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Emodin (3-methyl-1,6,8-trihydroxyanthraquinone) inhibits TNF-induced NF-κB activation, IκB degradation, and expression of cell surface adhesion proteins in human vascular endothelial cells

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Most inflammatory agents activate nuclear transcription factor-κB (NF-κB) which results in expression of genes for cytokines, adhesion molecules, and enzymes involved in amplification and perpetuation of inflammation. Emodin (3-methyl-1,6,8-trihydroxyanthraquinone) is an active component from the roots of *Polygonum cuspidatum* that has been reported to exhibit antiinflammatory properties but the mechanism is not known. In the present study we investigated the effects of emodin on the activation of NF-κB in human umbelical vein endothelial cells (EC). Treatment of EC with TNF activated NF-κB; preincubation with emodin inhibited this activation in a dose- and time-dependent manner. Emodin did not chemically modify NF-κB subunits but rather inhibited degradation of IκB, an inhibitory subunit of NF-κB. Since the promoter regions of ICAM-1, VCAM-1, and ELAM-1 contain NF-κB binding sites and these adhesion molecules are involved in the attachment of leukocytes to EC, the effect of emodin on the adhesion of monocytes to EC and the expression of these adhesion molecules was also studied. Treatment of EC with TNF for 6 h increased the adhesion of monocytes to EC, which correlated with increases in cell surface expression of ICAM-1, VCAM-1 and ELAM-1. Pretreatment of EC for 1 h with emodin inhibited both monocyte-EC attachment and expression of ICAM-1, ELAM-1 and VCAM-1. These results indicate that emodin is a potent inhibitor of NF-κB activation and expression of adhesion molecules and thus could be useful in treating various inflammatory diseases.

Keywords: emodin; TNF; NF-κB; adhesion; IκBα; endothelial cells

Introduction

Emodin has been shown to display a number of biological activities such as antiviral, antimicrobial, immunosuppressive hepatoprotective, anti-inflammatory, and antiulcerogenic (Huang *et al.*, 1992; Lin *et al.*, 1996). Although the molecular mechanism(s) by which emodin produces its biological effects remain unknown, it inhibits the activity of protein kianse C and of c-*src*, p56[lck], and HER-2 protein tyrosine kinases (Frew *et al.*, 1994; Jayasuriya *et al.*, 1992;

Zhang and Hung, 1996). Emodin has also been reported to induce the differentiation of HER-2/neu-overexpressing breast cancer cells (Zhang *et al.*, 1995) and inhibits the growth of *v-ras*-transformed human bronchial epithelial cells (Chan *et al.*, 1993).

Tumor Necrosis factor (TNF) is a multifunctional cytokine that modulates cell growth, inflammation, autoimmunity and septic shock (reviewed by Aggarwal and Natarajan, 1996). TNF mediates its cellular responses through two distinct receptors, the p60 receptor (TNFR-I) and the p80 (TNFR-II). The cytoplasmic domain (CD) of the p60 receptor via its death domain interacts with TNF-receptor associated death domain (TRADD) which recruits TNF receptor-associated factor-2 (TRAF-2) (Hsu *et al.*, 1996). TRAF-2 then associates with NF-κB inducing kinase (NIK) which activates IκB-specific kinases leading to NF-κB induction (reviewed by Stancovski and Baltimore, 1997). The activation of NF-κB can protect cells from TNF-induced apoptosis (for references see Baichwal and Baeuerle, 1997). NF-κB activation has also been shown to play a major role in TNF-induced inflammation (reviewed by Baeuerle and Baichwal, 1997). TNF induces the expression of NF-κB-dependent genes such as intercellular adhesion molecule (ICAM)-1 (also called CD54), vascular cell adhesion molecule (VCAM-1), and endothelial leukocyte adhesion molecule (ELAM)-1 (also called E selectin) which are involved in inflammation (Iademarco *et al.*, 1992; Schindler and Baichwal, 1994; Voraberger *et al.*, 1991).

NF-κB is a ubiquitous heterodimeric transcription factor that exists in an inactive form in the cytoplasm bound to the inhibitory proteins referred to as IκB (reviewed by Baeuerle and Baichwal, 1997). Treatment of cells with various inducers, including TNF results in phosphorylation, ubiquitination and subsequent degradation of IκB proteins, thus releasing the bound NF-κB, which translocates to the nucleus and induces gene expression.

Because of immunosuppressive and anti-inflammatory effects of emodin, we became interested in how it may affect NF-κB activation pathway leading to inflammation. In the present study we investigated the effects of emodin on the activation of NF-κB by TNF in human umbilical vein endothelial cells (EC), on the expression of adhesion molecules on EC and on the adhesion of monocytes to EC. The maximum concentration of emodin (50 μg/ml) and the time of incubation (6 h) used in these studies decreased cell viability by less than 4%. To examine the effect of emodin on TNF-induced NF-κB activation, EC were

pretreated with different concentrations of emodin for 1 h, then activated with TNF (1 nM) for 30 min, and examined for NF-κB activation by EMSA. The results in Figure 1a clearly indicate that emodin inhibited the TNF-dependent activation of NF-κB in a dose-dependent manner, with maximum effect occurring at a concentration of 50 μg/ml. No activation of NF-κB was observed in the cells treated with vehicle (DMSO) alone or with emodin alone (data not shown). The kinetics of inhibition of TNF-induced NF-κB activation was also studied. EC were incubated with 50 μg/ml emodin for 60 or 30 min before the addition of

TNF, at the same time as TNF, and 15 min after TNF. The cells were treated with TNF for 30 min. As shown in Figure 1b emodin inhibited the TNF-induced activation of NF-κB even when added at the same time as TNF, however, maximum inhibition was seen when it was added 30 or 60 min before TNF.
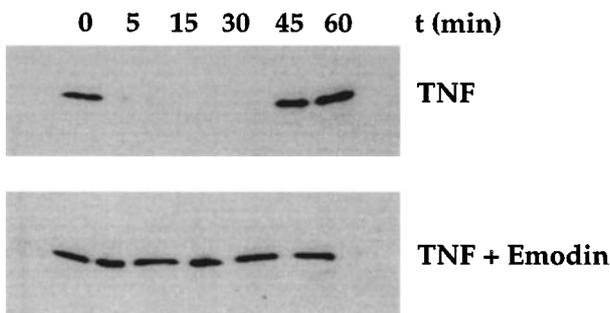
To determine whether emodin directly modifies NF-κB proteins, the nuclear extracts obtained from TNF-treated cells were incubated with different concentrations of emodin for 30 min, and then EMSA was conducted. Figure 1c shows that emodin did not modify the ability of NF-κB to bind to the DNA.
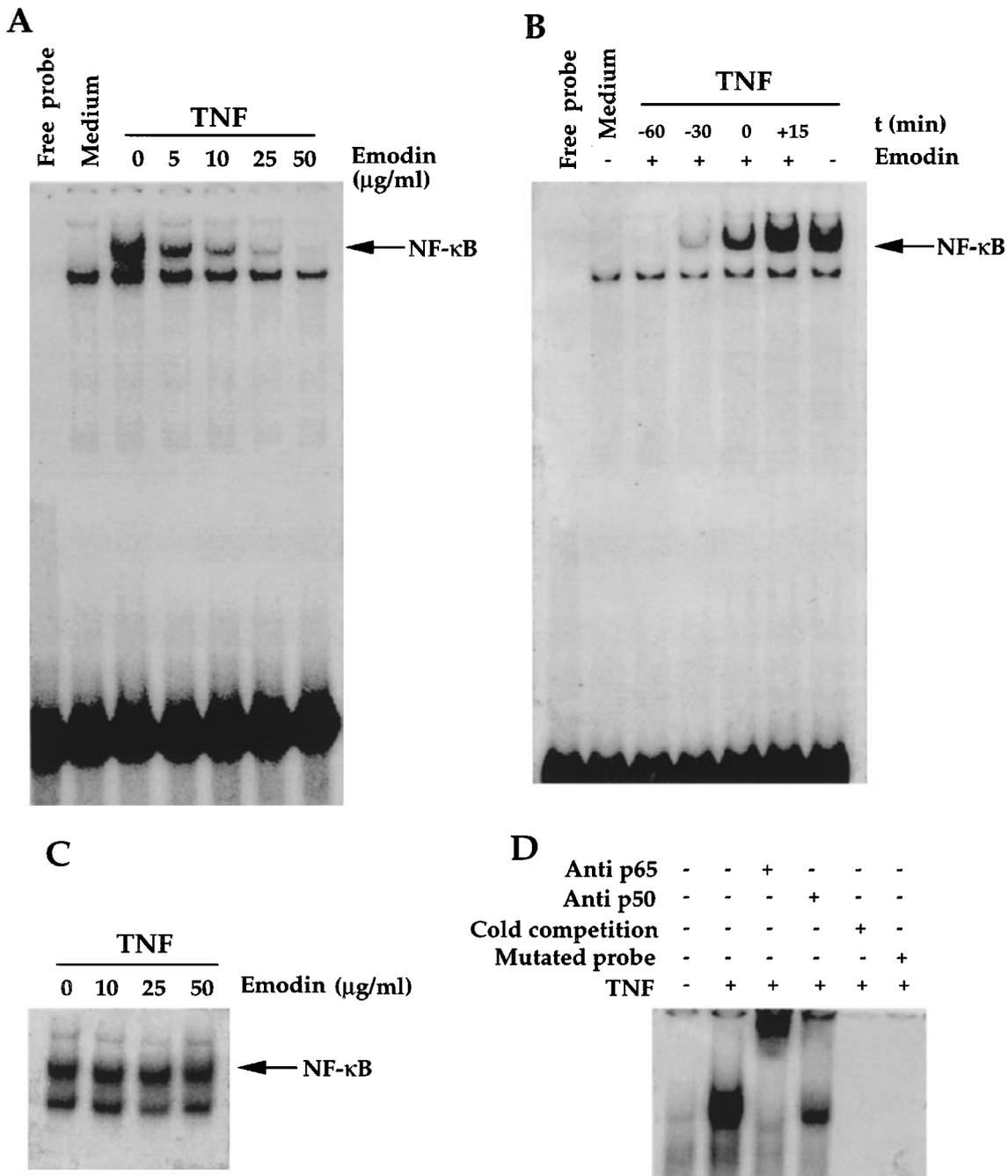
Figure 1 Effect of emodin on TNF-dependent activation of NF-κB. (a) EC (2×10^6 cells/well) were preincubated at 37°C for 1 h with different concentrations of emodin and then treated for 30 min with 1 nM TNF. (b) EC were preincubated with 50 μg/ml of emodin for different intervals of time and then treated with 1 nM TNF for 30 min. (c) Nuclear extracts prepared from TNF-treated cells were incubated at 37°C with indicated concentrations of emodin for 30 min. (d) For supershift and specificity analysis of NF-κB activation, nuclear extracts from TNF-treated cells, were incubated for 30 min with antibodies, and then assayed for NF-κB by EMSA

The DNA-binding ability of other transcription factors (AP-1 and Oct-1) was also not affected by emodin (data not shown). Thus these results indicate that emodin does not chemically modify NF-κB, AP-1 or Oct-1 proteins such that it affects their ability to bind the DNA. To show that the retarded band observed by EMSA in TNF-treated cells was indeed NF-κB, nuclear extracts were incubated with antibodies either to the p50 (NF-κB-1) or to p65 (RelA) subunits followed by EMSA. Antibodies to either subunit shifted the band to a higher molecular weight, thus suggesting that the TNF-activated complex consisted of p50 and p65 subunits. In addition, the retarded band observed by EMSA in TNF-treated cells disappeared when unlabeled oligonucleotide (100-fold in molar excess) was used, and it did not bind to the mutated probe (Figure 1d). We next studied the effect of emodin on the degradation of IκBα. The results shown in Figure 2 indicate that the treatment of EC with TNF decreased the IκBα band within 5 min, but it reappeared by 45 min. Pretreatment of the cells with emodin for 1 h abolished the degradation of IκBα. Treatment of cells with emodin alone had no effect on IκB level (data not shown).

Since the NF-κB activation is known to induce the expression of adhesion molecules which promotes the adhesion of EC to monocytes, we examined the effect of emodin on the binding of monocytes to EC. Human promyelomonocytic, HL-60 cells were labeled with tritiated thymidine and then incubated with EC monolayers pretreated with different concentrations of emodin in the presence or absence of TNF. The results presented in Figure 3a show that emodin inhibited the adhesion of HL-60 cells to monocytes in a dose-dependent manner. A concentration of 50 μg/ml, which inhibited NF-κB activation completely, was found to inhibit most of the binding of HL-60 cells to TNF-treated EC. The kinetics of emodin-mediated inhibition of adhesion of monocytes to TNF-treated endothelial cells was also studied. EC were treated with TNF for 6 h, and the 50 μg/ml emodin was added at the indicated times before or after the start of TNF treatment. As shown in Figure 3b, emodin was most effective in inhibiting TNF-induced adhesion of monocytes to EC when added 1 h before TNF, although a marginal inhibition could be seen even when it was added 1 h after TNF treatment.
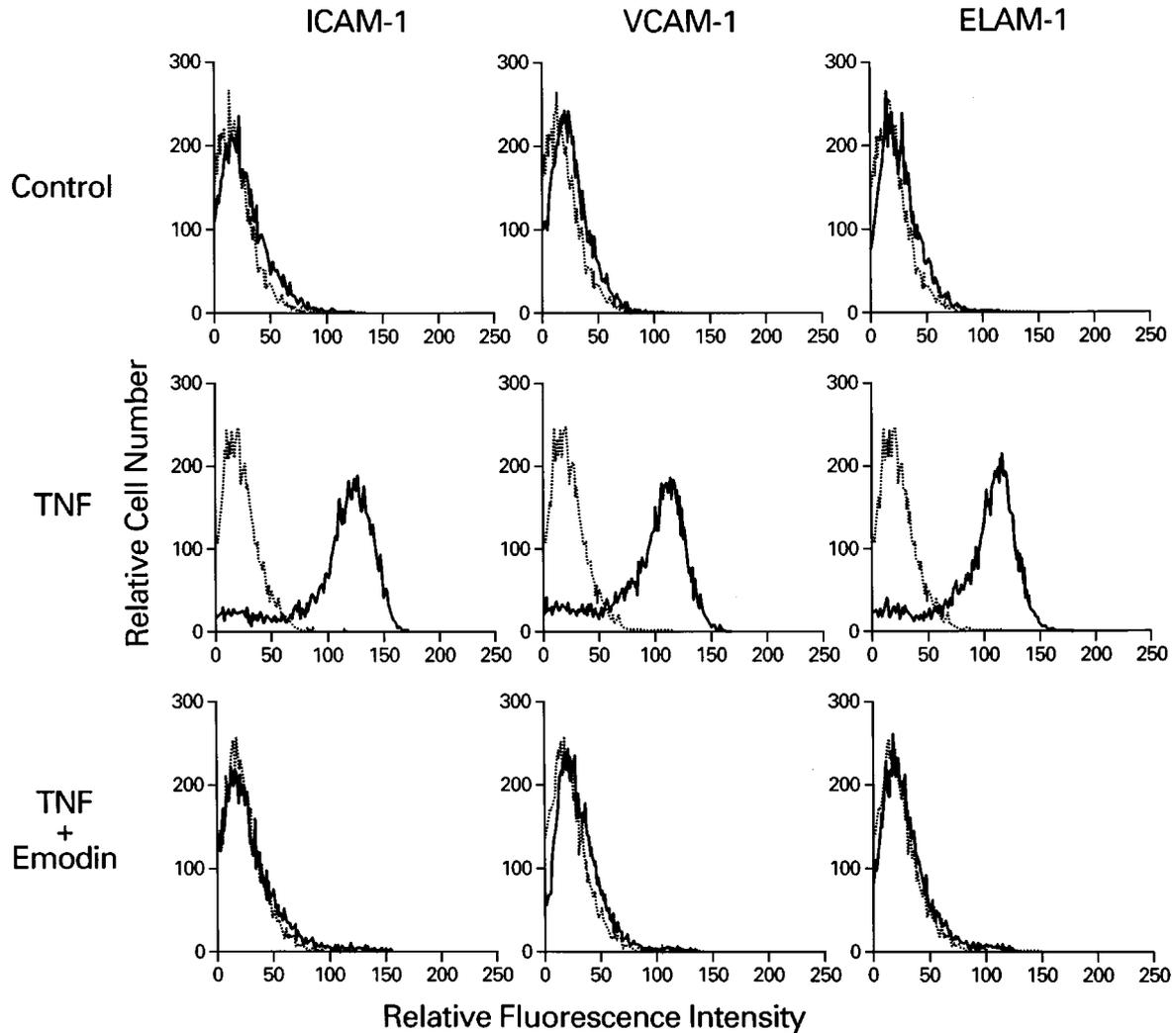
Because TNF-induced adhesion was blocked by antibodies against either ICAM-1 or VCAM-1 or ELAM-1 (data not shown), it indicates the role of these adhesion molecules in the adherence of HL-60 to

A

B
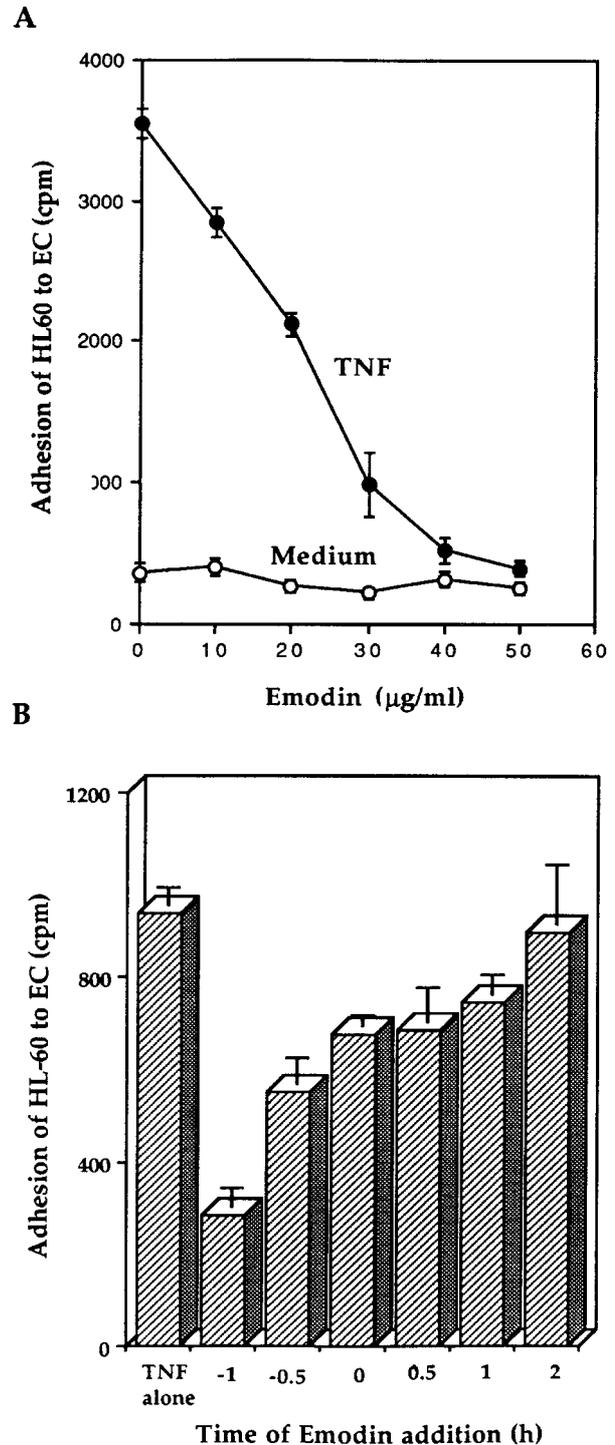
Figure 3 Effect of emodin on the adhesion of monocytes to endothelial cell monolayers. (**a**) EC grown to confluence in 96-well plastic culture plates were incubated with indicated concentrations of emodin for 1 h at 37°C and then treated with 100 ng/ml of TNF for 6 h. (**b**) EC were treated with 100 ng/ml of TNF for 6 h and emodin (50 μg/ml) was added at indicated time intervals before and after the start of TNF treatment. At the end of incubation, cells were washed and the adhesion of HL-60 to endothelial cells was determined as described (Kumar *et al.*, 1998)

Figure 2 Effect of emodin on TNF-induced degradation of IκBα. EC (2×10^6 cells) either untreated or pretreated for 1 h with emodin (50 μg/ml) were incubated for different times with TNF (1 nM) and then assayed for IκBα in the cytosolic fraction by Western blot as described previously (Reddy *et al.*, 1994)

Figure 4 Effect of emodin on TNF-induced cell surface expression of adhesion molecules on EC. Cells were preincubated with 50 μg/ml of emodin for 1 h, washed, treated with TNF for 6 h at 37°C and then analysed by FACS for expression of adhesion molecules as described (Dhawan et al., 1997). Broken lines is for non-immune IgG binding and solid line is for antibodies to adhesion molecules

EC. We therefore also examined the effect of emodin on the TNF-induced expression of ICAM-1, VCAM-1 and ELAM-1. EC were preincubated with 50 μg/ml emodin for 1 h and then treated with TNF for 6 h at 37°C. As shown in Figure 4, emodin completely inhibited the TNF-induced expression of ICAM-1, VCAM-1 and ELAM-1 on EC.

Thus these results indicate that emodin inhibits TNF-induced activation of NF-κB, the degradation of IκB, adhesion of monocytes to EC and cell surface expression of ICAM-1, VCAM-1 and ELAM-1. The inhibition of expression of these adhesion molecules by emodin seems to be a result of the inhibition of NF-κB activation by TNF because same concentrations of emodin suppressed the activation of NF-κB as well as expression of adhesion molecules. The concentrations of emodin used in our study (18–180 μM) are slightly higher than those employed previously (up to 80 μM) (Chan et al., 1993; Zhang et al., 1995, 1996) for in vitro and in vivo studies.

How emodin inhibits the activation of NF-κB and the expression of ICAM-1, VCAM-1 and ELAM-1 in response to TNF is not understood at this stage. The

activation of NF-κB by TNF is a complex phenomenon that involves the participation of several intermediates such as ceramides, protein serine-threonine kinase, protein tyrosine kinases (PTK), protein tyrosine phosphatases (PTPase), reactive oxygen intermediates (ROI) and proteases. In addition association of the CD of the p60 TNF receptors with TRADD, TRAF-2, NIK, Iκκα, Iκκβ and RIP also play an important role in TNF-induced NF-κB activation (for references see Stancovski and Baltimore, 1997). Inhibitors of protein tyrosine kinases and tyrosine phosphatases have been shown to block TNF-induced NF-κB activation (Singh and Aggarwal, 1995; Singh et al., 1996; Natarajan et al., 1998). Since emodin has also been reported to inhibit the activity of several PTK (Frew et al., 1994; Zhang et al., 1996; Chan et al., 1993; Fredenhagen et al., 1995), it is possible that inhibition of NF-κB activation is through PTK. The observation that emodin inhibits the degradation of IκB suggests that emodin impairs a step in the signal transduction pathway before the serine phosphorylation of IκB.

Several reports indicate that the NF-κB activation plays a pivotol role in regulating the cytokine-induced

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expression of these adhesion molecules (for references see Carlos and Harlan, 1994, Springer, 1994). Analysis of the promoter region of these adhesion molecules showed that ELAM-1, VCAM-1 and ICAM-1 contains 1, 2, and 3 decameric sequences, respectively, that are recognized by NF-κB (Iademarco *et al.*, 1992; Schindler and Baichwal, 1994; Voraberger *et al.*, 1991). It is therefore possible that emodin suppress the expression of these adhesion molecules by inhibiting NF-κB. Whether the inhibition of NF-κB alone is sufficient to block the expression of these molecules, however, is not clear. NF-κB-independent regulation of expression of VCAM-1 has also been observed in EC (McCarty *et al.*, 1995), suggesting that the expression of adhesion molecules can be regulated at different steps. In summary, the present observation that emodin inhibits the activation of NF-κB and expression of adhesion molecules suggests that emodin has potential as a treatment for pathological conditions in which NF-κB has been shown to play an important role such as graft *versus* host reaction, inflammation, HIV replication, cancer metastasis, acute phase response, and radiation damage.

Materials and methods

Materials

Emodin was purchased from Calbiochem (San Diego, CA). For initial studies emodin was kindly supplied by Dr Ching-Jer Chang of Purdue University (West Lafayette, IN). Emodin was dissolved at 30 mg/ml in 100% DMSO and then diluted for all experiments to the desired concentrations in the medium containing 10% FCS. Antibodies against the adhesion receptors ICAM-1, VCAM-1 and ELAM-1 were purchased from Becton Dickinson (Mountain View, CA). Oligonucleotide probes for NF-κB, AP-1 and Oct-1 were from Santa Cruz Biotechnology (Santa Cruz, CA).

Culture of endothelial cells

EC (Clonetics Corp. San Diego, CA) were cultured in endothelium basal medium (EBM) supplemented with 10 μg/ml bovine brain extract (endothelial cell growth factor), 0.5 μg/ml hydrocortison, 50 μg/ml gentamicin, 50 μg/ml amphotericin B, and 10% FCS (Clonetics Inc., San Diego, CA). 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 NF-κB and in cell adhesion assays.

Electrophoretic mobility shift assays for NF-κB

These assays were performed as described by Chaturvedi *et al.* (1997). Briefly, nuclear extracts were incubated with 16 fmoles of ^{32}P-end-labeled 45-mer double-stranded NF-κB oligonucleotide from the HIV-1 long terminal repeat (5′-TTGTTACAA**GGGACTTTC**CGCTG**GGGGAC-TTTC**CAGGGAGGCGTGG-3′; NF-κB binding sites are underlined) for 20 min at 37°C. The DNA-protein complex formed was separated from free oligonucleotide on a 7.5% native polyacrylamide gel, and then the gel was dried. A double-stranded oligonucleotide with mutant NF-κB sites (5′-TTGTTACAA**CTCACTTTC**CGCTG**CTCACTTTC**C-AGGGAGGCGTGG-3′) was used to examine the specificity of binding of NF-κB to the DNA. Binding specificity was also examined by competition with unlabeled oligonucleotide. For supershift assays, nuclear extracts prepared from TNF-treated cells were incubated with antibodies against either the p50 or p65 subunit of NF-κB for 30 min at 37°C before the complex was analysed by EMSA.

Abbreviations

EC, endothelial cells; NF-κB, nuclear factor-kappa B; EMSA, electrophoresis mobility shift assay; EBM, endothelial basal medium

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