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Curcumin (Diferuloylmethane) Inhibition of Tumor Necrosis Factor (TNF)-Mediated Adhesion of Monocytes to Endothelial Cells by Suppression of Cell Surface Expression of Adhesion Molecules and of Nuclear Factor-κB Activation

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ABSTRACT. Recruitment of leukocytes by endothelial cells and their subsequent migration from the vasculature into the tissue play major roles in inflammation. In the present study, we investigated the effect of curcumin, an antiinflammatory agent, on the adhesion of monocytes to human umbilical vein endothelial cells (EC). Treatment of EC with tumor necrosis factor (TNF) for 6 hr augmented the adhesion of monocytes to EC, and this adhesion was due to increased expression of intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and endothelial leukocyte adhesion molecule-1 (ELAM-1). Pretreatment of EC for 1 hr with curcumin completely blocked their adhesion to monocytes, as well as the cell surface expression of ICAM-1, VCAM-1, and ELAM-1 in EC. Although curcumin inhibited adhesion even when administered 1 hr after TNF treatment, maximum inhibition occurred when added either 1 hr before or at the same time as TNF. As the induction of various adhesion molecules by TNF requires activation of the transcription factor NF-κB, the effect of curcumin on the activation of this factor in the EC was also investigated. A 30-min treatment with TNF activated NF-κB; the activation was inhibited in a concentration-dependent manner by pretreatment with curcumin, indicating that NF-κB inhibition may play a role in the suppression of expression of adhesion molecules in EC. Our results demonstrate that the antiinflammatory properties of curcumin may be attributable, in part, to inhibition of leukocyte recruitment. BIOCHEM PHARMACOL **55**;6:775–783, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. curcumin; adhesion proteins; NF-κB; TNF; endothelial cells

Curcumin (diferuloylmethane) is a major chemical component of turmeric (*Curcuma longa*) and is used as a spice to give a specific flavor and yellow color to curry. It is also used as a cosmetic and in some medical preparations [1]. Curcumin has been shown to display antiinflammatory and anticarcinogenic properties [2–6]. The antiinflammatory effects of curcumin are most likely mediated through its ability to inhibit cyclooxygenase and lipoxygenase [7, 8]. Curcumin also inhibits TNF¶ and phorbol ester-stimulated human immunodeficiency virus long terminal repeat-directed gene expression [9]. Recently, curcumin has also been shown to inhibit the activation of a nuclear transcription factor, NF-κB, in different cell types [10, 11]. Although the antiinflammatory properties of curcumin are known, its molecular basis is not understood. Recruitment of leukocytes by EC and their subsequent migration are known to play a critical role in inflammation.

TNF is a multifunctional cytokine shown to be involved in inflammation through expression of various inflammatory molecules [12]. It induces the expression of adhesion molecules on EC involved in transendothelial cell migration of leukocytes [13–15]. In particular, TNF has been shown to induce *de novo* synthesis of ICAM-1 (also called CD54), VCAM-1, and ELAM-1 (also called E-selectin). ICAM-1 and VCAM-1 are 95 and 110 kDa proteins, respectively, and both belong to the immunoglobulin superfamily. Besides EC, ICAM-1 is also expressed by mono-

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¶ *Abbreviations:* TNF, tumor necrosis factor; HUVEC, human umbilical vein endothelial cells; EC, endothelial cell(s); ICAM-1, intracellular adhesion molecule-1 (also called CD54); VCAM-1, vascular cell adhesion molecule-1; ELAM-1, endothelial leukocyte adhesion molecule-1 (also called E-selectin); FBS, fetal bovine serum; NF-κB, nuclear factor-kappa B; EMSA, electrophoresis mobility shift assay; IL, interleukin; IFN, interferon; EBM, endothelial basal medium; and HBSS, Hanks' balanced salt solution.

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cytes, B and T cells, keratinocytes, chondrocytes, and epithelial cells. In addition to EC, monocytes, and dendritic cells, VCAM-1 is also expressed in myoblast and bone marrow fibroblasts. The 115 kDa protein ELAM-1, which belongs to the selectin family, is expressed exclusively on EC. Recent evidence indicates that the promoter region of the gene for these adhesion molecules contains NF-κB binding sites and that these sites are essential for the expression of these genes [16].

In the present report, we examined the effects of curcumin on the adhesion of leukocytes to human EC. We found that curcumin inhibits the TNF-induced adhesion of monocytes to EC by inhibiting the expression of adhesion molecules and NF-κB activation.

MATERIALS AND METHODS

Materials

Penicillin, streptomycin, RPMI-1640 medium, and FBS were obtained from GIBCO. Glycine, NaCl, BSA, and curcumin were obtained from the Sigma Chemical Co. Bacteria-derived recombinant human TNF, purified to homogeneity with a specific activity of 5×10^7 units/mg, was provided by Genentech, Inc. The HUVEC used in these experiments were obtained from the Clonetics Corp. Antibodies against adhesion receptors ICAM-1, VCAM-1, and ELAM-1 were purchased from Becton Dickinson. The double-stranded oligonucleotides having AP-1 and Oct-1 consensus sequences were obtained from Santa Cruz Biotechnology.

Culture of EC

EC were cultured in endothelium basal medium supplemented with 10 μg/mL bovine brain extract (EC growth factor), 0.5 μg/mL hydrocortisone, 50 μg/mL gentamicin, 50 μg/mL amphotericin B, and 10% fetal calf serum (Clonetics Corp.). Confluent monolayers were harvested by treatment with trypsin-EDTA and subcultured 1:3. Cells were plated onto 96-well culture plates coated with 5 μg/mL fibronectin and grown to confluence for use in cell adhesion assays. The effect of curcumin on cell viability was examined by the crystal violet method as described [17]. For most studies, cells at passage 3 were used. All experiments were repeated at least two times, and typical results are shown.

Monocyte Adherence to EC

Monocyte adherence to HUVEC was assayed according to a protocol described earlier [18]. Briefly, promyelomonocytic HL-60 cells were suspended at 0.75×10^6 cells/mL in RPMI 1640 medium containing 10% FBS and incubated with 0.2 μCi of [^{3}H]thymidine overnight at 37°. Labeled monocytes were washed and resuspended to approximately 10^6 cells/mL in EBM. Endothelial monolayers were incubated without or with indicated concentrations of curcumin for 1 hr. The cells were washed with medium and then incubated again in medium alone or medium containing 100 ng/mL TNF for 6 hr at 37°. The medium was aspirated, and 100 μL of monocyte suspension was added to each endothelial monolayer-containing well and incubated for 60 min at 37°. Nonadherent cells were aspirated, and the monolayers were washed three times with HBSS, pH 7.4. The adherent cells were solubilized and counted in a beta scintillation counter (Packard Co.).

Detection of Cell Surface Adhesion Receptor Expression on EC

HUVEC were harvested by a brief exposure to trypsin-EDTA and fixed by incubation for 30 min at 4° in 4% paraformaldehyde, washed three times with PBS, pH 7.4, and resuspended in FACS binding buffer (PBS, pH 7.4, containing 2% FBS, 0.1% NaN_3, 0.1% BSA, and 1 mg/mL human IgG). Cells were incubated with mouse monoclonal antibodies to ICAM-1, VCAM-1, and ELAM-1 at 4° for 30 min and washed three times with binding buffer. The washed cells were incubated with goat anti-mouse $F(ab)_2$ secondary antibodies conjugated to fluorescein isothiocyanate (FITC) for 30 min at 4°. EC were washed three times with PBS, pH 7.4, fixed in 4% paraformaldehyde, and analyzed for fluorescence on a FACScan fluorescence cytometer (Becton Dickinson).

Electrophoretic Mobility Shift Assays for NF-κB

HUVEC (1.2×10^6 cells/mL) were incubated in suspending medium or with different concentrations of curcumin for 1 hr followed by treatment with TNF for 30 min at 37°. Then nuclear extracts were prepared and run for EMSA according to the adopted procedure previously described in detail ([17] and references therein). Briefly, nuclear extracts prepared from 2×10^6 cells were assayed for protein content and then incubated with 16 fmol of ^{32}P end-labeled 45-mer double-stranded NF-κB oligonucleotide from the HIV-1 long terminal repeat (5′-TTGTTACAAGGGACTTTCC GCTGGGGACT TTCCAGGGAGGCGTGG-3′) for 20 min at 37°. The DNA–protein complex formed was separated from free oligonucleotide on 7.5% native polyacrylamide gel, and then the gel was dried. A mutated oligonucleotide was used to examine the specificity of binding of NF-κB to the DNA. Radioactive bands were visualized by a PhosphorImager (Molecular Dynamics) using Imagequant software.

The EMSAs for AP-1 and Oct-1 were performed as described for NF-κB using ^{32}P end-labeled double-stranded oligonucleotides. Specificity of binding was determined routinely by using an excess of unlabeled oligonucleotide for competition.

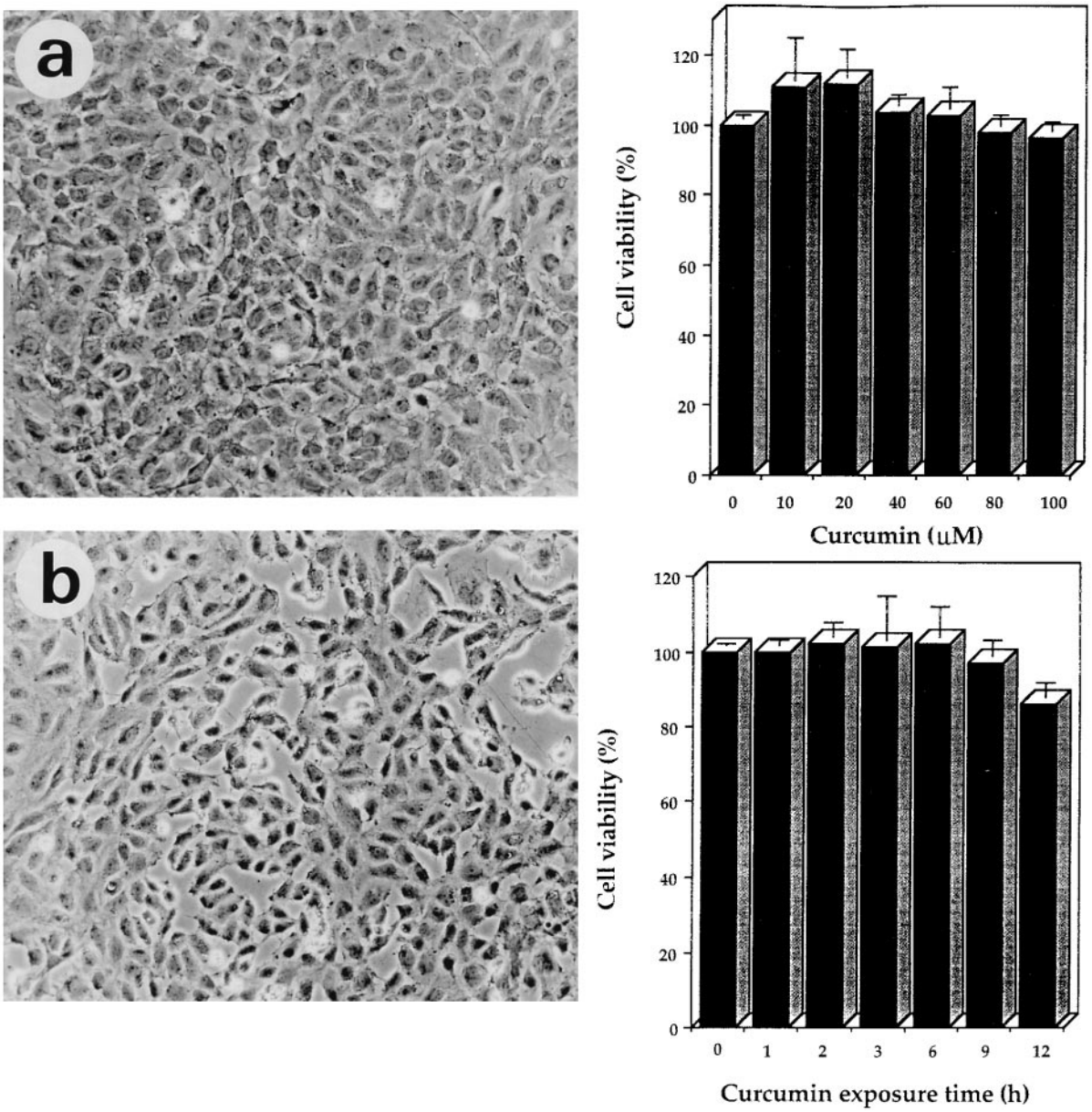

FIG. 1. Right panel: Effect of different concentrations of curcumin (top) and of different times (bottom) on the viability of EC. Cells (1×10^4) were treated with either 0–100 μM curcumin for 9 hr or with 50 μM curcumin for 0–12 hr and then examined for cell viability by the crystal violet method. Data are reported as means ± SD and were derived from three independent experiments. Left panel: Effect of curcumin on the morphology of EC. Cell monolayers were incubated with TNF (10 ng/mL) in (a) the absence or (b) the presence of curcumin (50 μM) for 6 hr at 37°, washed, fixed with 4% paraformaldehyde, and then examined by phase contrast microscopy. These data represent one of two replicate experiments.

RESULTS

In the present report, we investigated the effect of curcumin on TNF-induced adhesion of monocytes to EC. We first examined the effect of curcumin on the viability of human EC. Cell viability was determined by the crystal violet method. The results of cell treatment with different concentrations of curcumin for 9 hr or with 50 μM curcumin for different times are shown in the right panel of Fig. 1. Neither 100 μM curcumin for 9 hr nor 50 μM curcumin for 12 hr had any significant effect on cell viability. Cell viability at the highest time and concentration of curcumin was greater than 90%. The effect of curcumin on the morphology of EC is shown in the left panel of Fig. 1. For this experiment, cells were treated with TNF (10 ng/mL) in the absence or presence of 50 μM curcumin for 6 hr. The curcumin-treated cells appeared less dense than the curcumin-untreated cells. When the supernatant was examined, it was found that curcumin had caused the detachment of 10–20% of the cells. However, by the dye exclusion method, the detached cell fraction in the supernatant was found to be viable.

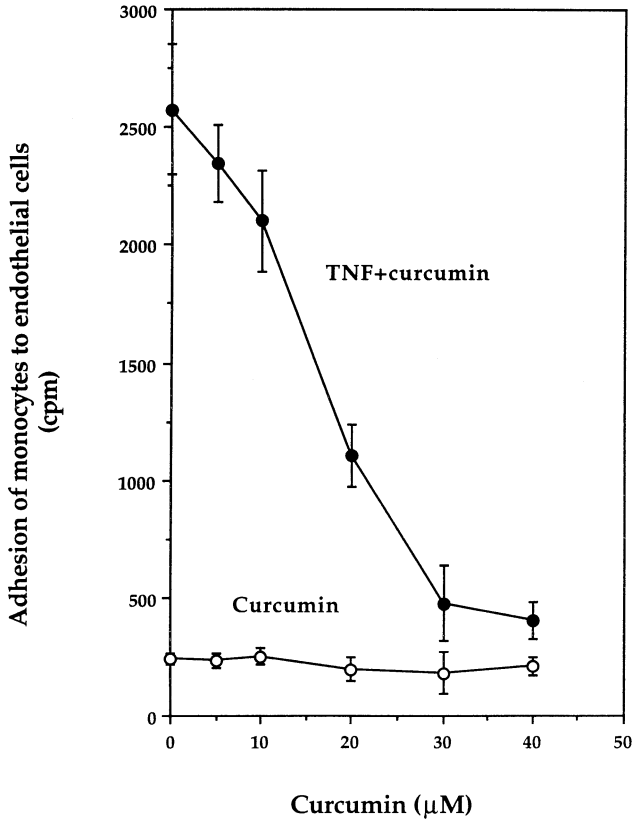

FIG. 2. Effect of curcumin on the adherence of monocytes to EC monolayers. HUVEC grown to confluence in 96-well plastic culture plates were incubated with or without curcumin for 1 hr at 37°. After 1 hr, the cells were washed and then treated with 100 ng/mL of TNF for 6 hr at 37°. At the end of the incubation, the cells were washed gently with medium and incubated with [^{3}H]thymidine-labeled HL-60 cells for 1 hr at 37°. Nonadherent cells were aspirated, and the monolayers were washed three times with HBSS, pH 7.4. The number of adherent cells was determined by solubilizing the cells and counting in a beta scintillation counter. Data are reported as means ± SD and were derived from three independent experiments done in triplicate. Zero curcumin concentration represents either cells with medium (○) or cells with TNF (●).

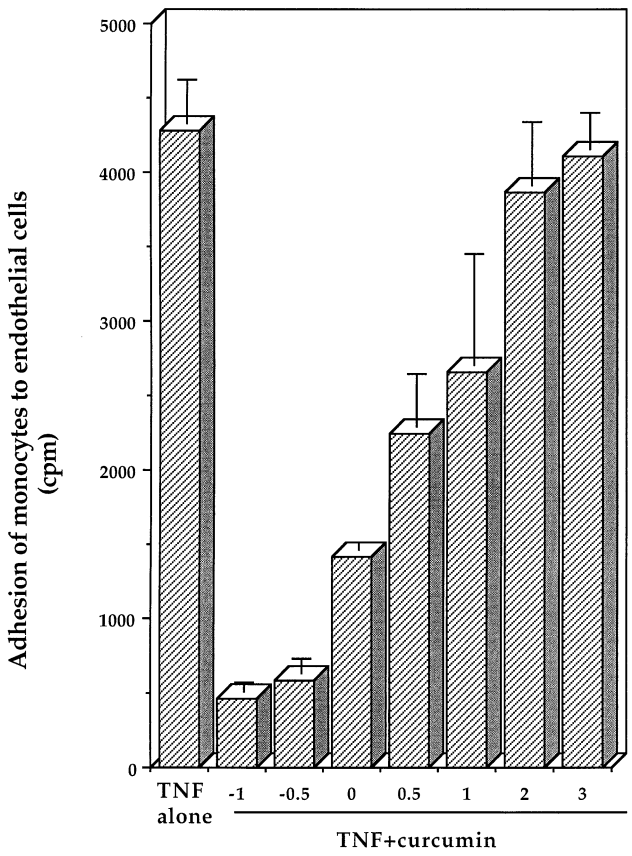

FIG. 3. Kinetics of curcumin-mediated inhibition of adhesion of HL-60 cells to EC. EC were treated with 100 ng/mL TNF or medium for 6 hr, and curcumin (40 μM) was added at different times before or after the start of TNF treatment. At the end of incubation, the cells were washed three times with HBSS, and the adhesion of HL-60 cells to EC was determined as described in Materials and Methods. Data are means ± SD and are representative of two independent experiments done in triplicate. Adhesion of monocytes to EC treated with medium alone was 387 ± 91 cpm.

Effect of Curcumin on the Adhesion of Monocytes to TNF-Treated EC

To study the effect of curcumin on the binding of monocytes to TNF-treated EC, HL-60 cells were labeled with tritiated thymidine and then incubated with endothelial monolayers pretreated with different concentrations of curcumin in the presence or absence of TNF. The results are shown in Fig. 2. Curcumin inhibited the adhesion of HL-60 cells to monocytes in a concentration-dependent manner with maximum inhibition at 40 μM curcumin.

The kinetics of curcumin-mediated inhibition of adhesion of monocytes to EC was also studied. EC were treated with TNF for 6 hr, and curcumin was added at the indicated times before or after the start of TNF treatment. As shown in Fig. 3, curcumin inhibited adhesion even when added 1 hr after the addition of TNF. These results indicate that curcumin is a potent inhibitor of cellular adhesion.

Effect of Antibodies Against ICAM-1, VCAM-1, and ELAM-1 on Adherence of Monocytes to EC

To determine whether adhesion molecules induced by TNF are involved in cellular adhesion, EC were treated with TNF (100 ng/mL) for 6 hr and then incubated with antibodies against ICAM-1, VCAM-1, or ELAM-1 for 1 hr at room temperature. Then adhesion to HL-60 cells was assayed. As shown in Fig. 4, TNF-induced adhesion was blocked significantly by antibodies against all the adhesion proteins. Under these conditions, control IgG had no effect. These results confirm the role of these adhesion molecules in adherence of HL-60 cells to EC.

Effect of Curcumin on TNF-Mediated Induction of ICAM-1, VCAM-1, and ELAM-1

Induction of adhesion molecules is essential for the interaction of endothelial cells with monocytes [19, 20]. We

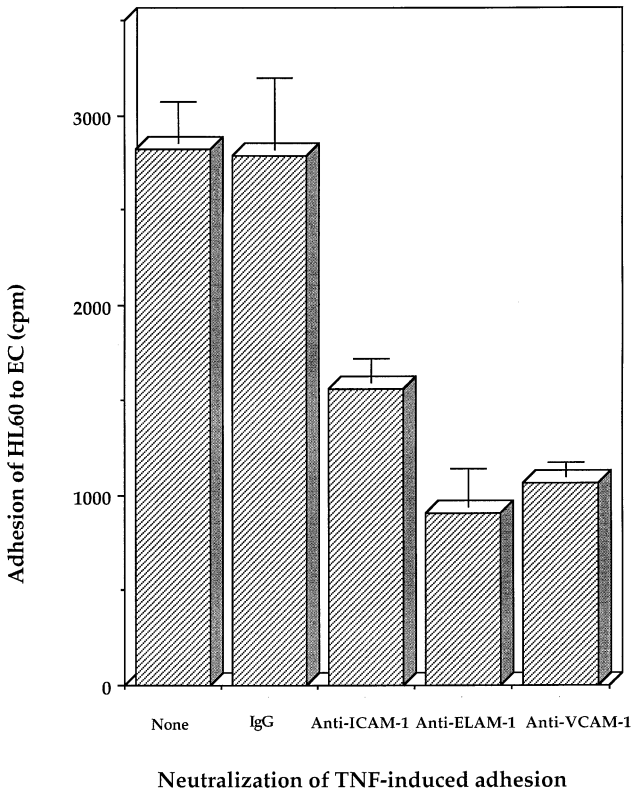

FIG. 4. Neutralization of TNF-induced adhesion of HL-60 cells to EC by antibodies against adhesion proteins. EC were incubated with 100 ng/mL of TNF for 6 hr at 37°, washed, treated with the indicated antibodies (1:200 dilution) for 1 hr at room temperature, and then examined for adhesion to HL-60 cells as described in Fig. 2. All determinations were made in triplicate, and the data shown are means ± SD.

therefore examined the effects of curcumin on the TNF-dependent expression of ICAM-1, VCAM-1, and ELAM-1. For this, EC were preincubated with 40 μM curcumin for 1 hr and then treated with TNF (100 ng/mL) for 6 hr at 37°. As shown in Fig. 5A, curcumin completely abolished the TNF-induced expression of ICAM-1 and ELAM-1. The expression of VCAM-1 was also inhibited significantly by curcumin. When examined for the concentration–response, it was found that 40 μM curcumin was sufficient to inhibit most of the expression of all the three cell surface adhesion proteins (Fig. 5B).

Effect of Curcumin on TNF-Dependent Activation of NF-κB in EC

The expression of adhesion molecules by TNF requires the activation of NF-κB [15]. We have shown previously that curcumin inhibits activation of NF-κB in myeloid cells [10]. However, it is not known whether curcumin inhibits the expression of adhesion proteins through suppression of NF-κB activation in EC. The signalling pathway that leads to activation of NF-κB may differ from one cell type to another and among inducers within a cell type [21–24]. To study the effect of curcumin on the activation of NF-κB, EC were preincubated with different concentrations of curcumin for 1 hr and then treated with TNF for 30 min at 37°. The results in Fig. 6A show that curcumin inhibited the activation of NF-κB in a concentration-dependent manner, with approximately 90% suppression occurring at 40 μM. The band was specific to NF-κB, as it disappeared when unlabeled oligonucleotide (100-fold excess) was added, and it did not bind mutated oligo probe. No activation of NF-κB was noted in untreated cells or those treated with either the vehicle (DMSO) or curcumin alone (data not shown). Among the transcription factors, the effect of 40 μM curcumin on NF-κB activation was specific, as this concentration inhibited neither AP-1 nor Oct-1 (Fig. 6B).

DISCUSSION

In the present study, we investigated the effects of curcumin on the adhesion of monocytes with EC. The results presented here clearly demonstrate that curcumin blocked the TNF-mediated attachment of monocytes to EC by inhibiting the EC surface expression of the adhesion molecules ICAM-1, VCAM-1, and ELAM-1. Curcumin also suppressed the TNF-mediated activation of NF-κB in EC.

Previous studies have demonstrated that several adhesion molecules play a critical role in the recruitment and migration of leukocytes to inflammation sites in various diseases [20, 25–27]. Recruitment of leukocytes from peripheral blood into tissue requires a series of cell-adhesion-molecule-mediated interactions between the vascular endothelium and the leukocyte cell surface. It has been reported that expression of adhesion molecules on HUVEC is increased or induced by stimulation with inflammatory cytokines, including TNFα, IL-1β, IL-4, and IL-13 [28].

Exactly how curcumin inhibits the expression of these adhesion molecules is not known. It has been reported that the promoter regions of the genes for ICAM-1, VCAM-1, and ELAM-1 contain 1, 2, and 3 NF-κB binding sites respectively, which are critical for the expression of these proteins on EC [15, 20, 29, 30]. Inasmuch as we found that curcumin also blocks TNF-mediated activation of NF-κB, it is conceivable that curcumin suppresses the expression of these molecules by inhibiting the activation of NF-κB in response to TNF. As the concentration of curcumin that inhibited NF-κB was similar to that which blocked the expression of adhesion proteins and cell adhesion, it suggests a critical role for NF-κB activation. Indeed, some other inhibitors of NF-κB are also known to inhibit the expression of these adhesion proteins on EC. Among these inhibitors are a wide variety of pharmacological agents with diverse mechanisms of action, including such immunosuppressive agents as cyclosporin A, such antiinflammatory agents as alkoxybenzo[*b*]thiophene-2-carboxamide, the protein kinase C inhibitor *N*,*N*,*N*-trimethylsphingosine, protease inhibitors, such antioxidants as pyrrolidine dithiocarbamate, inducers of intracellular cyclic AMP, such protein tyrosine kinase inhibitors as genistein and herbimy-

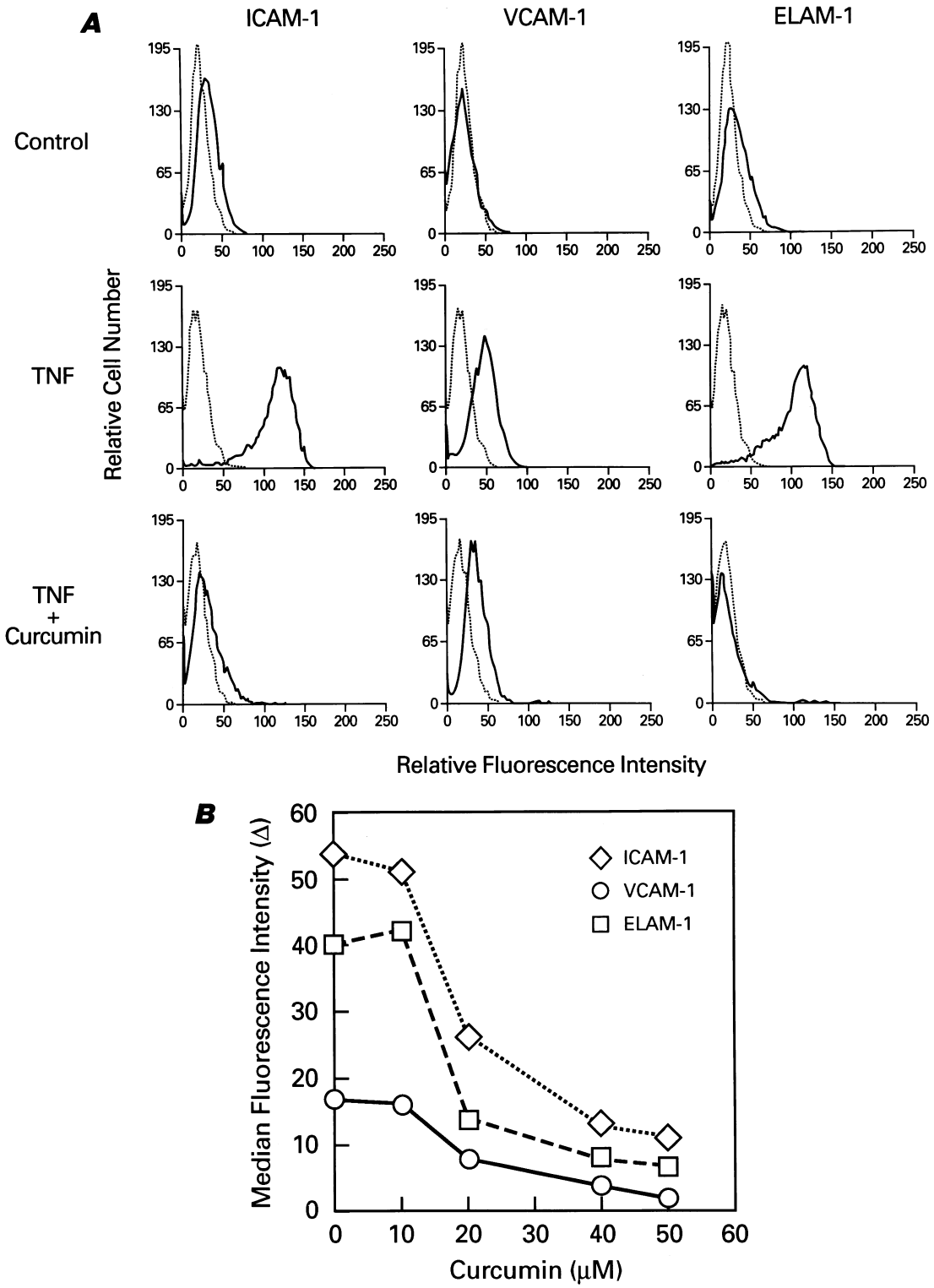

FIG. 5. (A) Effect of curcumin on TNF-induced cell surface expression of adhesion molecules on EC. EC cultured in plastic tissue culture flasks were preincubated with 40 μM curcumin for 1 hr. The cells were washed gently and then treated with TNF for 6 hr at 37°. At the end of the incubation, the expression of adhesion molecules on the surface of HUVEC was determined by flow cytometry, as described in Materials and Methods. The dotted line in the fluorescence profile is for the non-immune IgG binding, and the solid line is for antibodies to adhesion molecules (ICAM-1, VCAM-1, and ELAM-1). The data represent one of three separate replicate experiments. (B) Effect of different concentrations of curcumin on TNF-induced cell surface expression of adhesion molecules on EC. EC cultured with 0–50 μM curcumin and TNF (10 ng/mL) for 6 hr at 37° were examined by flow cytometry for the expression of adhesion molecules, as described in Materials and Methods. The data represent one of two separate replicate experiments.

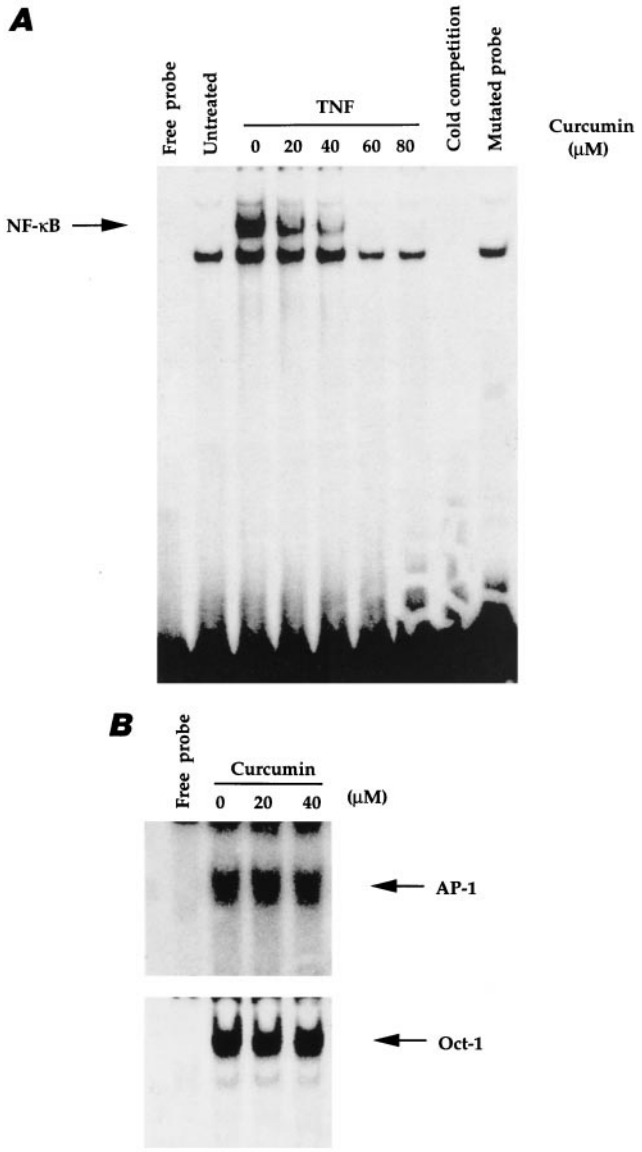

FIG. 6.(A) Effect of curcumin on the TNF-dependent activation of NF-κB. EC (2 × 10^6/mL) were incubated at 37° with different concentrations of curcumin followed by activation with 0.1 nM TNF. Nuclear extracts were made, and NF-κB was assayed as described in Materials and Methods. (B) Effect of curcumin on AP-1 and Oct-1 transcription factors. EC (2 × 10^6/mL) were treated with different concentrations of curcumin for 60 min at 37°; then nuclear extracts were prepared and used for EMSA of AP-1 and Oct-1 transcription factors as described.

cin A, nitric oxide, such cytokines as IFN-α, and the antisense oligonucleotide specific to the p65 subunit of NF-κB [31–41]. However, it is not clear at this stage whether these agents inhibit the expression of adhesion protein by inhibiting NF-κB. Indeed NF-κB-independent induction of VCAM-1 has been demonstrated in EC [32, 35, 39], and there is a report that proteosome inhibitor can block IL-1-induced VCAM-1 and ICAM-1 gene expression in EC without inhibiting NF-κB activation [35]. However, in our study, curcumin inhibited the TNF-induced expression of all three adhesion molecules, as well as NF-κB activation. These results are consistent with a number of other reports that inhibitors of NF-κB activation block gene expression of adhesion proteins in human EC [33, 34, 36, 38, 40–42].

NF-κB can be activated by a variety of signals including TNF, relevant to EC physiology. Although the early events of NF-κB activation differ, all may converge to phosphorylate IκBα, which is essential for its degradation and the subsequent translocation of p50 and p65 polypeptides to the nucleus [21]. However, the kinase responsible for IκBα phosphorylation and the protease involved in degradation have not been identified. However, several serine/threonine protein kinases, including protein kinase C, Raf-1 protein kinase, and double-stranded RNA-activated protein kinase have been implicated.

The present observation that curcumin inhibited the activation of NF-κB induced by TNF in EC suggests that curcumin impairs a step in the signal transduction after the diverse signals converge and before the phosphorylation of IκBα. Indeed, curcumin has been shown to inhibit the degradation of IκBα in U937 cells [10]. Recent studies also implicate protein tyrosine phosphatases and protein tyrosine kinases in the induction of expression of these adhesion proteins. The inhibitors of PTPase have been shown to inhibit the TNF-mediated induction of expression of ICAM-1, ELAM-1, and VCAM-1 (Dhawan *et al.*, unpublished observation). Protein tyrosine kinase inhibitors such as herbimycin A and genistein were also found to suppress TNF-stimulated induction of EC adhesion molecules [37, 38]. It is possible, therefore, that curcumin interferes with the activity of some of these enzymes, which would, in turn, result in the inhibition of their induction. We have shown that curcumin inhibits both serine/threonine kinase and protein tyrosine kinase activities [43], which provides additional support for the above assumption.

The concentration of curcumin used to block cell adhesion in our studies *in vitro* has been employed previously by other investigators [44–47]. Furthermore, concentrations of curcumin in the range of several millimolar have been used safely in animal studies to block carcinogenesis and inflammation [2–6, 48, 49]. These observations suggest that the concentrations used in our studies *in vitro* are achievable *in vivo* without any side-effects. It has been shown that curcumin blocks colon tumorigenesis by modulating arachidonic acid metabolism [2] dependent on phospholipase A_2, cyclooxygenase, and lipoxygenase. The synthesis of all these enzymes requires NF-κB activation [50–52]. In addition, 92 kDa type IV collagenase gene expression, which is associated with the invasiveness of tumor cells, is also regulated by NF-κB [53]. Therefore, it is possible that *in vivo* curcumin inhibits tumorigenesis by different mechanisms, including inhibition of expression of cell adhesion proteins as described here.

Overall, our data establish that curcumin can inhibit the adhesion of monocytes to EC by inhibiting the expression of ICAM-1, ELAM-1, and VCAM-1 on EC. Because of the

low toxicity of curcumin, its ability to inhibit the induction of adhesion molecules suggests that curcumin should be explored for its therapeutic value in atherogenesis, bacterial sepsis, inflammation, and tumor metastasis.

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