized using enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK). Negative control incubations had primary antibody replaced by normal mouse serum at the same g protein) were analyzed by electrophoretic mobility shift assay (EMSA).

**Electrophoretic Mobility Shift Assay (EMSA)**

Transcription factor activity in the nuclear extracts of control and activated cells was examined using consensus sequence oligonucleotides for AP-1 and NFκB (Promega, Southampton, UK). The corresponding mutant oligonucleotides for these consensus sites were purchased from Santa Cruz Biotechnologies, Heidelberg, Germany (Table 2).

Each probe was labeled with [γ32P]-ATP by incubation with 10 units of T4 polynucleotide kinase (New England Biolabs) for 15 min at room temperature and the binding reaction carried out on each nuclear extract as described previously (49). After electrophoresis in 5% polyacrylamide gels, the gels were dried and autoradiographed.

**Statistical Analyses**

Results were expressed as mean ± SD and statistically significant differences were evaluated using the Mann-Whitney U test, with \( P < 0.05 \) considered significant.

**Results**

The Induction of ICAM-1 and RANTES after ICAM-1 Cross-Linking Is Transcriptionally Regulated and \([Ca^{2+}]_i\)-Dependent

Analysis by RT PCR (Fig 1A) or Northern blotting (Fig 1B) of the levels of ICAM-1 and RANTES mRNA, normalized to those of the actin housekeeping gene, showed that there was a rapid increase following cross-linking, which was maximal at 1 to 2 h and which was maintained up to 12 h. This increase was inhibited by chelation of \([Ca^{2+}]_i\) with BAPTA-AM (Fig 2A). Protein expression was maximal for both proteins at 24 to 48 h following cross-linking and was also inhibited by incubation with BAPTA-AM. In the case of RANTES this inhibition reached 72%, while that for ICAM-1 was 100% (Fig 2B). In contrast, despite a similar time-dependency, the synthesis of both proteins induced by TNFα was independent of a flux in \([Ca^{2+}]_i\) (Fig 3).

We have previously established that the newly expressed ICAM-1-induced ICAM-1 protein is functional (26). A modified chemotactic assay was used to demonstrate functionally active RANTES induction. Using three preparations of mononuclear cells each from a separate donor, approximately 45% of the monocyte chemotactic activity in the medium of cells, 24 h after cross-linking, was due to RANTES (Table 3). To confirm that the cross-linking-induced up-regulation of both genes of interest occurred at the transcriptional level, cells were pre-treated with actinomycin D and the cross-linking was carried out for 24 h in the presence of actinomycin D. There was a dose-dependent inhibition of the peak expression of both ICAM-1 and RANTES, first evident at between 50 and 100 ng/ml and which became significantly different to control at 150 ng/ml for ICAM-1 and 200 ng/ml for RANTES (Fig 4).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Product Size</th>
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<tbody>
<tr>
<td>ICAM-1</td>
<td>5'-TCAGAGGTTGAAGACTGG-3'</td>
</tr>
<tr>
<td>RANTES</td>
<td>5'-AGCCTGCTGCTATCCTAT-3'</td>
</tr>
<tr>
<td>β-ACTIN</td>
<td>5'-GGAGCCATGATCTGGATCTT-3'</td>
</tr>
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</table>

Table 1. PCR primers
of the intracellular levels of ATP demonstrated that there was no toxic effect on the cells at any of the concentrations of actinomycin D used (not shown).

ICAM-1 Cross-Linking Activates MAP Kinases in a 
$[\text{Ca}^{2+}]_{i}$-Dependent Manner

Previous studies have reported that MAP kinase activation is involved in the ICAM-1 signaling cascade. The specific kinases activated, however, depend on cell type. Initially cell extracts were analyzed by western blotting using antibodies specific for the phosphorylated forms of p38, ERK 1 and ERK 2. There was a time-dependent increase in the levels of phospho-p38 in the extracts, which was maximal at 1 to 3 min (Fig 5A). The blots were then stripped and re-probed with antibody recognizing both the non-phosphorylated and phosphorylated forms of the kinase (Total p38). Re-probing the blots demonstrated that there was no increase in the overall levels of p38 protein (Fig 5B). This relative increase in the ratio of phospho-p38 to Total-p38 was inhibited by BAPTA-AM (Fig 5C and 5D) at the same concentrations which had inhibited ICAM-1 mRNA induction. Furthermore, using the same blots, we observed similar changes in the levels of phospho-ERK 1 and 2 (Fig 6A) without any change in the levels of total ERK protein (Fig 6B). This increase in phosphorylation was also inhibited in the presence of BAPTA-AM.
The presence of BAPTA-AM (Fig 6C and D). These results suggest that there was a causal link between the changes in $[\text{Ca}^{2+}]_i$ and the activation of the MAP kinases.

**p38 MAP Kinase Activation Is Involved in ICAM-1 but not RANTES Upregulation after ICAM-1 Cross-Linking**

The increase in ICAM-1 mRNA levels seen at 3 h following ICAM-1 cross-linking was inhibited by the p38 inhibitor SB203580 (Fig 7), at a concentration, which maximally inhibits p38 MAP kinase activity without affecting other kinases (50). There was, however, no effect of the MEKK inhibitor, PD 98059 up to a maximum concentration of 50 $\mu$M (a concentration 25 times its IC$_{50}$). This suggests that it is specifically the p38 MAP kinase that is linked to the induction of ICAM-1. In contrast, neither inhibitor affected the upregulation of RANTES mRNA, suggesting a separate distinct pathway of $[\text{Ca}^{2+}]_i$-dependent RANTES induction.

**NF-κB and AP-1 Binding Activity Induced by ICAM-1 Cross-Linking**

Analysis of the promoter regions of the ICAM-1 and RANTES genes has identified several potential sites for AP-1 and NFκB binding. We have therefore investigated whether ICAM-1 cross-linking triggered any changes in the binding activity for these two transcription factors. EMSA using probes with consensus binding sequences for AP-1 and NFκB was used to analyze nuclear extracts from fibroblasts at defined times following cross-linking. There was a time-dependent increase in specific binding activity for both NFκB and AP-1, when compared to that of mutant probe, which was maximal at 15 min following cross-linking (Fig 8).

To test whether the induction of binding activity initiated by ICAM-1 cross-linking was $[\text{Ca}^{2+}]_i$ and p38-dependent we incubated cells with BAPTA-AM or SB203580 during cross-linking as above. Nuclear extracts of the cells were then analyzed by EMSA. Both BAPTA-AM and SB203580 induced a significant inhibition of the maximal AP-1 binding activity induced by ICAM-1 cross-linking (Fig 9). This was not accompanied, however, by an inhibition of NFκB binding activity.

**Discussion**

The transcription of several genes is triggered as a result of ICAM-1 ligation. These include intercellular adhesion molecules such as VCAM-1 and ICAM-1 itself and pro-inflammatory cytokines such as RANTES and IL-8. The ligation of ICAM-1 therefore represents an important event with the potential for greatly amplifying inflammation (15,51). Furthermore, in a recent study, signaling through ICAM-1 was shown to provide a co-stimulatory signal with that of CD3 leading to T cell proliferation and the induction of Th1 but not Th2 cytokines (52). In the present study, the pathways induced by binding to ICAM-1 were examined in detail to better understand the intracellular mechanisms involved in this response.

Our previous work has identified the triggering of an increase in $[\text{Ca}^{2+}]_i$, due to an influx from the extracellular environment, as an immediate response to ICAM-1 ligation (26). This increase was essential for the initiation of ICAM-1 transcription, which was blocked by an intracellular chelator of $[\text{Ca}^{2+}]_i$. The present study confirmed that both the mRNA and protein for ICAM-1 and RANTES were induced by ICAM-1 cross-linking and that this was transcriptionally regulated in a manner sensitive to actinomycin D. We previously demonstrated that the newly-induced ICAM-1 protein was functional (26); we now show that the RANTES induced is chemotactically active. The results generated, however, suggest that RANTES only makes up approximately 45% of the chemotactic activity for monocytes generated following ICAM-1 liga-
tion. The identification of the other molecules involved is currently being investigated.

ERK 1 and 2 and p38 MAP kinase underwent phosphorylation in response to ICAM-1 cross-linking. The phosphorylation of each of these kinases was blocked by the addition of BAPTA-AM, suggesting that one or all of them may be involved in the signal transduction pathway leading to ICAM-1 and RANTES induction. However, incubating cells with the p38 MAP kinase inhibitor, SB203580 or the MEK inhibitor, PD98059 demonstrated that there were two distinct pathways activated. One pathway, leading to the transcription of ICAM-1, was dependent on p38 but not ERK activation, while the other was not inhibited by either SB203580 or PD98059. These findings suggest that a [Ca^{2+}]_r-dependent p38 activation pathway is triggered by ICAM-1 ligation which leads to the induction of ICAM-1 transcription but that a separate [Ca^{2+}]_r-dependent pathway leads to RANTES transcription. The demonstration of the importance of [Ca^{2+}]_r, in triggering these signaling pathways, extends the findings of a recent study investigating ICAM-1 signaling in astrocytes, in which p38 MAP kinase was activated and led to the induction of IL-6 (53).

Other studies examining ICAM-1 activation have identified the involvement of a variety of downstream signaling molecules. ERK 1 and 2, for example, were activated in human umbilical vein endothelial cells and in these cells this was directly related to the induction of IL-8 and RANTES (28). In contrast, there was no ERK activation detected following ICAM-1 cross-linking in endothelial cells isolated from the brain microvasculature (31). Others have reported that cross-linking ICAM-1 on cells of the B lymphoma line A20 induced a number of cellular responses (30). These included the activation of members of the Src family of kinases, and an increase in the kinase activity of Raf-1. Raf-1 is a serine-threonine kinase, which phosphorylates MEK and thus initiates ERK 1 and 2 activation. In these cells there was, therefore, also ERK-1 and ERK-2 activation. In our cells, however, while there was ERK activation, it was not linked to the transcription of ICAM-1 or RANTES.

The promoter region of ICAM-1 contains several potential binding sites for a variety of transcription factors (36). Among these are four putative AP-1 sites and two potential sites for NFκB binding. Previous work has demonstrated that ICAM-1 cross-linking on the surface of E11 human synovial cells induced the transcription of IL-1β (32). Analysis of the upstream 5'-untranslated region of the IL-1 β gene demonstrated that an AP-1 (Jun/Fos) complex was essential for this response. A further study using HUVEC also demonstrated activation of ERK 1 leading to the induction of AP-1 activity (27). There was no detectable change, however, in NFκB activity. In the present study of primary human fibroblasts, using consensus sequences for the AP-1 and NFκB binding sites, nuclear binding activity for both transcription factors was found to be increased following ICAM-1 cross-linking. Interestingly, however, only the binding activity of AP-1 was reduced by chelating [Ca^{2+}]_r, or blocking p38 activation. This finding supports the observations of Lawson et al., that, since there was no JNK-1 activity detected following cross-linking in their study (27), the p38 pathway was that by which AP-1 was activated. We now further demonstrate that this occurs through a [Ca^{2+}]_r-dependent mechanism. The activation of AP-1 transcriptional activity by p38 MAP kinase has also been demonstrated previously in response to co-stimulation of CD3 and CD28 in T lymphocytes (54). A role for the mediation of this response by changes in [Ca^{2+}]_r, however, was not addressed in this study. Furthermore, p38 phosphorylation is a controlling factor in the induction of binding activity for a number of transcription factors with potential binding sites in the ICAM-1 promoter, such as SP-1 (55), STAT (56) or Ets (57). Thus there may be additional transcription factor activity also induced by ICAM-1 cross-linking which will affect the transcription of other as yet unidentified genes.

Although there was induction of NFκB binding activity following ICAM-1 cross-linking, this was not necessary for ICAM-1 or RANTES induction, since it was not inhibited by either BAPTA-AM or SB203580. This was similar to the TNFα-induced upregulation of NFκB (not shown (27,51)). The cells therefore, had the mechanisms intact for the activation of this transcription factor but its activation was not essential for the observed effect on ICAM-1 and RANTES.

The results presented herein were generated using monoclonal antibody cross-linking to mimic the interaction of ICAM-1

### Table 3. Results of modified chemotactic assay

<table>
<thead>
<tr>
<th></th>
<th>Medium from Control Cells</th>
<th>Medium from ICAM-1 Cross-Linked Cells</th>
<th>Medium + RANTES (1 μg/ml)</th>
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</thead>
<tbody>
<tr>
<td>Cells migrated</td>
<td>115 ± 27</td>
<td>12998 ± 987</td>
<td>10254 ± 659</td>
</tr>
<tr>
<td>Cells migrated + RANTES</td>
<td>109 ± 31</td>
<td>7149 ± 639</td>
<td>1082 ± 231</td>
</tr>
<tr>
<td>Percentage RANTES-Specific</td>
<td>N/A</td>
<td>45 ± 8%</td>
<td>91.7 ± 12%</td>
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a Peripheral blood mononuclear cells were added to the upper chamber of the chemotaxis plate as described in Materials and Methods. Chemotactic activity in serum-free conditioned medium from cells to which irrelevant antibody had been added.

b in the medium from cells on which ICAM-1 had been cross-linked.

c or in serum-free medium to which recombinant RANTES (1 μg/ml) had been added.

d was then assayed over a period of 2 h. Parallel migration assays were carried out in the presence of excess RANTES in the upper well to abolish the RANTES chemotactic gradient.

f Results are presented as mean ± SD for three separate experiments.
with its physiologic receptors on the surface of leukocytes. We have previously shown that the adherence of neutrophils to fibroblasts or to endothelial cells induced a rapid increase in \([\text{Ca}^{2+}]_i\). This increase was blocked by antibodies to the leukocyte \(\beta_2\) integrins and was mimicked by cross-linking ICAM-1 on the fibroblast or endothelial cell surface. Cross-linking also induced the expression of ICAM-1 and VCAM-1 proteins, which were functional and led to the increased binding of both neutrophils and mononuclear cells (26). To permit the optimal response to cross-linking, however, a minimal expression of ICAM-1 must be induced on the surface of the target cell. Others have incubated the cells in medium containing low concentrations of serum to maximize the expression of ICAM-1 while minimizing activation of the cell (27,51). To ensure that cells were synchronous in terms of their cell cycle and to co-ordinate the subsequent low-level expression of ICAM-1 on each cell, fibroblasts in the current study were made quiescent in serum-free culture for 72 h, before treatment with a low concentration of TNF\(\alpha\) for a further 24 h. This dose induced ICAM-1 mRNA (peaking at 1 to 3 h) (26) and the sub-maximal expression of ICAM-1 protein on the cell surface at 18 to 24 h. This period of sub-maximal pro-inflammatory cytokine treatment was designed to mirror the \textit{in vivo} situation in that the release of cytokines such as IL-1\(\beta\) and TNF\(\alpha\) is the event mediating the initial up-regulation of chemokine and adhesion molecule synthesis at inflammatory sites. This pre-treatment also induced NF\(\kappa\)B and AP-1 transcription factor binding activity, which had decreased after 24 h but which was still present at low levels in the cells at the time of cross-

Figure 4. The increase in ICAM-1 and RANTES expression induced by ICAM-1 cross-linking was regulated at the level of transcription. Growth arrested fibroblasts were stimulated for 24 hours with TNF\(\alpha\) (10\(^{-12}\)M). They were then incubated with anti-ICAM-1 antibody in the presence of actinomycin D (10–500 ng/ml). Cross-linking antibody with actinomycin D at the same concentration was added after washing the cells and the incubation continued for 24 hours. ICAM-1 protein expression on the surface and RANTES in the conditioned medium were then measured as described. The results are presented as mean ± SD for three independent experiments. (Significance of inhibition: * = \(P < 0.05\), ** = \(P < 0.01\)).

Figure 5. ICAM-1 cross-linking-induced p38 MAP kinase activation was inhibited by the chelation of [Ca\(^{2+}\)]. Growth arrested fibroblasts were stimulated for 24 hours with TNF\(\alpha\) (10\(^{-12}\)M), prior to incubation with anti-ICAM-1 antibody in the absence (A and B) or presence (C and D) of BAPTA-AM (2\(\mu\)M) for 1 hour. Cross-linking antibody with or without the inhibitor was then added and incubated with the cells for up to 10 minutes. Following this incubation the cells were lysed and the soluble cytosolic proteins isolated. Fifty micrograms of protein were loaded into each well and were then analyzed by SDS-PAGE, followed by immunoblotting with anti-phospho-p38 antibody (A and C). Antibody binding was visualized by enhanced chemiluminescence. Subsequently the membranes were stripped of bound antibody (manufacturer’s recommended protocol), and were then re-probed with a total-p38 antibody (B and D). The results shown are representative of those obtained in 3 separate experiments.
linking. Interestingly, our own studies and those of others have shown that, although cytokine treatment and ICAM-1 cross-linking both trigger the induction of chemokines and adhesion molecules, they do so through different signaling pathways (26,27). There thus exist separate mechanisms controlling the acute and the chronic phases of inflammation.

At sites of infection and inflammation, leukocyte interaction with ICAM-1 through β2 integrin ligation is essential for infiltration of the site and resolution of injury. Indeed those individuals in which there is a genetic deficiency in β2 integrin expression and function suffer repeated bacterial infections, which are not cleared and are often fatal [reviewed in (58)]. Our previous studies and the present report demonstrate that leukocyte binding to fibroblasts and endothelial cells through the β2 integrin/ICAM-1 interaction, is mimicked by antibody cross-linking of ICAM-1. We now show that this interaction induced p38 MAP kinase activity leading to the activation of the AP-1 transcription factor and that this was calcium-dependent. In addition, there was activation of NFκB although the mechanism of induction was not examined. This differs from a report describing the induction of VCAM-1 in endothelial cells, following ICAM-1 cross-linking (27). In these cells there was no NFκB activation and AP-1 activation was through the ERK-1 and ERK-2 pathways. Thus, there appear to be cell-specific differences in the pathways and responses triggered by ICAM-1 ligation that may be important in understanding the cytokine-independent amplification of inflammation. Our results suggest that the mechanisms in the renal cortical interstitium may differ to those in the endothelium and this would provide potentially important therapeutic options for selectively affecting the course of persistent inflammation.

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References


