

# Expression of Functional ICAM-1 on Cultured Human Keratocytes Induced by Tumor Necrosis Factor- $\alpha$

Naoki Kumagai\*, Ken Fukuda\*<sup>,†</sup>, Youichiro Fujitsu\* and Teruo Nishida\*

*Departments of \*Biomolecular Recognition and Ophthalmology; <sup>†</sup>Ocular Pathophysiology, Yamaguchi University School of Medicine, Ube City, Yamaguchi, Japan*

**Purpose:** Leukocytes such as neutrophils contribute to the pathogenesis of corneal ulcer. The effect of the proinflammatory cytokine tumor necrosis factor (TNF)- $\alpha$  on the expression of intercellular adhesion molecule (ICAM)-1 by cultured human keratocytes was investigated because the interaction of leukocytes with ICAM-1 expressed on the surface of structural cells mediates leukocyte infiltration into tissue at sites of inflammation.

**Methods:** Cultured human keratocytes were incubated with various concentrations of TNF- $\alpha$ . The surface expression of ICAM-1 was evaluated by whole-cell enzyme-linked immunosorbent assay, flow cytometry, and immunohistochemistry. The abundance of ICAM-1 mRNA in cell lysate was determined by quantitative reverse transcription and polymerase chain reaction analysis. Adhesion of neutrophils to corneal fibroblasts was assayed by measuring the fluorescence of Calcein-AM-labeled neutrophils.

**Results:** Incubation of keratocytes with TNF- $\alpha$  induced increased expression of ICAM-1 on the surface of keratocytes in a dose- and time-dependent manner. The abundance of ICAM-1 mRNA in keratocytes was increased by the incubation of cells with TNF- $\alpha$ . Exposure of keratocytes to TNF- $\alpha$  increased the adherence of human neutrophils to these cells.

**Conclusions:** Stimulation of keratocytes with TNF- $\alpha$  resulted in an increase in the abundance of ICAM-1 mRNA, the cell surface expression of ICAM-1 protein, and enhanced adhesion of neutrophils to these cells. The expression of ICAM-1 on human keratocytes may thus contribute to leukocyte infiltration into the corneal stroma of individuals with inflammatory ocular diseases. **Jpn J Ophthalmol 2003;47:134–141** © 2003 Japanese Ophthalmological Society

**Key Words:** Human eye, intercellular adhesion molecule-1, keratocyte, neutrophil, tumor necrosis factor- $\alpha$ .

## Introduction

Neutrophils and other leukocytes play important roles in the pathogenesis of corneal ulcer, regardless of whether this condition is infectious or noninfectious in nature. We recently showed that keratocytes secrete chemokines that might facilitate the chemotaxis of leukocytes to the corneal stroma.<sup>1</sup> At sites of inflammation, the interaction of leukocytes with resident fibroblasts, vascular endothelial cells, or epithe-

lial cells promotes the activation and transmigration of these white blood cells.<sup>2</sup> Intercellular adhesion molecule (ICAM)-1, a cell surface glycoprotein of 76 to 114 kDa and a member of the immunoglobulin superfamily of proteins,<sup>2</sup> contributes to the local infiltration of leukocytes in immune responses by mediating the adhesion and activation of these cells through interaction with the  $\beta$ 2 integrin chain expressed on their surface.<sup>3</sup> In vascular tissue, the expression of ICAM-1 on the surface of endothelial cells facilitates the transmigration and local infiltration of leukocytes.<sup>3</sup> In the cornea, which is an avascular tissue, the infiltration and activation of leukocytes are thought to be regulated by the resident cells of the corneal stroma, the keratocytes.

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Correspondence and reprint requests to: Naoki KUMAGAI, MD, PhD, Department of Ophthalmology, Yamaguchi University School of Medicine, 1-1-1 Minami-Kogushi, Ube City, Yamaguchi 755-8505, Japan

In addition to their effects on leukocytes, cytokines modulate the functions of structural cells. The cytokine tumor necrosis factor (TNF)- $\alpha$  plays an important role in the pathogenesis of both infectious and noninfectious inflammatory disorders.<sup>4</sup> Culture of the entire human cornea in the presence of TNF- $\alpha$  induces the expression of ICAM-1 immunoreactivity in keratocytes.<sup>5</sup> However, the characteristics and mechanism of this effect, including its time course and dose-response relation, whether it represents a direct action of TNF- $\alpha$  on keratocytes, whether it is mediated at the transcriptional or translational level, and whether the newly expressed ICAM-1 is biologically active, have remained unclear. To provide insight into the mechanism of leukocyte infiltration into the cornea, we have now examined the effects of TNF- $\alpha$  on the abundance of ICAM-1 protein and mRNA in cultured human keratocytes with the use of whole-cell enzyme-linked immunosorbent assay (ELISA), flow cytometry, immunocytochemistry, and quantitative reverse transcription and polymerase chain reaction (RT-PCR) analysis. The biological activity of ICAM-1 expressed by keratocytes was also assessed by measurement of neutrophil adherence.

## Materials and Methods

### Materials

Eagle's minimum essential medium (MEM), OPTI-MEM, Roswell Park Memorial Institute 1640, trypsin (0.5%)-ethylenediamine-N,N,N',N'-tetraacetic acid (0.53 mM), Hanks' balanced salt solution, fetal bovine serum, and nonenzymatic cell dissociation solution were from Gibco BRL (Grand Island, NY, USA). Cell culture dishes and 96-well cell culture plates (Costar 3596) were from Corning (Corning, NY, USA), and 8-well chamber slides (Lab-tek) were from Nalge Nunc International (Naperville, IL, USA). Dispase was from Sanko Junyaku (Tokyo), and *Clostridium histolyticum* collagenase was from Sigma (St. Louis, MO, USA).

A mouse monoclonal antibody to ICAM-1 was obtained from Pharmingen (San Diego, CA, USA), and fluorescein isothiocyanate-conjugated goat antibodies to mouse immunoglobulin G (IgG) and horseradish peroxidase-conjugated goat antibodies to mouse IgG were from Chemicon (Temecula, CA, USA). Human recombinant TNF- $\alpha$  was from Genzyme (Cambridge, MA, USA). The 3,3',5,5'-tetra-methylbenzidine (TMB) one-step substrate system was from DAKO (Carpinteria, CA, USA), RNeasy Mini Kit was from Qiagen (Hilden, Ger-

many), and TaKaRa RNA PCR Kit (AMV) was from Takara Shuzo (Shiga). LightCycler-DNA Master SYBR Green I was from Roche Molecular Biochemicals (Mannheim, Germany), ethidium bromide and DNA molecular size markers (Marker 11) were from Nippongene (Toyama), and agarose (NuSieve 3:1) was from FMC Bioproducts (Rockland, ME, USA). Bovine serum albumin (BSA) and dextran were from Nacalai Tesque (Kyoto). Ficoll Paque Plus was from Amersham Pharmacia Biotech (Uppsala, Sweden). Calcein-AM was from Molecular Probes (Eugene, OR, USA). All media and cytokines used for cell culture were endotoxin minimized.

### Isolation and Culture of Human Keratocytes

Four human corneas were obtained from Mid-America Transplant Service (St. Louis, MO, USA). The donors were white men and women ranging in age from 4 to 65 years. After the center of each donor cornea was punched out for corneal transplantation surgery, the remaining rim of tissue was used for the present experiments. The human tissue was used in strict accordance with the basic principles of the Declaration of Helsinki. Keratocytes were prepared and cultured as described previously.<sup>1</sup> In brief, the endothelial layer of the corneal rim was removed mechanically and the tissue was then incubated with dispase (2 mg/mL in MEM) for 1 hour at 37°C. After mechanical removal of the epithelial sheet, the tissue was treated with collagenase (2 mg/mL in MEM) at 37°C until a single-cell suspension of keratocytes was obtained. The cells from each cornea were cultured separately in MEM supplemented with 10% fetal bovine serum in 60-mm dishes until they had achieved approximately 90% confluence, and they were used for the present studies after four to six passages. The purity of the cell cultures was assessed on the basis of both the distinctive morphology of the keratocytes and their reactivity with antibodies to vimentin in immunofluorescence analysis.<sup>1</sup> No contamination by corneal epithelial cells was detected.

### Whole-cell ELISA for ICAM-1

Whole-cell ELISA for ICAM-1 was performed as described by Rothlein et al.,<sup>6</sup> with minor modifications. Keratocytes ( $1 \times 10^3$  cells per well) were grown in 96-well plates for 3 days. The culture medium was then changed to OPTI-MEM and the cells were cultured for an additional 3 days. The keratocytes were then cultured for various times in OPTI-MEM containing various concentrations of TNF- $\alpha$ , after which the medium was aspirated and the cells

were washed twice with phosphate-buffered saline (PBS). The cells were fixed for 15 minutes at room temperature with PBS containing 1% paraformaldehyde, washed with PBS, and incubated for 1 hour at 37°C with a monoclonal antibody to ICAM-1 (1:10,000 dilution) in PBS containing 1% BSA. After washing three times with PBS containing 0.1% BSA, the cells were incubated for 1 hour with horseradish peroxidase-conjugated goat antibodies to mouse IgG. The keratocytes were washed three times with PBS containing 0.1% BSA, and were then incubated for 20 minutes in the dark with 100  $\mu$ L of TMB solution. The reaction was terminated by the addition of 50  $\mu$ L of 1 M H<sub>2</sub>SO<sub>4</sub>, and the absorbance of each well was measured at 450 nm with a microplate reader (MPR A4i; Tosoh, Tokyo). The expression of ICAM-1 was calculated as a percentage of the control value as follows:  $100\% \times [\text{absorbance (TNF-}\alpha \text{ stimulated)} - \text{absorbance (background)}] / [\text{absorbance (unstimulated)} - \text{absorbance (background)}]$ .

#### *Immunocytochemical Analysis of ICAM-1 Expression*

Keratocytes ( $5 \times 10^3$ ) were transferred to 8-well culture slides and cultured for 3 days. The culture medium was changed to OPTI-MEM and the cells were cultured for an additional 3 days, after which they were incubated for 24 hours in OPTI-MEM containing TNF- $\alpha$  (1 ng/mL). The cells were washed twice with PBS and then fixed for 15 minutes at room temperature with PBS containing 1% paraformaldehyde. After washing three times with PBS containing 0.1% BSA, the cells were incubated for 1 hour at room temperature with a mouse monoclonal antibody to ICAM-1 (1:1000 dilution) or normal mouse IgG (control) in PBS containing 1% BSA. The keratocytes were washed three times with PBS containing 0.1% BSA, incubated for 1 hour with fluorescein isothiocyanate-conjugated goat antibodies to mouse IgG, washed with PBS, and observed under a fluorescence microscope (Axioskop 50; Zeiss, Oberkochen, Germany).

#### *Flow Cytometric Analysis of Cell Surface Expression of ICAM-1*

Flow cytometric analysis of cell surface ICAM-1 was performed as previously described.<sup>7</sup> In brief, keratocytes were cultured in 100-mm dishes for 3 days in MEM supplemented with 10% fetal bovine serum, and were then incubated first for 24 hours with OPTI-MEM alone and then for an additional 24 hours with OPTI-MEM containing TNF- $\alpha$  (10 ng/

mL). They were then detached from the culture dish with the use of a nonenzymatic cell dissociation solution and incubated for 30 minutes at 4°C in PBS containing 1.0% BSA and a saturating concentration of a mouse monoclonal antibody to ICAM-1. After washing, the cells were incubated for 30 minutes at 4°C with fluorescein isothiocyanate-conjugated goat antibodies to mouse IgG and then fixed with 1% formaldehyde in PBS. As a negative control, cells were incubated under similar conditions with an isotype-matched mouse IgG in place of the primary antibody. The cells were analyzed by flow cytometry with an EPICS-XL instrument (Beckman Coulter, Fullerton, CA, USA); at least 10,000 cells were analyzed for each sample.

#### *Quantitative RT-PCR Analysis of ICAM-1 mRNA*

The culture medium of keratocytes cultured in 100-mm dishes was changed to OPTI-MEM. After culture for 3 days, the OPTI-MEM was supplemented with TNF- $\alpha$  (1 ng/mL) and the cells were incubated for various times. The keratocytes were then washed with PBS, and total RNA was extracted with the use of RNeasy Mini Kit. The extracted RNA was subjected to reverse transcription with the use of TaKaRa RNA PCR Kit, and the abundance of ICAM-1 cDNA was then quantified by real-time PCR with a LightCycler Instrument (Roche Molecular Biochemicals) as described previously.<sup>8</sup> Rapid cycling in glass capillaries was performed with LightCycler-DNA Master SYBR Green I, which contains nucleotides, Taq DNA polymerase, buffer, SYBR Green I dye, and 10 mM MgCl<sub>2</sub>; template DNA, primers, sterile H<sub>2</sub>O, and additional MgCl<sub>2</sub> (final concentration, 2 mM) were added. SYBR Green I dye fluoresces only when it is bound to the outer surface of double-stranded DNA. The relative concentrations of PCR products can thus be measured by detecting the fluorescence of each capillary. The amplification protocol consisted of an initial denaturation step at 95°C for 30 seconds, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 55°C for 10 seconds, and elongation at 72°C for 20 seconds. SYBR Green I fluorescence was measured at the end of each elongation step in order to monitor the amount of PCR product formed during each cycle. After completion of the amplification protocol, melting curve analysis was performed to confirm the specificity of amplification by cooling the sample to 65°C at a rate of 20°C/second, maintaining a temperature of 65°C for 15 seconds,

and then heating at a rate of 0.2°C/second to 95°C, with continuous measurement of fluorescence. The fluorescence signal ( $F$ ) was plotted against temperature ( $T$ ) to generate a melting curve for each sample; the melting curve was then converted to a melting peak by plotting the negative derivative of fluorescence with respect to temperature against temperature ( $-dF/dT$  vs.  $T$ ). Each PCR product gives rise to a specific melting temperature. The size of the PCR products was verified by electrophoresis on a 4% agarose gel, staining with ethidium bromide (1  $\mu$ g/mL), and observation under a Nighthawk illumination system (pdi, Huntington Station, NY, USA), which comprises a charge-coupled device camera, an ultraviolet transilluminator, and an analysis program (Quantity One).

Real-time PCR data were analyzed with LightCycler Data software (Roche Molecular Biochemicals), which first normalizes the values of each sample by the subtraction of background fluorescence generated during the initial cycles. A fluorescence threshold is then set at 5% of full scale, and the software determines the cycle number at which each sample reached this threshold. This cycle number is inversely related to the log of the initial template concentration. Transcripts of the constitutively expressed gene for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were used to normalize the amount of cDNA in each sample.

The sequences of the PCR primers were as follows: ICAM-1 forward, 5'-CACAGTCACCTATG GCAACG-3'; ICAM-1 reverse, 5'-TTCTTGATCT TCCGCTGGC-3'; G3PDH forward, 5'-GCCAAA AGGGTCATCATCTC-3'; and G3PDH reverse, 5'-ACCACCTGGTGCTCAGTGTA-3'. These primers yielded PCR products of the expected sizes of 750 bp for ICAM-1 mRNA and 500 bp for G3PDH mRNA.<sup>9,10</sup>

### *Isolation and Labeling of Neutrophils*

Peripheral venous blood was collected from healthy adult volunteers into tubes containing heparin. Leukocytes were separated from erythrocytes by centrifugation of the blood through a 4% dextran solution. Neutrophils were isolated from other leukocytes by centrifugation through a density gradient of Ficoll Paque Plus. Contaminating erythrocytes were hypotonically lysed by exposure to 0.2% NaCl. Lysis was terminated by the addition of 1.6% NaCl, and the remaining neutrophils were suspended in Hanks' balanced salt solution. Only preparations consisting of >98% neutrophils were used for the experiments.

The neutrophils were labeled with Calcein-AM for 30 minutes at 37°C and then resuspended in RPMI medium in which the free concentrations of Mg<sup>2+</sup> and Ca<sup>2+</sup> were each adjusted to 1  $\mu$ M.

### *Assay of Neutrophil Adherence to Keratocytes*

Keratocytes ( $5 \times 10^3$  cells per well) were cultured and stimulated in 96-well microtiter plates as described above for whole-cell ELISA. The keratocytes were then washed with OPTI-MEM, and Calcein-AM-labeled neutrophils ( $1 \times 10^4$ ) were added to each well. After incubation of the plates for 1 hour at 37°C, nonadherent cells were removed by carefully washing three times with RPMI medium. The percentage of adherent neutrophils was determined by measuring the fluorescence of the wells with a fluorescence photometer (Cytofluor; Applied Biosystems, Foster City, CA, USA).

### *Statistical Analysis*

Data are expressed as means  $\pm$  SEM, and were analyzed with either the Mann-Whitney  $U$ -test, or the Dunnett test. A  $P$  value of  $< .05$  was considered statistically significant.

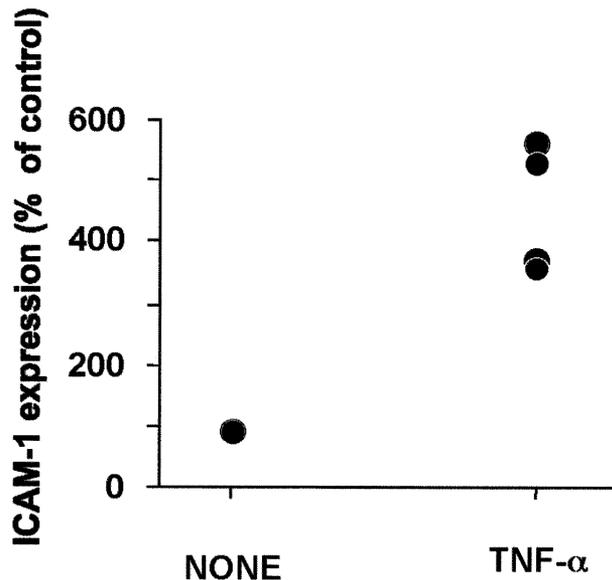
## **Results**

### *Effect of TNF- $\alpha$ on the*

### *Expression of ICAM-1 by Human Keratocytes*

We first examined the effect of TNF- $\alpha$  on the expression of ICAM-1 by human keratocytes isolated from four different donors. The cells were cultured for 24 hours with TNF- $\alpha$  at a concentration of 1 ng/mL, after which ICAM-1 expression was assessed by whole-cell ELISA. For all four keratocyte preparations examined, the expression of ICAM-1 was increased by exposure of the cells to TNF- $\alpha$  (Figure 1). No relation between the extent of ICAM-1 expression and the age or sex of the corneal donors was apparent (data not shown). Based on the findings that the responses of the cells from the four different donors were virtually identical, we performed subsequent experiments with keratocytes isolated from one donor.

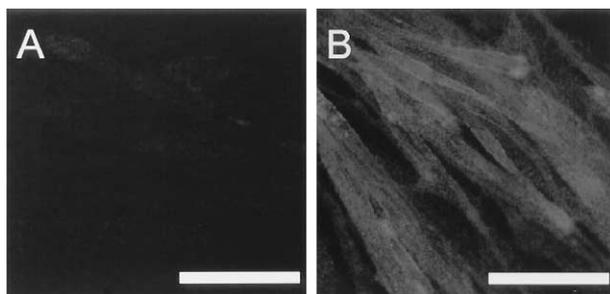
The expression of ICAM-1 by keratocytes incubated for 24 hours in the absence or presence of TNF- $\alpha$  (1 ng/mL) was also examined by immunocytochemical analysis. Although no specific fluorescence was detected with cells incubated in the absence of TNF- $\alpha$ , cells exposed to this cytokine exhibited marked ICAM-1-specific fluorescence (Figure 2). The fluorescence was most prominent



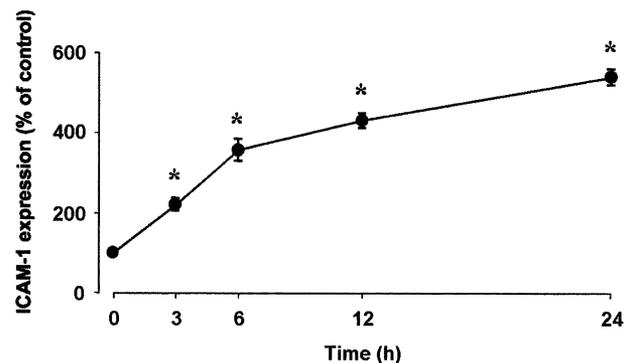
**Figure 1.** Effect of tumor necrosis factor (TNF)- $\alpha$  on intercellular adhesion molecule (ICAM)-1 expression by human keratocytes derived from different donors. Keratocytes prepared from four different donors were incubated separately for 24 hours in the absence or presence of TNF- $\alpha$  (1 ng/mL), after which the expression of ICAM-1 was examined by whole-cell ELISA. Data are expressed as a percentage of the control value (no addition), and are means of values from four separate experiments, each performed with four replicates, for each donor.

around the edges of the cells, indicative of surface expression of ICAM-1.

With the use of whole-cell ELISA, we next investigated the time course of the TNF- $\alpha$ -induced increase in ICAM-1 expression by keratocytes. Incubation of cells with TNF- $\alpha$  (1 ng/mL) for various times revealed that the effect of TNF- $\alpha$  on ICAM-1



**Figure 2.** Immunocytochemical analysis of intercellular adhesion molecule (ICAM)-1 expression by human keratocytes. Cells were incubated for 24 hours in the absence (A) or presence (B) of TNF- $\alpha$  (1 ng/mL), after which ICAM-1 expression was examined by immunocytochemistry with antibodies specific for this protein. Bars = 50  $\mu$ m.



**Figure 3.** Time course of the tumor necrosis factor (TNF)- $\alpha$ -induced increase in intercellular adhesion molecule (ICAM)-1 expression by human keratocytes. Cells were incubated for the indicated times in the presence of TNF- $\alpha$  (1 ng/mL), after which the expression of ICAM-1 was determined by whole-cell enzyme-linked immunosorbent assay. Data are expressed as a percentage of the control value (zero time point), and are mean  $\pm$  SEM ( $n = 6$ ) from a representative experiment that was repeated three times with similar results. \* $P < .05$  (Mann-Whitney  $U$ -test) versus the zero time point.

abundance was time-dependent, with statistically significant increases in this parameter being apparent at 3, 6, 12, and 24 hours (Figure 3). Similarly, incubation of keratocytes for 24 hours with various concentrations of TNF- $\alpha$  revealed that the effect of this cytokine on ICAM-1 expression was dose-dependent; the effect was statistically significant at a TNF- $\alpha$  concentration of 0.003 ng/mL and maximal at 1 ng/mL (Figure 4).

We also examined the expression of ICAM-1 by keratocytes with the use of flow cytometry. Such analysis revealed that ICAM-1 was expressed on the surface of unstimulated keratocytes (Figure 5). However, expression of ICAM-1 was significantly ( $P < .05$ , Mann-Whitney  $U$ -test) increased by stimulation of the cells with TNF- $\alpha$  (10 ng/mL); the fluorescence intensity values were  $1.05 \pm 0.03$ ,  $14.99 \pm 2.24$ , and  $183.53 \pm 9.86$  for the negative control, unstimulated cells, and TNF- $\alpha$ -stimulated cells, respectively (data are means  $\pm$  SEM of values from four independent experiments). The magnitude of the TNF- $\alpha$  effect observed by flow cytometry was similar to that detected by whole-cell ELISA.

#### *Effect of TNF- $\alpha$ on the Abundance of ICAM-1 mRNA in Human Keratocytes*

The effect of TNF- $\alpha$  on the amount of ICAM-1 mRNA in cultured human keratocytes was examined by quantitative RT-PCR analysis. ICAM-1

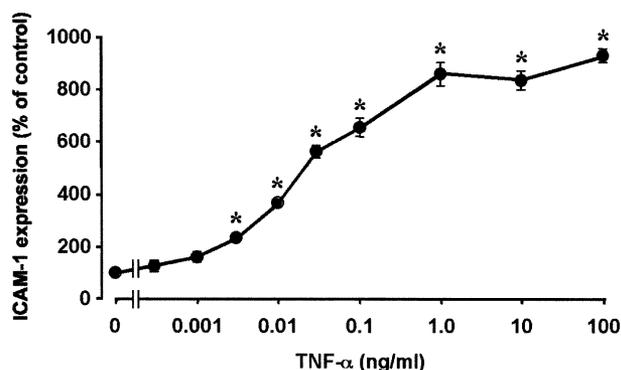
mRNA was detected in cells incubated in the absence of TNF- $\alpha$ , but the amount of this mRNA was increased markedly in a time-dependent manner by incubation of cells in the presence of TNF- $\alpha$  (1 ng/mL); the maximal effect was apparent at 2 hours, after which the amount of ICAM-1 mRNA gradually decreased (Figure 6).

#### Adherence of Neutrophils to TNF- $\alpha$ -stimulated Keratocytes

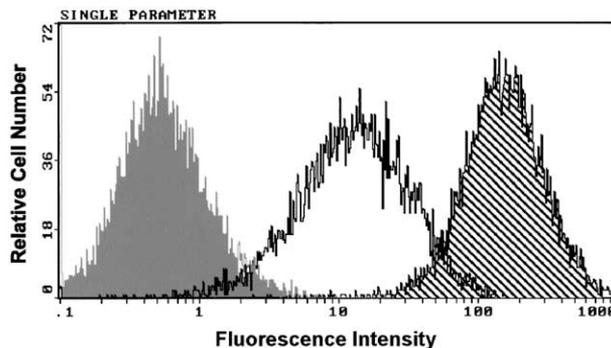
Finally, we examined the adherence of human neutrophils to keratocytes that had been incubated for 24 hours in the absence or presence of TNF- $\alpha$  (1 ng/mL). The two cell types were incubated together for 1 hour, after which the percentage of adherent neutrophils was determined. Prior exposure of keratocytes to TNF- $\alpha$  resulted in a significant (approximately twofold) increase in the number of neutrophils that adhered to the keratocytes (Figure 7).

### Discussion

We have shown that TNF- $\alpha$  directly increases the expression of ICAM-1 by human keratocytes at both the protein and mRNA levels, and that stimulation of keratocytes with TNF- $\alpha$  promotes the interaction of these cells with neutrophils. The effect of TNF- $\alpha$  on the expression of ICAM-1 by keratocytes was dose-dependent, being significant at a concentration of 3 pg/mL. The effect was also time-dependent, with



**Figure 4.** Dose dependence of the tumor necrosis factor (TNF)- $\alpha$ -induced increase in intercellular adhesion molecule (ICAM)-1 expression by human keratocytes. Cells were incubated for 24 hours with the indicated concentrations of TNF- $\alpha$ , after which the expression of ICAM-1 was assessed by whole-cell enzyme-linked immunosorbent assay. Data are expressed as a percentage of the control value (no TNF- $\alpha$ ), and are mean  $\pm$  SEM (n = 6) from a representative experiment that was repeated three times with similar results. \* $P$  < .01 (Dunnett test) versus the corresponding value for cells incubated in the absence of TNF- $\alpha$ .



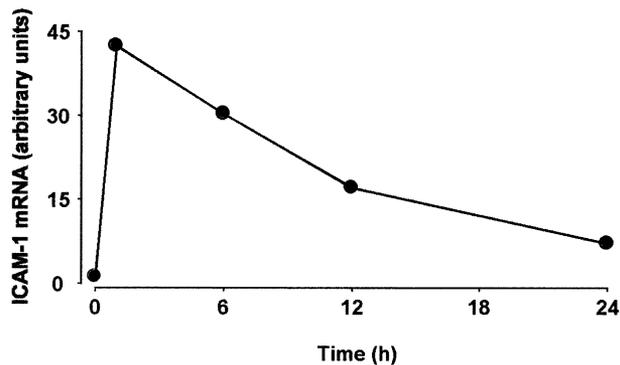
**Figure 5.** Flow cytometric analysis of the expression of intercellular adhesion molecule (ICAM)-1 on the surface of human keratocytes. Cells were incubated for 24 hours in the absence (open histogram) or presence (hatched histogram) of tumor necrosis factor (TNF)- $\alpha$  (10 ng/mL), after which the expression of ICAM-1 was assessed by flow cytometry. Control staining with an isotype-matched mouse immunoglobulin was also performed (solid histogram). Data are from a representative experiment that was repeated four times with similar results.

the amount of ICAM-1 protein increasing up to 24 hours and that of ICAM-1 mRNA peaking at 2 hours.

ICAM-1 has previously been shown to mediate leukocyte interaction with the cornea.<sup>5</sup> Our observation that exposure of keratocytes to TNF- $\alpha$  increased the adherence of neutrophils to these cells indicates that the ICAM-1 expressed by keratocytes in response to TNF- $\alpha$  is biologically active. Corneal epithelial cells also express functional ICAM-1 in response to stimulation with TNF- $\alpha$ .<sup>11</sup> Considering that ICAM-1 plays a central role in the infiltration and activation of inflammatory cells, ICAM-1 expressed on the surface of TNF- $\alpha$ -stimulated keratocytes and corneal epithelial cells might contribute to the local accumulation of inflammatory cells in the cornea.

Our observation that TNF- $\alpha$  directly increases the expression of ICAM-1 by keratocytes is consistent with the previous demonstration of the presence of TNF- $\alpha$  receptors and the downstream NF- $\kappa$ B signaling pathway in these cells.<sup>12</sup> Our data are also consistent with the demonstration that TNF- $\alpha$  increases the expression of ICAM-1 in cultured skin fibroblasts at both the protein and mRNA levels,<sup>13</sup> suggesting that this effect of TNF- $\alpha$  occurs in fibroblasts from both avascular tissue such as the cornea as well as vascular tissue such as the skin.

Leukocyte infiltration into the corneal stroma is apparent in many corneal inflammatory diseases, regardless of whether they are infectious or noninfec-

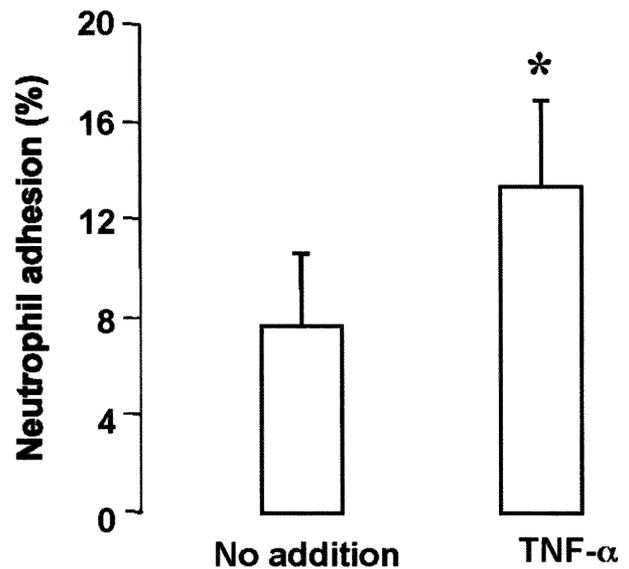


**Figure 6.** Time course of the effect of tumor necrosis factor (TNF)- $\alpha$  on the abundance of intercellular adhesion molecule (ICAM)-1 mRNA in human keratocytes. Cells were incubated for the indicated times in the presence of TNF- $\alpha$  (1 ng/mL), after which the amount of ICAM-1 mRNA in cell lysates was determined by quantitative reverse transcriptase-polymerase chain reaction. The amount of ICAM-1 mRNA was normalized by glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA and is presented in arbitrary units (with 1 unit corresponding to the value for the zero time point). Data are from a representative experiment that was repeated three times with similar results.

tious in origin. The concentration of TNF- $\alpha$  is increased in the tear fluid of individuals with such diseases,<sup>14–17</sup> and the corneal stroma might be directly exposed to this tear fluid as a result of defects in the corneal epithelium. Keratocytes in the corneal stroma might thus be exposed to the high concentrations of TNF- $\alpha$  present in the tear fluid of such individuals. The TNF- $\alpha$  concentration in the tear fluid of some patients with inflammatory corneal diseases exceeds 0.1 ng/mL, a concentration sufficient to induce ICAM-1 expression by keratocytes in our *in vitro* study.

The importance of keratocyte activation and the expression of humoral factors by these cells in response to stimulation with cytokines such as TNF- $\alpha$  had been demonstrated previously.<sup>1,18,19</sup> Our present results showing that TNF- $\alpha$  induces the expression in these cells of ICAM-1, which is a ligand of the  $\beta$ 2 integrin chain expressed on the surface of all leukocytes, further support the importance of keratocytes in controlling the local infiltration of leukocytes into the cornea.

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**Figure 7.** Adherence of neutrophils to keratocytes stimulated with tumor necrosis factor (TNF)- $\alpha$ . Keratocytes were incubated for 24 hours in the absence or presence of TNF- $\alpha$  (1 ng/mL), after which the cells were incubated for 1 hour in the presence of Calcein-AM-labeled human neutrophils and the percentage of adherent neutrophils was determined. Data are mean  $\pm$  SEM (n = 6) from a representative experiment that was repeated three times with similar results. \* $P < .05$  (Mann-Whitney *U*-test) versus the value for keratocytes incubated in the absence of TNF- $\alpha$ .

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