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Tumor Necrosis Factor (TNF)-α and Interleukin (IL)-1β Down-regulate Intercellular Adhesion Molecule (ICAM)-2 Expression on the Endothelium

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Leukocyte recruitment is a crucial step in inflammation. Inflammatory stimuli upregulate the expression of some endothelial adhesion molecules, such as E-selectin or ICAM-1, but not of others such as ICAM-2. ICAM-2, a constitutively expressed endothelial ligand for β2 integrins LFA-1 and Mac-1, is involved in leukocyte adhesion to resting endothelium and in transmigration *in vitro*, however its role in inflammation is unclear. We have studied the effect of TNF-α and IL-1β on ICAM-2 expression on human umbilical vein endothelial cells (HUVECs). Prolonged treatment (24 h) of HUVECs with TNF-α (10 ng/ml) or IL-1β (34 ng/ml) reduced ICAM-2 surface expression to 50% of control, while interferon (IFN)-γ had no effect. The loss in ICAM-2 surface expression correlated with a reduction of ICAM-2 mRNA to ≈40% of control after 24 h of cytokine treatment. The activity of an ICAM-2 promoter reporter plasmid transfected into HUVECs was down-regulated by TNF-α and IL-1β to similar values. Thus inflammatory cytokines inhibit ICAM-2 transcription, despite the absence of known cytokine-responsive elements in the promoter. Immunocytochemistry on HUVEC monolayers showed that ICAM-2 expression, mainly at the cell junctions in resting cells, was markedly decreased by cytokine treatment. This data suggest that ICAM-2 expression on the endothelium may be regulated during inflammation.

Keywords: Endothelium, vascular, cell adhesion molecules, gene expression, human, intercellular junctions

Abbreviations: ICAM-1, intercellular adhesion molecule-1; ICAM-2, intercellular adhesion molecule-2; LFA-1, lymphocyte function-associated antigen-1; HUVEC, human umbilical vein endothelial cell; TM, thrombomodulin; VEGF, vascular endothelial growth factor

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INTRODUCTION

Leukocyte recruitment into tissues is a multistep process that involves sequential interactions between members of several superfamilies of adhesion receptors (Granger and Kubes, 1994; Schleiffenbaum and Fehr, 1996; Hynes, 1992; Springer, 1994). This critical step of the inflammatory response is also a crucial event in the pathogenesis of many diseases (Buckley and Simmons, 1997; Menger and Vollmar, 1996). Several agents – inflammatory cytokines, viruses, bacterial toxins or oxidised lipoproteins, as well as mechanical forces generated by blood flow – are known to increase the endothelial expression of E-selectin, intercellular adhesion molecule (ICAM)-1 (See Abbreviations) and vascular cell adhesion molecule (reviewed in Ross, 1997) by transcriptional mechanisms which require the synergistic interaction of a small group of transcription factors, including NF-κB and AP-1 (Collins *et al.*, 1995).

Other adhesion molecules involved in leukocyte recruitment, such as ICAM-2 and CD31, are constitutively expressed on the endothelium and are not upregulated by inflammatory stimuli (Defougerolles *et al.*, 1991; Newman, 1997; Staunton *et al.*, 1989). While CD31 has been shown both *in vitro* and *in vivo* to be required for leukocyte transmigration (Muller *et al.*, 1993; Zocchi *et al.*, 1996), the role of endothelial ICAM-2 is less clear. ICAM-2 is a two-domain Ig superfamily member, with 35% identity to ICAM-1 (domains 1 and 2) which, like ICAM-1, binds β2 integrins LFA-1 and Mac-1 (Defougerolles *et al.*, 1991; Gahmberg *et al.*, 1991; Staunton *et al.*, 1989; Xie *et al.*, 1995). The crystal structure of ICAM-2 was recently solved (Casasnovas *et al.*, 1997) providing evidence for different architectures of integrin binding sites in Ig superfamily members. ICAM-2 is expressed on resting endothelial cells, platelets and at low levels on leukocytes, its expression pattern being more restricted than that of ICAM-1 (Defougerolles *et al.*, 1991). On endothelial cells, ICAM-2 is expressed at the junctions and also on the apical surface (Bradley *et al.*, 1995), and has therefore been suggested to be involved in leukocyte transendothelial migration (Defougerolles *et al.*, 1991). *In vitro* observations showed that anti-ICAM-2 monoclonal antibodies (mAb) partially inhibited T cell transmigration across endothelial monolayers induced by the chemokine MCP-1 (Roth *et al.*, 1995). The lack of upregulation of ICAM-2 by cytokines *in vitro* led to the suggestion that ICAM-2 may not play a role in leukocyte recruitment in inflammation, but may be involved instead in T cell recirculation in normal tissues (Nortamo *et al.*, 1991; Staunton *et al.*, 1989). Indeed binding to resting endothelium of purified LFA-1 or lymphoblastoid cell lines was partially inhibited by anti-ICAM-2 mAbs (Defougerolles *et al.*, 1991). However, in all systems tested so far, β2 integrin-dependent adhesion to endothelial cells appears to be mainly dependent on ICAM-1, leaving open the question of a specific role for ICAM-2. The suggestion for another function for ICAM-2, as a modulator of leukocyte adhesion, comes from the work of Gahmberg and colleagues (Li *et al.*, 1995; Li *et al.*, 1993a; Li *et al.*, 1993b; Somersalo *et al.*, 1995), which showed that a peptide from domain 1 of ICAM-2 regulates β2-integrin ligand binding. Recently, a role for ICAM-2 in the colonization of fetal brain by microglia has been proposed (Rezaoe *et al.*, 1997). Very little information is available on the role of ICAM-2 in *in vivo* models of inflammation: in a thioglycollate-induced mouse model of inflammation, antibodies to ICAM-2 did not reduce PMN recruitment into the peritoneum while ICAM-1 antibodies did (Fleetwood and Randi, unpublished).

To study the effect of endothelial activation on ICAM-2, we have used the inflammatory cytokines TNF-α and IL-1β on confluent HUVEC monolayers in culture, and found that both cytokines decreased surface ICAM-2 expression on HUVECs to 50% of the control values. This decrease correlates with inhibition of ICAM-2 promoter activity. Interestingly, the cytokine-induced down-regulation of ICAM-2 was more evident at the endothelial cell junctions. These

results suggest that endothelial ICAM-2 is regulated during inflammation.

METHODS

Cell Culture

HUVECs were purchased from BioWhittaker UK Ltd (Berkshire, UK) as pooled frozen aliquots. Cells (passage 2) were seeded at the recommended density of $2.5 \times 10^3/cm^2$ on tissue culture flasks coated with 0.1% gelatin. The cells were maintained in EGM Medium (Endothelial Cell Basal Medium, containing: 2% foetal calf serum, 12 μg/ml bovine brain extract, 10 ng/ml human recombinant epidermal growth factor, 1 μg/ml hydrocortisone, 50 μg/ml gentamycin, 50 ng/ml amphotericin-B, all purchased from BioWhittaker UK Ltd as EGM BulletKit). The monolayers were disrupted by brief treatment with trypsin/EDTA (trypsin 0.25%/Na_2EDTA 0.2%), subcultured at a 1 : 3–1 : 4 ratio and used between passage 3 and 4.

Antibodies, Cytokines and Reagents

The following monoclonal antibodies (mAb) were used: mouse IgG1 (clone B-T1) to human ICAM-2 (Serotec, UK); mouse IgG2a (clone CBR-IC2/2) to human ICAM-2 (Bender MedSystem, Germany); mouse IgG1 (clone 84H10) to human ICAM-1 (Serotec, UK); mouse IgG1 (clone 16803.1) to human TNF-α Receptor I (R&D Systems, UK); rabbit anti-human ZO-1 (Zymed, San Francisco, USA). Mouse IgG1, mouse IgG2a, anti-rabbit IgG control Abs and FITC-conjugated goat anti-mouse secondary Ab were from Sigma (St. Louis, MO, USA). Recombinant human TNF-α (activity 5×10^7 Units/mg) was purchased from Sigma; recombinant human IL-1β (activity 1.2×10^7 Units/mg) and IL-1 receptor antagonist (Eisenberg *et al.*, 1990) were a gift from Dr. Keith Ray (GlaxoWellcome). SYBRGreen nucleic acid stain was from Molecular Probes (Leiden, The Netherlands). Restriction enzymes, the luciferase reporter plasmid pGL3 basic, all the reagents for the luciferase detection assay and the AMV Reverse Transcription System were from Promega (Madison, WI, USA). The pCRScript cloning vector was from Stratagene (La Jolla, CA, USA). Total RNA extraction kit was from Qiagen (Chatsworth, CA, USA).

Flow Cytometry (FACS)

For FACS analysis, monolayers of resting or stimulated HUVECs were detached from culture flasks by brief treatment with trypsin/EDTA. The amount of ICAM-1 and ICAM-2 measured by FACS was similar when cells were detached by treatment with PBS containing 3 mM EDTA. The cells were washed and resuspended in PBS containing 10% FCS and 0.01% NaAzide (FACS buffer). The cells were then incubated with saturating amounts of anti-ICAM-1, anti-ICAM-2 or control mAb for 10 min at RT, washed 2× in cold FACS buffer and incubated with FITC-conjugated goat anti-mouse secondary Ab at 1 : 30 dilution in FACS buffer for 10 min at RT. After 2 washes, flow cytometry was performed using the EPICS XL cell sorter (Coulter Corporation, Miami, Florida) and analysed by the WinMDI software. In some experiments, cells were also stained after the final wash with 10 μg/ml of propidium iodide to monitor cell viability.

RNA Extraction, Reverse Transcription Polymerase Chain Reaction (RT-PCR) and Northern Blotting

Total cellular RNA was isolated from HUVECs using the Qiagen total RNA extraction kit. Semi-quantitative RT-PCR was performed as described (Su *et al.*, 1997). Briefly, cDNA was synthesized from 0.5 μg of total RNA using the Promega AMV Reverse Transcription System, in 5 mM $MgCl_2$, 1 mM each dNTP, 0.5 U ribonuclease inhibitor, 0.5 μg OligodT, 15 U AMV reverse transcriptase, for 15 min at 42°C. The enzyme was inactivated by incubation at 99°C for 5 min. PCR reactions were designed so that the same aliquot of cDNA was

used as template for ICAM-2 and GAPDH amplification in each reaction. PCR reactions were set up with all components, including 10 µl of reverse transcribed cDNA, except the primers. The reaction was then divided into two tubes, and ICAM-2 or GAPDH primers (200 ng) were added to each tube. ICAM-2 primer sequences: P1: TCGGATGAGAAGGTATTCGAG; P2: CATCTGGCTGTCCGACACA; GAPDH primer sequences: CT1: TGAAGGTCGGAGTCAACGGATTTGGT; CT2:CATGTGGGCCATGAGGTCCACCAC. PCR conditions were established by using serial dilutions of ICAM-2 cDNA (0.125 to 1 µg) as template for amplification, so that the amount of product obtained was proportional to the amount of ICAM-2 present as template. 2–5 µl of PCR product were analysed by electrophoresis on a 2% agarose gel, which was post-stained with SYBR-Green for 20 min, before analysis on a fluorimager (Molecular Dynamics, USA). Each individual DNA band was scanned and the number of pixels quantitated using the ImageQuant software package. For each experiment, three separate gels were quantified; for each experiment, PCR was performed on two separate occasions on freshly prepared cDNA.

Northern blotting was performed as previously described (Sambrook *et al.*, 1989). Briefly, 40 µg of total RNA were loaded on a 1% agarose–0.66 M formaldehyde gel, transferred on to nylon membrane (Boehringer Mannheim, Germany), UV cross-linked and hybridized with ^{32}P labelled full length human ICAM-2 cDNA and autoradiographed. The membranes were then reprobed with a ^{32}P labelled β-actin probe (838 bp PCR product, Clontech, USA). Densitometry of ICAM-2 and β-actin levels on Northern blots was carried out using a STORM phoshoimager and the ImageQuant software package.

Reporter Gene Constructs

The luciferase reporter vector pGL3 basic (Promega) was used to construct the ICAM-2 promoter plasmid. A 384 bp fragment of the human ICAM-2 promoter was obtained from human genomic DNA (Promega) by PCR, using primers IP1: CCAGGCATGACTCCAACAATGC; IP2: GACATCTCTGGCAGTGTCCACG (Cowan *et al.*, 1996). This fragment (nucleotides 13-397: accession number m32331) was cloned into pCRScript (Stratagene), released with *EagI*/*SmaI*, and cloned into the *SmaI* site of the pGL3 basic plasmid. The 384 bp fragment was sequenced and found to be identical to that published by Cowan *et al.* (1996). pGL3 basic was used as a negative control in all experiments. The SV40 control plasmid from Promega, containing the SV40 promoter and enhancer, was used as a positive control for luciferase activity. The SV40 enhancer contains a consensus NF-κB binding site and was therefore used also as a positive control for cytokine activation of HUVECs.

HUVECs Transfection and Luciferase Reporter Assay

HUVECs at passage 3 were seeded at 40% confluency the day before electroporation. On the day of transfection the cells were trypsinized and resuspended at a density of 2.5×10^6 cells/ml in RPMI containing 5% FCS. Five µg reporter plasmid, 0.5 µg β-galactosidase control plasmid and 10 µg carrier plasmid were used per point. Cells were electroporated at 350 V, 1500 µF using an Equibio electroporator and seeded into 3 ml of EGM medium in 35 mm dishes. For time course experiments, cells were left to recover for 24 h after electroporation, then were treated with TNF-α (10 ng/ml) or IL-1β (34 ng/ml). Twenty-four hours later cells were washed twice with PBS, harvested in 400 µl reporter lysis buffer (Promega) and assayed for luciferase and β-galactosidase activity. For luciferase assays, 40 µl cell lysate was added to 100 µl luciferase assay reagent at room temperature in a 96 well plate and light intensity measured for 1 s in a Wallac MicroBeta Luminometer. For β-galactosidase assays, 50 µl cell lysate was added to 50 µl 2× assay buffer (containing *o*-nitrophenyl-β-D-galactopyroanoside) in a 96 well plate. Samples were incubated at 37°C until colour developed

and absorbance was measured at 414 nm in a Titertek Multiskan spectrophotometer. Luciferase values were normalised for transfection efficiency using β-galactosidase values.

Fluorescence Confocal Microscopy of HUVEC Monolayers

Confluent HUVEC monolayers, grown on 0.1% gelatin coated plastic flaskettes, were fixed in 4% paraformaldehyde in PBS for 15 min and then washed in PBS, pH 7.4. To prevent non-specific antibody binding, cells were incubated with BSA 0.25% in PBS for 30 min. To permeabilize the cells for ZO-1 and phalloidin staining, 0.2% Triton X-100 was added to the blocking solution. After washing in PBS/BSA, the cells were stained for 1 h with 5 μg/ml anti-ICAM-2 mAb, 100 μg/ml anti-ICAM-1 mAb, 2 μg/ml rabbit anti-ZO-1 polyclonal Ab, or the equivalent amount of control Ab (IgG1, IgG2a or normal rabbit serum). After washing, cells were incubated for 1 h with 20 μg/ml of FITC-conjugated goat anti-mouse or anti-rabbit IgG in PBS/BSA. Cells were washed and nuclei counterstained at 37°C for 20 min with 2 μg/ml propidium iodide in the presence of 200 μg/ml RNase. Actin filaments were stained with 200 ng/ml phalloidin-TRITC for 20 min. After washing, the slides were mounted in Citifluor anti-fade mountant. All procedures, except for the propidium iodide staining, were performed at room temperature. Confocal microscopy for fluorescence was carried out with a Leica TCS4D confocal microscope (Leica Ltd, UK) with a 63× objective lens, which produces confocal sections approximately 0.4 μm thick. FITC fluorescence was imaged at 488 nm excitation and 530 nm emission; propidium iodide at 488 nm excitation and 600 nm emission, and TRITC at 548 nm excitation and 600 nm emission. A series of 12 confocal sections was taken from the apical to the basal surface of the cells, and used for three-dimensional image reconstructions. Low magnification phase microscope images were also taken with a 10× objective lens using 488 nm excitation and the non-confocal transmission detector of the confocal microscope.

RESULTS

TNF-α and IL-1β Decrease ICAM-2 Surface Expression on Endothelial Cells

To determine the effect of cytokines TNF-α and IL-1β on ICAM-2 expression, confluent HUVEC monolayers at passage 4 were treated with TNF-α (10 ng/ml), IL-1β (34 ng/ml) or IFN-γ (20 ng/ml) for 24 h, and surface expression of ICAM-1 and ICAM-2 was analysed by flow cytometry. Treatment with TNF-α or IL-1β decreased ICAM-2 surface expression by about 50% (Fig. 1A top panel and 1B), while IFN-γ had no effect on ICAM-2 expression, even at higher concentrations (200 ng/ml, data not shown). All three cytokines upregulated ICAM-1 expression (Fig. 1A bottom panel) as previously reported (Pober and Cotran, 1990). The combination of TNF-α and IL-1β did not decrease ICAM-2 expression any further (data not shown), suggesting that similar mechanisms are involved in TNF-α and IL-1β-dependent down-regulation of ICAM-2 expression. The effect of the cytokines on ICAM-2 expression was dose-dependent, as shown in Fig. 2. After 24 h incubation, ICAM-2 levels decreased to about 50% of control with 1 ng/ml TNF-α or 3.4 ng/ml IL-1β; no further decrease in ICAM-2 levels was obtained with higher cytokine concentrations. A time course of TNF-α or IL-1β showed that ICAM-2 expression decreased gradually over a period of 24 h, reaching about 50% of control levels (Fig. 3A). No further decrease was observed at 48 h (data not shown). Cytokine-induced increase in ICAM-1 expression was more rapid (Fig. 3B), as previously described (Pober and Cotran, 1990). ICAM-2 down-regulation by TNF-α was inhibited by an anti-TNF-Receptor I mAb; similarly, the IL-1β-induced down-regulation was inhibited by IL-1 receptor antagonist (data not shown), indicating that the effects on endothelial cells were specific for both cytokines. The decrease in ICAM-2 surface

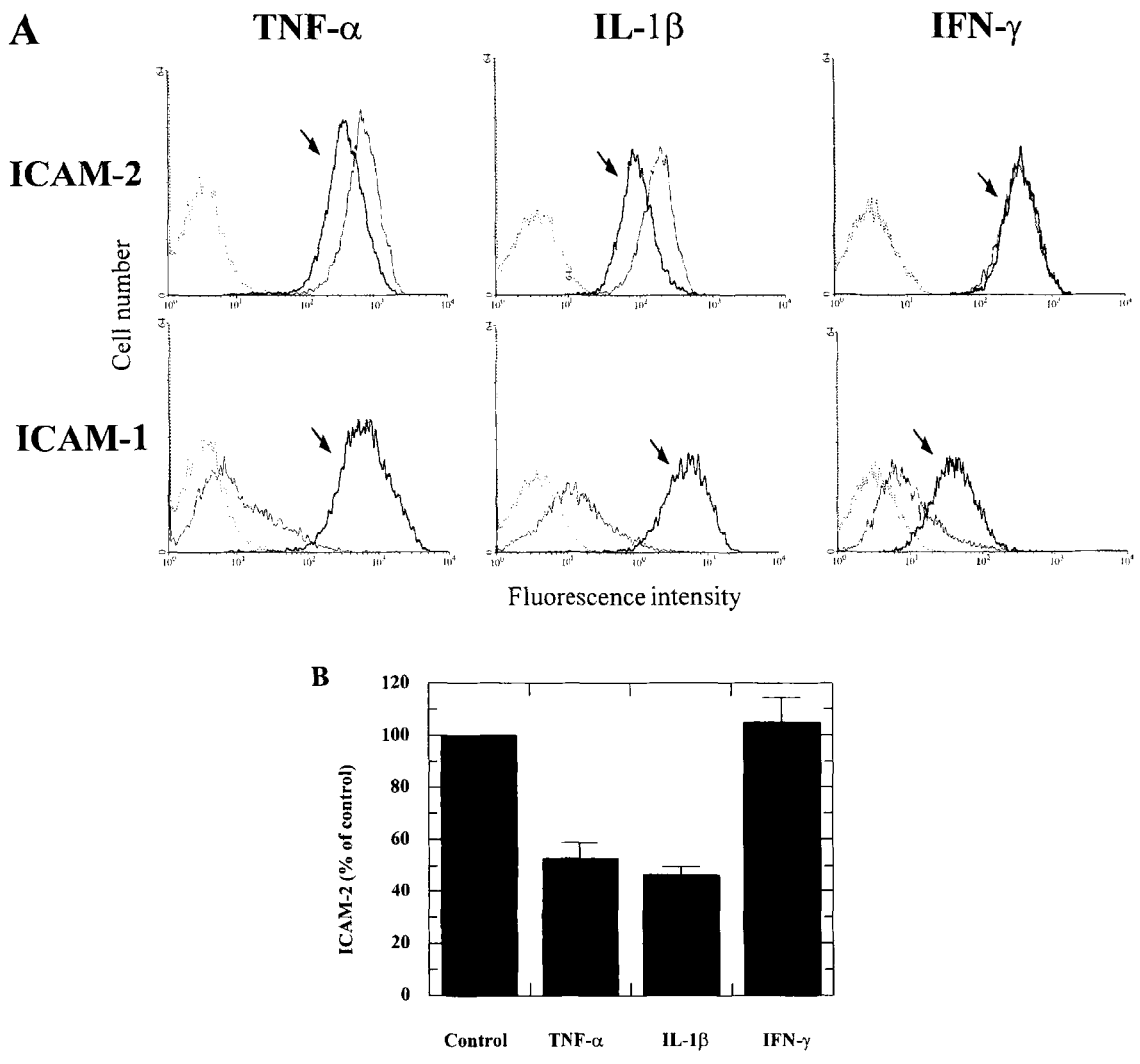

FIGURE 1 TNF-α and IL-1β down-regulate ICAM-2 expression on HUVECs. (A) Total surface expression of ICAM-2 (top) or ICAM-1 (bottom) on resting HUVECs (fine line) or HUVECs treated with TNF-α (10 ng/ml), IL-1β (34 ng/ml) or IFN-γ (20 ng/ml) for 24 h (thick line, indicated by arrows), determined by FACS analysis, using an anti-ICAM-2 or anti ICAM-1 mAb. Both TNF-α and IL-1β down-regulate ICAM-2 expression to 50% of control levels, while IFN-γ has no effect. All three cytokines upregulate ICAM-1 expression as expected. The FACS profile of a matching control Ab on resting HUVECs is shown in each panel (dotted line). One experiment representative of 5. (B) Down-regulation of ICAM-2 surface expression by TNF-α, IL-1β or IFN-γ. Cell surface expression was determined by FACS after 24 h incubation with TNF-α (10 ng/ml), IL-1β (34 ng/ml) or IFN-γ (20 ng/ml). Values are shown as mean $\pm$ SD of 5 separate experiments.

expression was not due to the procedure employed to disrupt the monolayer (see Methods), since the same results were obtained by resuspending the cells with PBS/EDTA 3 mM (data not shown). This data indicates that ICAM-2 expression on endothelial cells can be down-regulated by pro-inflammatory cytokines TNF-α and IL-1β.

TNF-α and IL-1β Decrease ICAM-2 Steady-State mRNA Levels

To determine whether the decrease in surface expression of ICAM-2 following treatment with TNF-α or IL-1β was due to decreased ICAM-2 synthesis, we examined the effect of the cytokines

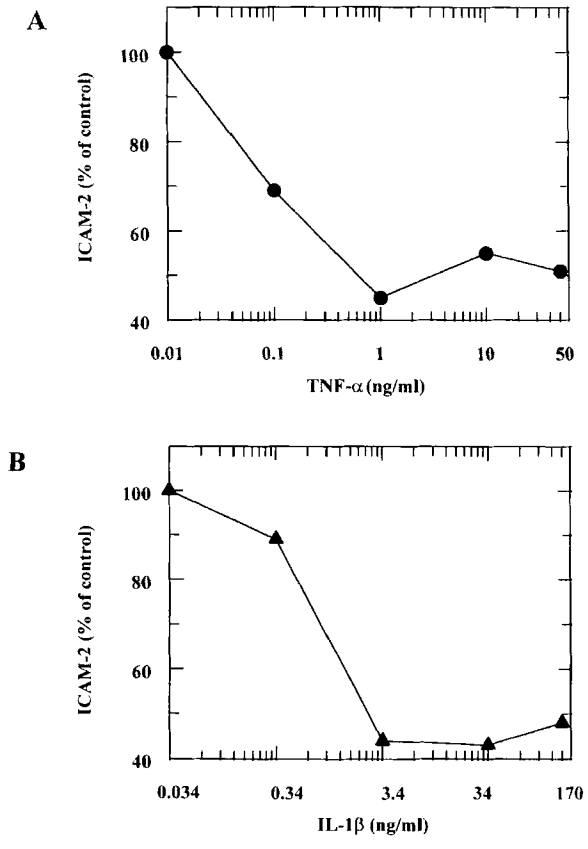

FIGURE 2 Dose-dependent down-regulation of ICAM-2 surface expression on HUVECs. HUVECs were treated with increasing concentrations of TNF-α (panel A, 0.01–50 ng/ml) or IL-1β (panel B, 0.034–170 ng/ml) for 24 h. Cell surface expression of ICAM-2 was determined by FACS analysis. Values are expressed as percentage of ICAM-2 levels in control HUVECs, from one experiment representative of three.

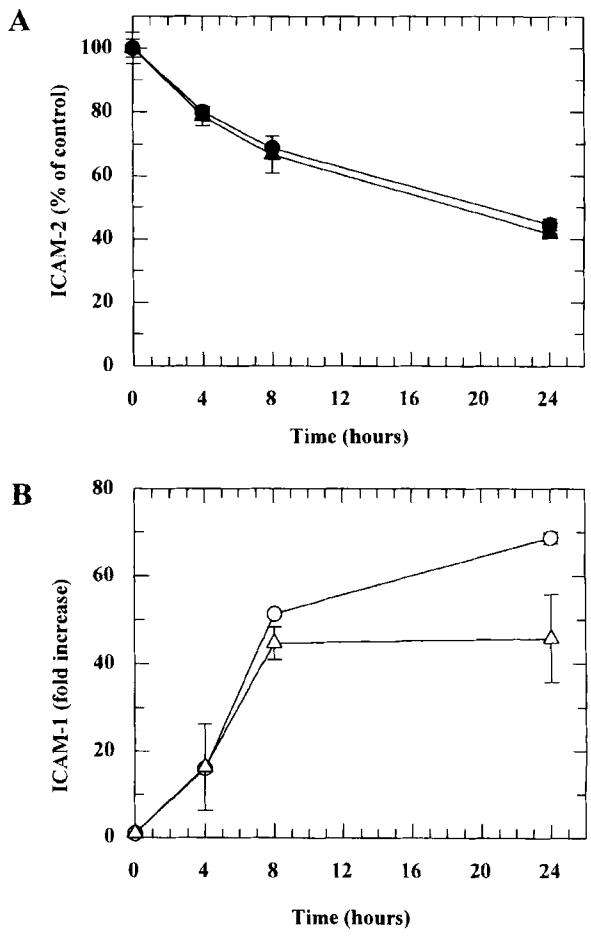

FIGURE 3 Time-course of ICAM-2 down-regulation (panel A) and ICAM-1 upregulation (panel B) by cytokines TNF-α and IL-1β. Confluent HUVEC monolayers were treated with either TNF-α (circles, 10 ng/ml) or IL-1β (triangles, 34 ng/ml) for the indicated times. Cell surface expression of ICAM-2 or ICAM-1 was determined by FACS analysis (see Methods). The data shown are the mean ± SEM of duplicate determination from one experiment representative of three separate experiments. ICAM-2 values are shown as percentage of expression on control cells; ICAM-1 values are shown as fold increase of expression over control cells.

on ICAM-2 mRNA by RT-PCR and by Northern blotting. Total RNA from resting or cytokine-treated cells was extracted and analysed by semi-quantitative RT-PCR (Su *et al.*, 1997) using primers spanning the extracellular domain of ICAM-2 (see Methods). A GAPDH fragment amplified from the same samples was used to normalize results. Figure 4A shows the RT-PCR products generated by ICAM-2 primers (top) and GAPDH primers (bottom) from total RNA extracted from HUVECs stimulated with TNF-α for 0, 4, 8 and 24 h. The same pattern was obtained from IL-1β-treated cells (not shown). In order to quantify the RT-PCR fragments, agarose gels were stained with SYBR Green, and each band was quantified by analysis on a fluorimager. Analysis of HUVECs treated with either TNF-α (10 ng/ml) or IL-1β (34 ng/ml) for 0, 4, 8 and 24 h revealed that ICAM-2 expression decreased gradually over time, to levels of 35–40% of control values by 24 h after cytokine treatment (Fig. 4B). For each experiment, three separate gels were quantified; for each experiment, PCR was

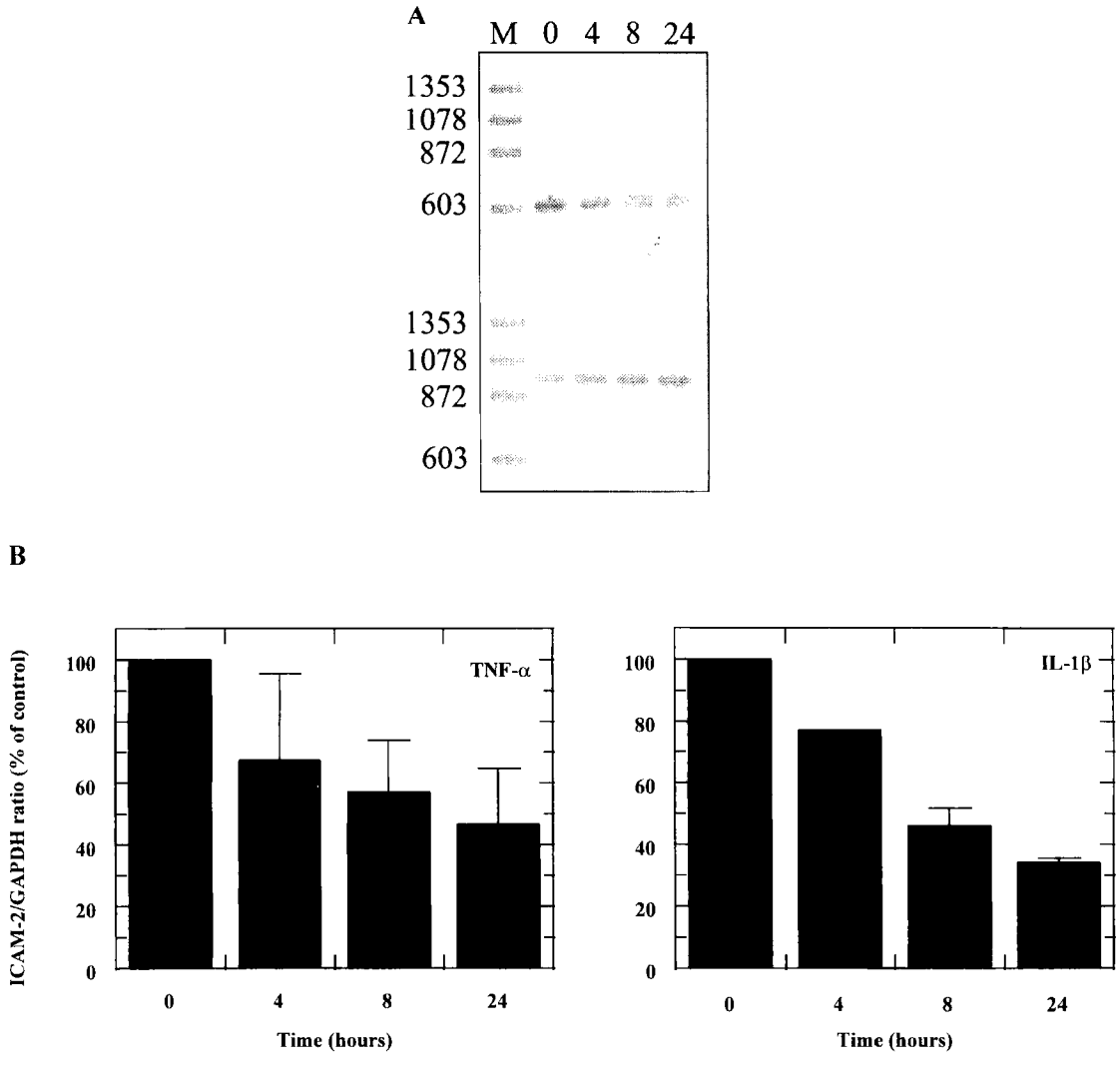

FIGURE 4 TNF-α and IL-1β down-regulate ICAM-2 mRNA in HUVECs: RT-PCR. (A) Gel analysis of RT-PCR of steady-state ICAM-2 (top, 622 bp) and GAPDH (bottom, 983 bp) mRNA in resting cells (0) or cells treated with TNF-α for 4, 8 or 24 h. Total RNA was extracted from resting HUVECs or cells stimulated with TNF-α (10 ng/ml) or IL-1β (34 ng/ml). 0.5 µg of total RNA was used as RT-PCR template. RT-PCR was performed as described Su *et al.*, 1997, using primers for ICAM-2 or GAPDH (see Methods). PCR products (4 µl) were run on a 2% agarose gel, post-stained with SYBRGreen and then visualized on a Fluorimager 575. M: molecular weight marker ϕX174 DNA-*HaeIII* digest; band sizes are indicated in bp. (B) Semi-quantitative RT-PCR analysis of ICAM-2 mRNA in HUVECs. RT-PCR bands visualized on a Fluorimager, as shown in Fig. 4A, were quantified using the ImageQuant software package. Each individual DNA band was scanned and the number of pixels counted. For each experiment, three separate gels were quantified; for each experiment, PCR was performed on two separate occasions on freshly prepared cDNA. Values are expressed as percentage of ICAM-2 mRNA levels in control cells. The data shows mean ± SD of three separate experiments.

performed on two separate occasions on freshly prepared cDNA.

Northern blot analysis was performed on samples treated with TNF-α or IL-1β (Fig. 5A) for 0 and 24 h. The ratio between ICAM-2 and β-actin mRNA (Fig. 5B) confirmed that both cytokines decrease ICAM-2 mRNA levels to about 50% after 24 h treatment, as shown by RT-PCR. Therefore

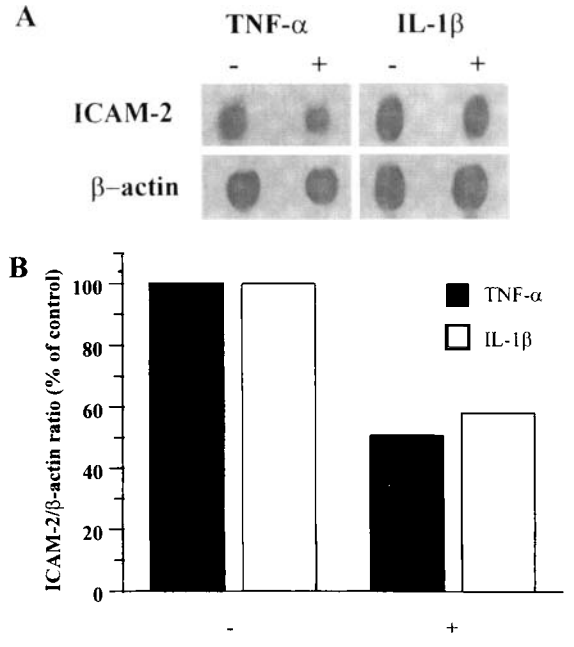

FIGURE 5 TNF-α and IL-1β down-regulate ICAM-2 mRNA in HUVECs: Northern blot analysis. (A) Total RNA was extracted from resting HUVECs or HUVECs treated with TNF-α (10 ng/ml) or IL-1β (34 ng/ml) for 24 h, transferred onto nitrocellulose filters and hybridised with ICAM-2 cDNA and β-actin probes. A representative autoradiograph is shown from 2–3 cytokine treatments. (B) Densitometric analysis of ICAM-2 mRNA levels, normalized against β-actin levels. Northern blots were scanned using a phosphoimager and band intensity was measured using the ImageQuant software package. The values are expressed as a percentage of the ICAM-2/ β-actin mRNA ratio in resting cells.

TNF-α and IL-1β down-regulate ICAM-2 mRNA levels in HUVECs; levels and time course of inhibition are comparable to that observed at the protein level.

The ICAM-2 Promoter is Down-regulated by TNF-α and IL-1β

Cytokines TNF-α and IL-1β down-regulate the expression of many endothelial antigens, including adhesion molecules (reviewed in Pober and Cotran, 1990). Several mechanisms have been implicated in cytokine-dependent down-regulation: decreased transcription in the case of vascular endothelial growth factor (VEGF) receptor 2/flk-1 (Patterson *et al.*, 1996) and thrombomodulin (TM) (Conway and Rosenberg, 1988; von der Ahe *et al.*, 1993), decreased mRNA stability for CD31 and CD34 (Delia *et al.*, 1993; Stewart *et al.*, 1996), reduced secretion for protein S (Hooper *et al.*, 1994). To determine the mechanism of ICAM-2 down-regulation in HUVECs, we analysed the effect of cytokines TNF-α and IL-1β on the activity of the ICAM-2 promoter. Figure 6 shows an alignment of the homologous regions between human and mouse ICAM-2 promoters. No TATA box is present in the promoters. Consensus binding sites for transcription factors ETS and GATA are conserved between mouse and human ICAM-2 promoters; an SP1 consensus site is present in the human but not in the mouse sequence. The ICAM-2 promoter does not contain a consensus site for transcription factors NF-κB and AP-1, which are involved in the cytokine-dependent upregulation of E-selectin and ICAM-1 (Collins *et al.*, 1995). A 384 bp fragment of the human ICAM-2 promoter (nucleotides 13-397, accession n. m32331) has been shown to drive tissue-specific expression in a mouse transgenic model (Cowan *et al.*, 1996). This fragment was cloned upstream of a luciferase reporter gene, and the construct was transiently transfected into HUVECs. Figure 7 shows the ICAM-2 promoter activity in resting cells (0 h) and in cells treated with TNF-α (Fig. 7A) or IL-1β (Fig. 7B) for 8 or 24 h. TNF-α (10 ng/ml) and IL-1β (34 ng/ml) inhibited the basal activity of the ICAM-2 promoter to about 25–40% of control, similar to the levels of ICAM-2 mRNA as measured by RT-PCR. The IL-1β-induced inhibition was slightly more pronounced than the TNF-α-induced one, however the difference between the two was not significant at either 8 h ($p = 0.129$, unpaired T-test) or 24 h ($p = 0.133$, unpaired T-test). In resting cells, the activity of the ICAM-2 promoter was similar to that of the SV40 control (Fig. 7C). The SV40 enhancer contains a consensus site for NF-κB, which has been shown to respond to IL-1β (Espel *et al.*, 1990); thus TNF-α and IL-1β-induced upregulation of the SV40 reporter activity in transfected HUVECs was used as

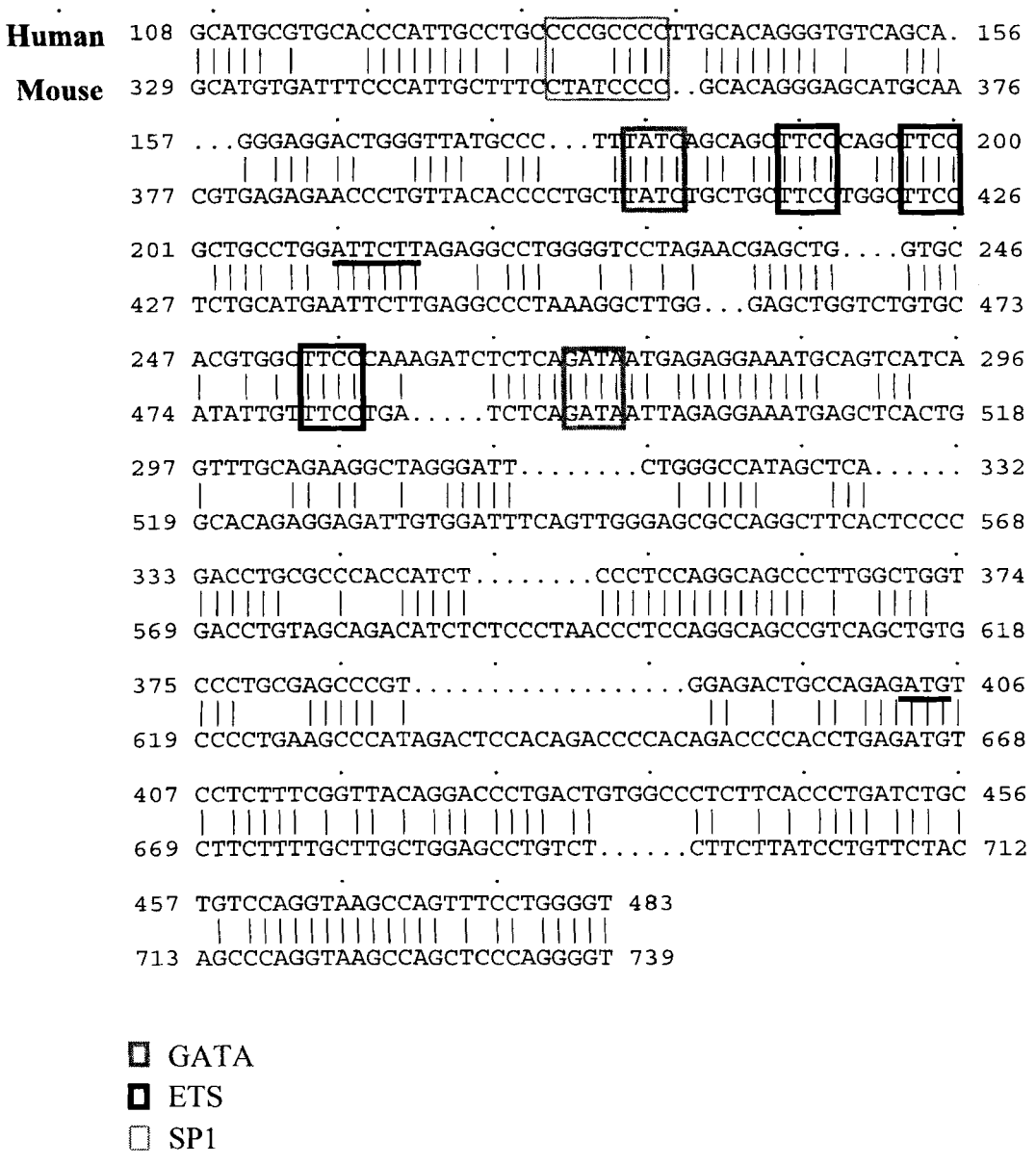

FIGURE 6 Alignment of human and mouse ICAM-2 promoters. The 384 bp fragment of the human ICAM-2 promoter has been shown to drive tissue-specific expression in amouse transgenic model Cowan *et al.*, 1996. Consensus binding sites for transcription factors GATA, ETS and SP1 are boxed. The SP1 site is present in human ICAM-2 only, while the GATA and ETS sites are conserved in both the human and mouse sequence. A potential transcriptional initiator element and the translation start site are underlined. No TATA box is present in the ICAM-2 promoter sequence.

positive control for cytokine treatment (Fig. 7C). Treatment with TNF-α (Fig. 7C) or IL-1β (not shown) had no effect on the basic pGL3 reporter plasmid activity. Therefore, this data shows that the down-regulation of ICAM-2 surface expression on HUVECs induced by TNF-α and IL-1β correlates with the transcriptional down-regulation of the ICAM-2 promoter.

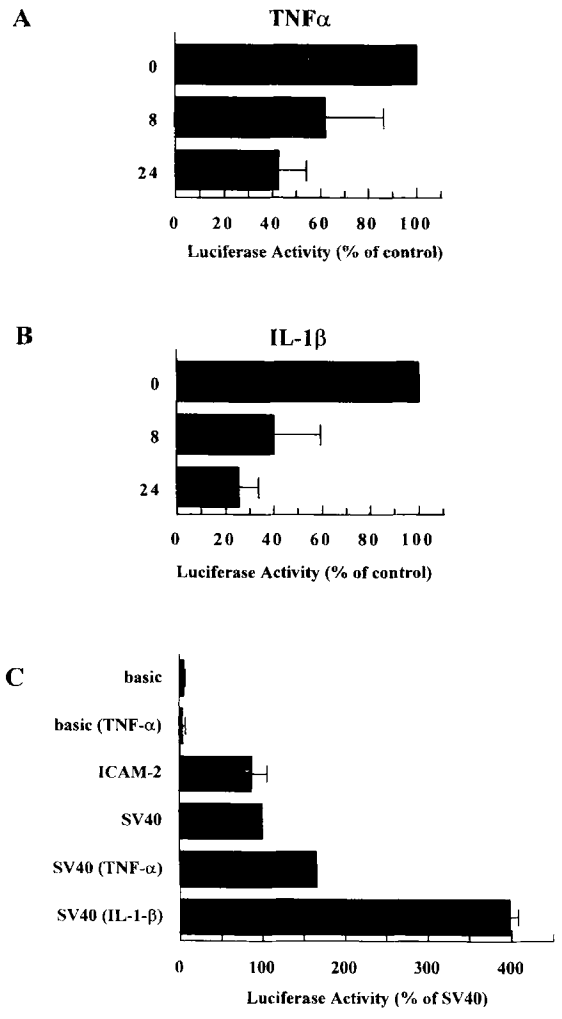

FIGURE 7 Cytokines inhibit the ICAM-2 promoter activity. The human ICAM-2 promoter was cloned into the luciferase reporter plasmid pGL3 (see Methods). The construct was transiently transfected into HUVECs and luciferase activity measured after 0, 8 and 24 h of TNF-α or IL-1β treatment. TNF-α (panel A) and IL-1β (panel B) down-regulate the ICAM-2 promoter activity respectively to 40% and 25% of control values. Luciferase activity was normalized against β galactosidase activity. Values are expressed as percentage of ICAM-2 promoter activity in control cells (0 h), as mean ± SD of 3–4 experiments. (C) Activity of the pGL3 luciferase plasmid in resting HUVECs (basic) and in HUVECs treated with TNF-α (10 ng/ml) [basic (TNF-α)]. Treatment with IL-1β (34 ng/ml) had a similar effect (data not shown). Also shown are the ICAM-2 promoter-luciferase plasmid (ICAM-2), the SV40 control plasmid (SV40) [containing a consensus site for the transcription factor NF-κB (Espel *et al.*, 1990)] in resting HUVECs and in HUVECs treated with TNF-α (10 ng/ml) or IL-1β (34 ng/ml) for 24 h. Luciferase activity was normalized against β galactosidase activity; values are expressed as percentage of the SV40 control plasmid, as mean ± SD of three separate experiments.

Cytokines Decrease ICAM-2 Staining at the Cell Junctions

Little is known about the effects of cytokines on the localization of adhesion molecules which are constitutively expressed on the endothelium. TNF-α, but not IL-1β, was shown to induce CD31 redistribution from the cell junctions to the apical surface (Romer *et al.*, 1995). We set out to investigate the effects of TNF-α and IL-1β on ICAM-2 distribution on HUVECs. TNF-α and IL-1β are known to induce morphological changes in endothelial cells in culture (Pober and Cotran, 1990); since culture conditions can drastically affect the endothelial phenotype, we first examined the morphological and cytoskeletal changes induced by the cytokines in our experimental system. After 24 h incubation with TNF-α (10 ng/ml) or IL-1β (34 ng/ml), HUVEC monolayers lose their cobblestone-like appearance and become elongated and enlarged (Fig. 8(a)–(c)). This correlates with a reorganisation of the actin filaments: in resting cells, actin rings are found around the periphery of the cell (Fig. 8(d)), while in TNF-α or IL-1β-treated cells actin filaments are organized in bundles, which run along the longitudinal axis of the cells (Fig. 8(e) and (f)). These changes are similar to those reported by several investigators (Pober and Cotran, 1990). To determine whether cytokines affect ICAM-2 distribution on endothelial cells, we performed fluorescent confocal microscopy. Confluent HUVEC monolayers were labelled with anti-ICAM-2 mAb followed by FITC-conjugated anti-mouse Ab (green) and with propidium iodide (red) to visualize the nuclei. In resting HUVECs, intense ICAM-2 staining was observed at the cell junctions (Fig. 9(a)), with some apical expression. Treatment of HUVECs with either cytokine for 24 h resulted in reduced overall ICAM-2 staining (Fig. 9(b) and (c)). The effect appeared more pronounced at the cell junctions: the intense linear staining for ICAM-2 was almost completely absent in IL-1β-treated cells, and only occasionally present at some junctions in TNF-α-treated cells. Resting HUVEC monolayers stained for ICAM-1 (green) showed very low

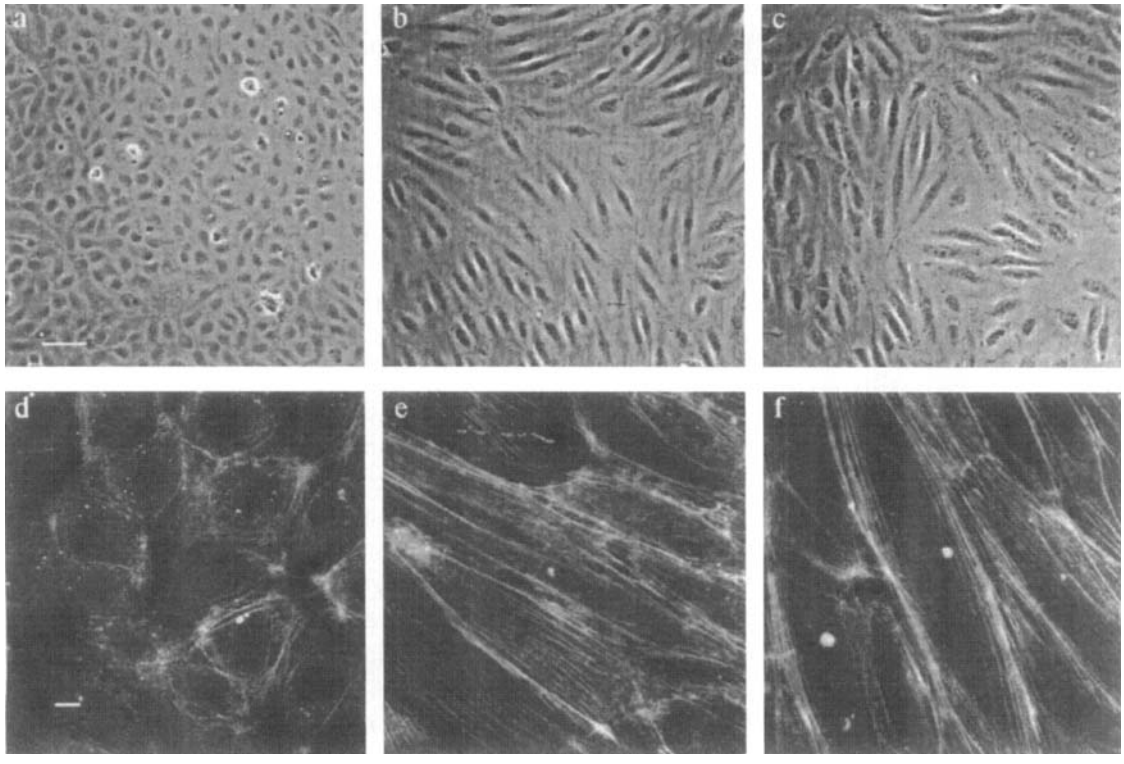

FIGURE 8 Morphological changes in HUVECs monolayers induced by cytokine treatment, here shown by phase microscopy (a)–(c) and actin immunofluorescence (d)–(f). Treatment with IL-1β or TNF-α induced similar structural changes, although slightly more pronounced in the IL-1β-treated cells. (a) Confluent monolayer of resting HUVECs showing the typical cobblestone morphology; treatment with TNF-α (10 ng/ml) (b) or with IL-1β (34 ng/ml) (c) for 24 h results in cell elongation and enlargement. (d) Single confocal sections showing that actin microfilaments, stained here with phalloidin-FITC, form cortical rings just beneath the lateral cell membrane. Treatment with TNF-α (10 ng/ml) (e) or IL-1β (34 ng/ml) (f) for 24 h results in the formation of stress fibers, which run in bundles along the longitudinal axis of the cells, just below the lateral cell membrane; adhesion plaques are visible where the cells narrow and make contact. Bar line 100 μm in (a) (c) and 10 μm in (d) and (e).

constitutive expression (Fig. 9(d)); cytokine treatment upregulated ICAM-1 expression on the apical surface of the cells. Occasionally, ICAM-1 staining had a dotted appearance (Fig. 9(e) and (f)), consistent with ICAM-1 being concentrated on the microvilli (Almenar-Queralt Angels *et al.*, 1995). In order to rule out that the decrease in ICAM-2 junctional expression was due to disruption of the endothelial junctions, monolayers were stained with antibodies to the tight junction marker ZO-1. As shown in Fig. 9(g)–(i), ZO-1 staining is localized to the junctions in both resting and cytokine-stimulated HUVECs, suggesting that the disappearance of ICAM-2 from the endothelial junctions is not due to disruption of the overall integrity of the junctions. To further characterize the pattern of ICAM-2 distribution after cytokine treatment, individual confocal sections of HUVECs monolayers were analysed. Comparison of serial sections from the top (a) to the bottom (i) of resting (Fig. 9) and TNF-α or IL-1β treated monolayers (Figs. 11 and 12 respectively) shows differences in the distribution of ICAM-2 on the cell surface. In the top sections, ICAM-2 staining forms a cap over the apical surface of both resting (Fig. 10(a)–(c)) and cytokine-treated cells (Figs. 11 and 12(a)–(c)); the staining is moderately

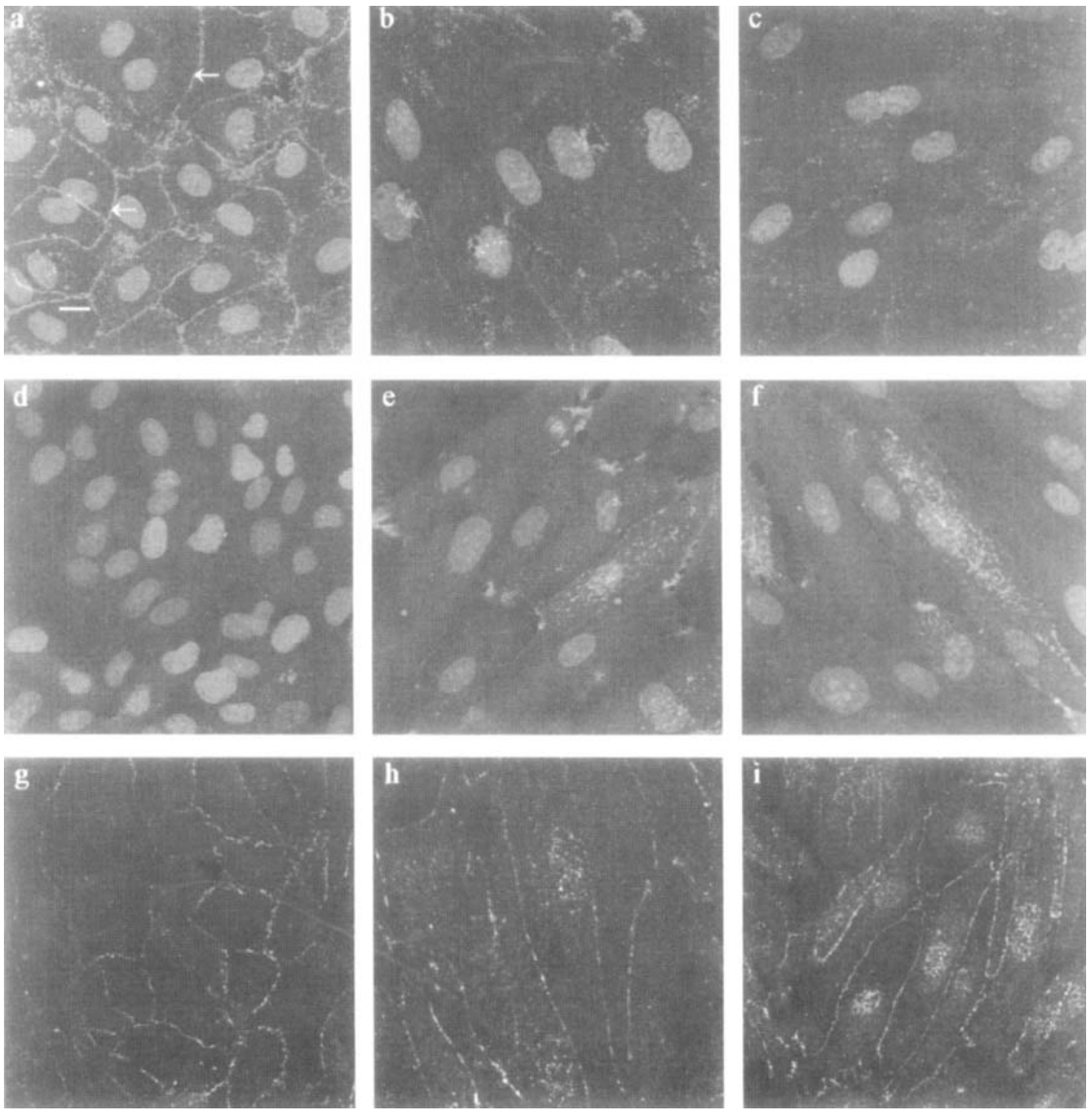

FIGURE 9 Confocal 3D reconstructions of HUVECs monolayers, made from 12 sections, stained for ICAM-2 (top), ICAM-1 (middle) or ZO-1 (bottom). All cytokine treatments were as follows: TNF-α (10 ng/ml), IL-1β (34 ng/ml) for 24 h. (a) In resting cells, ICAM-2 staining (green) is concentrated mainly at the cell junctions (arrows), with some staining on the apical surface of the cells. Treatment with TNF-α (b) or IL-1β (c) for 24 h results in decreased overall staining for ICAM-2, as well as loss of ICAM-2 localization at the cell junctions, particularly in IL-1β-treated cells. Nuclei are stained in red by propidium iodide (see Methods). (d) ICAM-1 staining (green) in confluent resting HUVECs is very weak, however treatment with TNF-α (e) or IL-1β (f) results in a upregulation of ICAM-1 expression, which is mainly concentrated on the apical surface. Note the intense immunostaining on isolated cells, likely to be due to ICAM-1 localization on microvilli (Almenar-Queralt, Angels *et al.*, 1995). Nuclei are stained in red by propidium iodide. (g) (i) Staining for ZO-1 in confluent resting HUVEC cells (nuclei are not shown). ZO-1 is concentrated at the cell junctions, and appears as a narrow line both in resting (g) and cytokine-treated cells (h, TNF-α; i, IL-1β). Note the elongated and enlarged shape of HUVECs treated with either cytokine (h), (i). Bar line 10 μm. (See Color Plate III.)

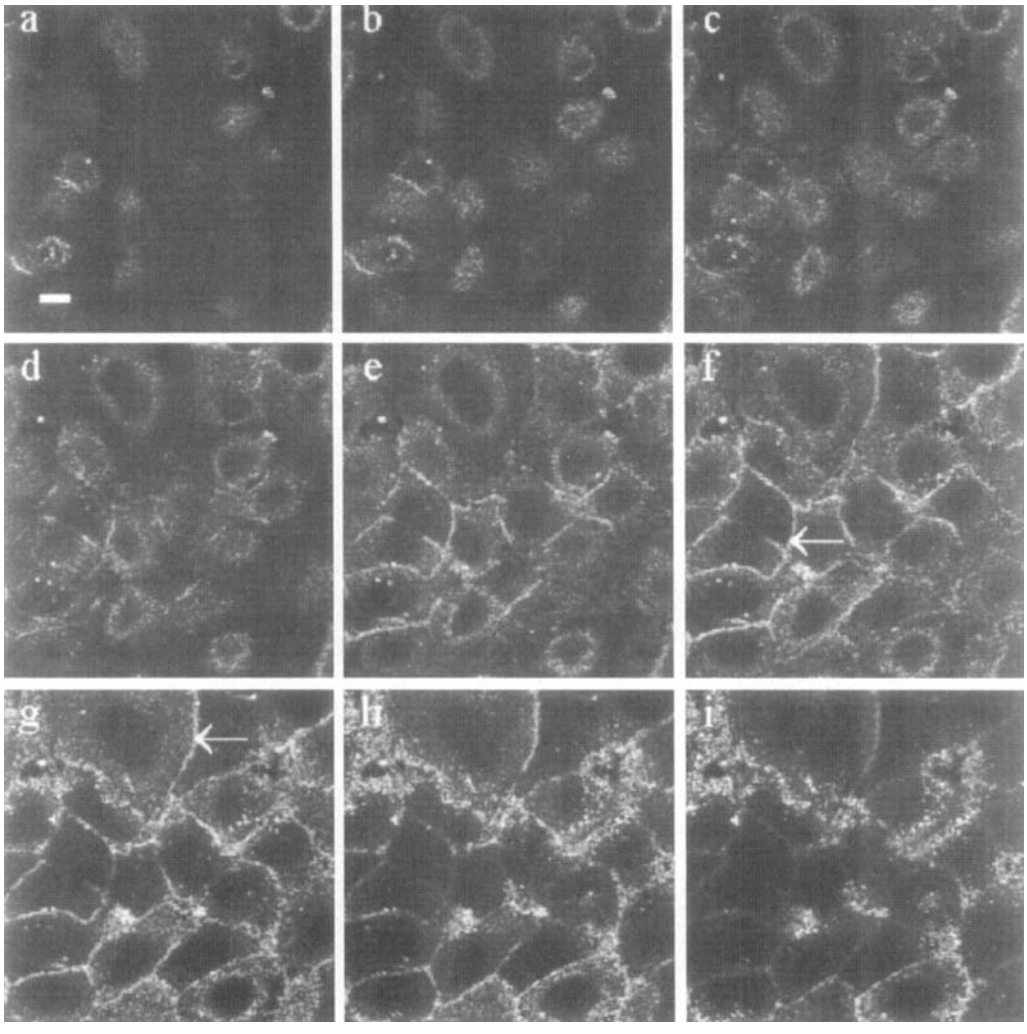

FIGURE 10 Serial confocal sections of a monolayer of resting HUVECs, stained with anti-ICAM-2 mAb. ICAM-2 expression is present both on the apical surface of the cells (a)–(c) as well as at the junctions (e)–(i), where it forms an intense line at the points of cell–cell contact. One representative experiment of three is shown. Bar line 10 μm.

reduced in cytokine-treated cells. However, ICAM-2 junctional staining, visible in resting cells as an intense line at the points of cell–cell contact (Fig. 10(e)–(i)), is reduced in cells treated with TNF-α (Fig. 11(e)–(i)) or IL-1β (Fig. 12(e)–(i)), and is replaced by diffuse staining. Therefore the decrease in ICAM-2 expression observed at the protein and mRNA level correlates with decreased surface staining, with a marked reduction in ICAM-2 expression at the endothelial cell junctions.

DISCUSSION

ICAM-2, a ligand for β2 integrins LFA-1 and Mac-1 on endothelial cells, platelets and leukocytes, is constitutively expressed on the endothelium (Defougerolles *et al.*, 1991; Springer, 1994; Staunton *et al.*, 1989) and has been implicated in lymphocyte recirculation (Defougerolles *et al.*, 1991); however its role in inflammation is still unclear. In this paper, we show that prolonged

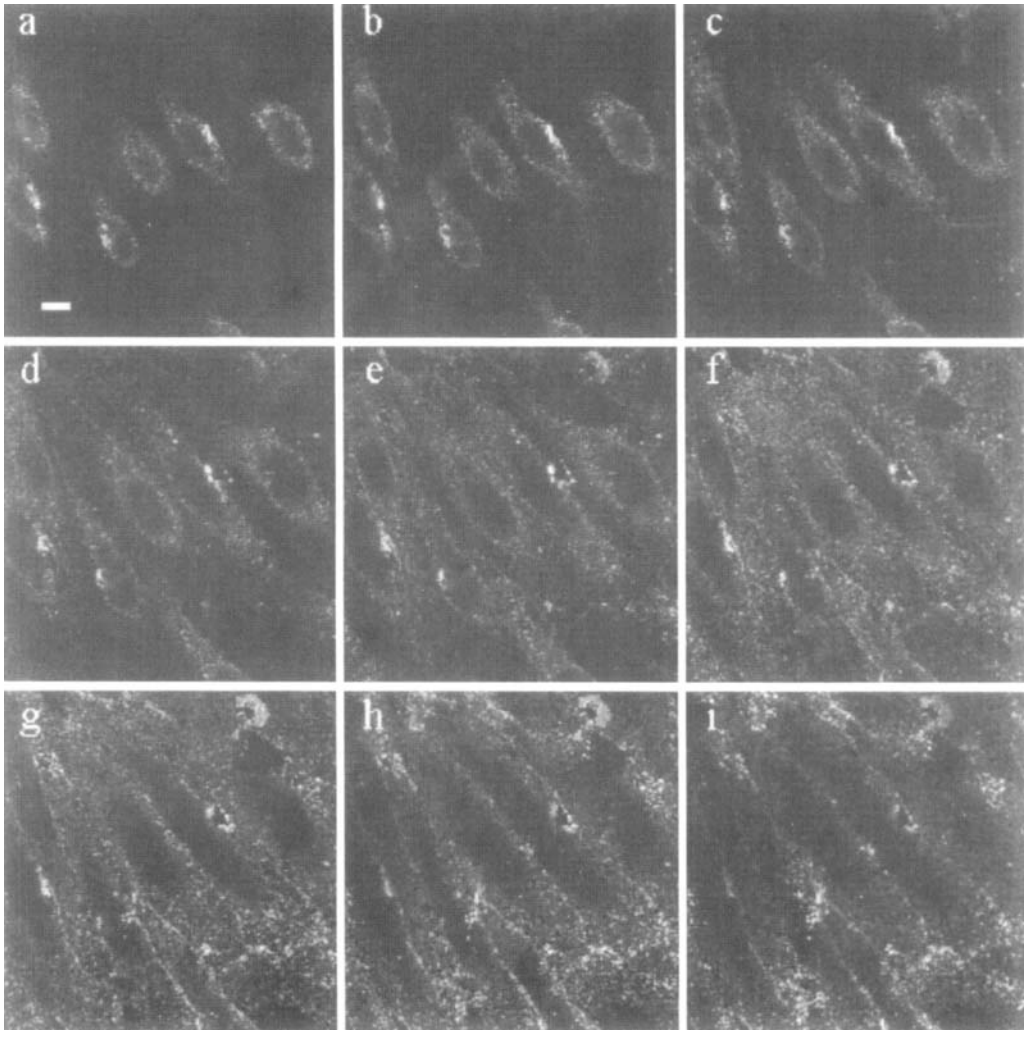

FIGURE 11 Serial confocal sections of a monolayer of HUVECs treated with TNF-α (10 ng/ml) for 24 h, stained with an anti-ICAM-2 mAb. ICAM-2 staining is present on the apical surface of the cells (a)–(c), moderately reduced compared to resting cells (Fig. 9). The intense ICAM-2 staining at the endothelial junctions observed in resting cells (Fig. 9) is markedly decreased, although still present in some areas of the monolayer (e)–(i). One representative experiment of three is shown. Bar line 10 μm.

exposure (24 h) to inflammatory cytokines TNF-α and IL-1β down-regulates ICAM-2 surface expression on endothelial cells *in vitro*, particularly at the cell junctions. Thus ICAM-2 expression is susceptible to regulation by inflammatory cytokines, suggesting that ICAM-2 may play a role in the endothelial response to inflammation.

ICAM-1 and ICAM-2 are the two endothelial ligands involved in LFA-1 dependent adhesion and transmigration. Several *in vitro* studies have investigated the role of ICAM-1 and ICAM-2 in leukocyte adhesion to endothelial cells, without being able to define a specific and distinct role for ICAM-2 (Defougerolles *et al.*, 1991; Roth *et al.*, 1995). Our results show that the endothelial expression of these two Ig superfamily members, which share ligands and functions, is reciprocally regulated by inflammatory cytokines. The magnitude of

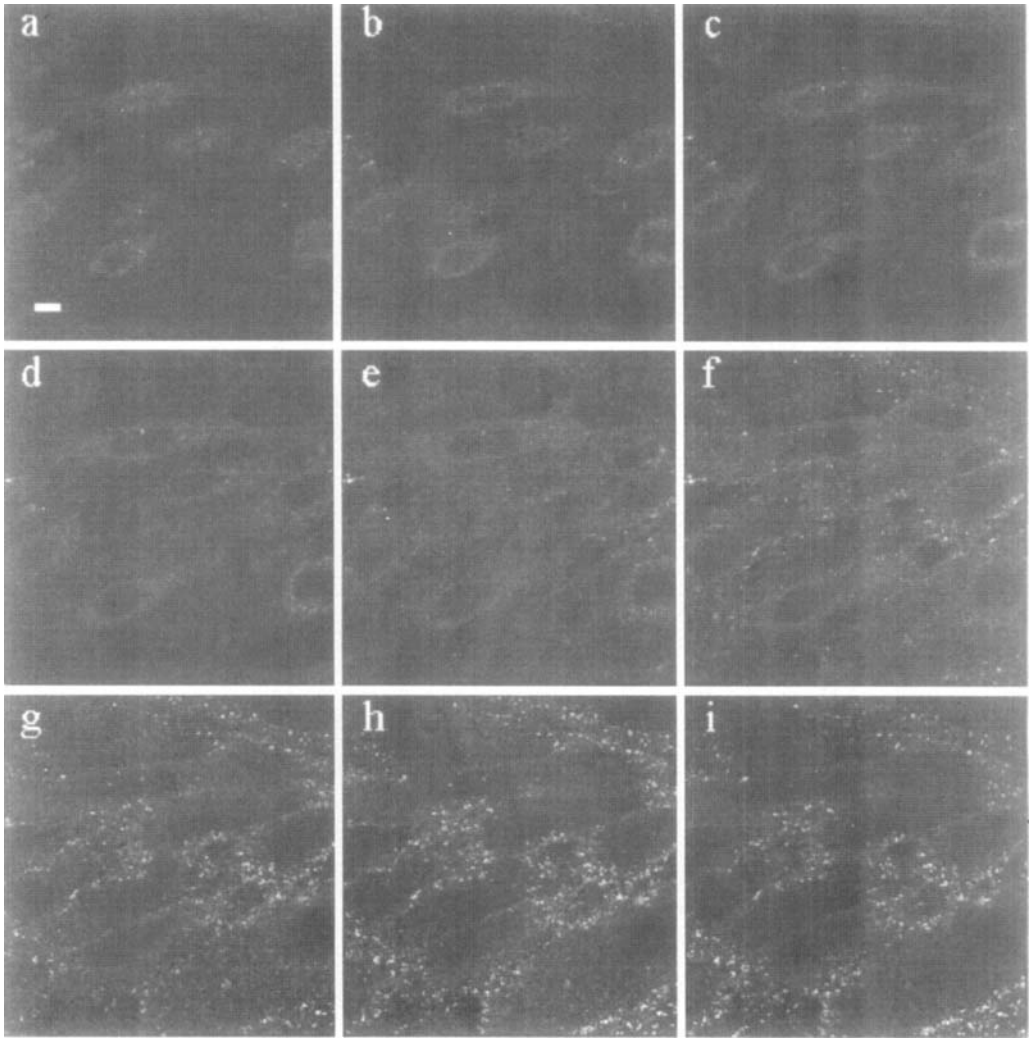

FIGURE 12 Serial confocal sections of a monolayer of HUVECs treated with IL-1β (34 ng/ml) for 24 h, stained with an anti-ICAM-2 mAb. ICAM-2 staining is present on the apical surface of the cells (a)–(c), although reduced compared to control cells (Fig. 9). Very little staining is visible at the endothelial junctions throughout the monolayer (e)–(i). One representative experiment of three is shown. Bar line 10 μm.

the response and the time course of ICAM-1 and ICAM-2 regulation by cytokines are very different: whether the two events are functionally correlated remains to be established. The different *in vitro* response to cytokines may reflect variations in the pattern of *in vivo* expression of adhesion molecules at early and late stages during an inflammatory response (Granger and Kubes, 1994; Schleiffenbaum and Fehr, 1996). Selectivity and specificity of leukocyte influx in the various stages of inflammation could therefore be partially regulated by differential expression of endothelial adhesion molecules.

Other adhesion molecules constitutively expressed on the endothelium involved in leukocyte recruitment are down-regulated by inflammatory cytokines. Treatment of HUVEC for 72 h with IL-1β or TNF-α in combination with IFN-γ was

shown to down-regulate the expression of CD34, a ligand for L-selectin (Puri *et al.*, 1995), by decreasing mRNA levels (Delia *et al.*, 1993). The effect of inflammatory cytokines on endothelial CD31 is less clear. CD31 is an Ig superfamily member constitutively expressed at the endothelial cell junctions (reviewed in Newman, 1997) with a staining pattern similar to ICAM-2. CD31 expression on bovine endothelial cells has been shown to be down-regulated by TNF-α (100 ng/ml) and IFN-γ, but not by IL-1β (Stewart *et al.*, 1996). In HUVECs however TNF-α, but not IL-1β, was shown to induce CD31 redistribution from the cell junctions to the apical surface, without affecting the levels of expression (Romer *et al.*, 1995). In our study, TNF-α and IL-1β, but not IFN-γ, were found to affect both ICAM-2 expression levels and the cellular localization. Endothelial junctions control leukocyte extravasation, therefore regulation of the expression of adhesion molecules at the cell junctions (such as CD31 or ICAM-2) will be crucial in regulating leukocyte transmigration. This data also suggest that different mechanisms may be involved in the cytokine-dependent down-regulation of adhesion molecules CD31, CD34 and ICAM-2.

Why inflammatory cytokines should decrease the expression of molecules involved in leukocyte recruitment, such as CD34, CD31 or ICAM-2, is not clear. The gradual down-regulation of adhesion molecules required for leukocyte adhesion and transmigration could contribute to limit the extent of the inflammatory infiltrate over time. Alternatively, these molecules may be exerting an inhibitory effect on leukocyte adhesion to the endothelium. No such evidence is available for CD31 or CD34; however other adhesion molecules such as ICAM-3, N-CAM (Acheson *et al.*, 1997) and CD43 (Ardman *et al.*, 1992) have been shown to have both pro-adhesive and anti-adhesive activity. ICAM-3 is, like ICAM-2, a ligand for β2 integrins and is constitutively expressed on leukocytes but not on endothelial cells (Fawcett *et al.*, 1992; Vazeux *et al.*, 1992). Monoclonal antibodies to ICAM-3 have been shown to promote T cell adhesion to HUVEC via activation of β2 integrin function (Campanero *et al.*, 1993; 1994), but also to inhibit neutrophil adhesion to HUVECs by preventing neutrophil activation and upregulation of Mac-1 (Skubitz *et al.*, 1997). In the case of ICAM-2, Gahmberg and colleagues showed that a peptide from domain 1 induced LFA-1/ICAM-1-dependent T cell aggregation (Li *et al.*, 1993a) and Mac-1 binding to fibrinogen and iC3b (Li *et al.*, 1995), however it also inhibited the ICAM-1-dependent adhesion of leukocytes to the endothelial cell line Eahy926 (Li *et al.*, 1993b). This suggests that ICAM-2 could have both pro-adhesive and anti-adhesive activities, possibly inducing different integrin activation states depending on the cellular background, the presentation of the ligand, the glycosylation state of the molecules or other unknown variables. The *in vivo* relevance of these observations is unknown. It is tempting to speculate that ICAM-2 modulates leukocyte adhesion and transmigration via regulation of the affinity state of β2 integrins, and that variations in the levels of ICAM-2 expression on endothelial cells, particularly at the cell junctions, could influence such mechanism. Support for this model comes from the recent observations on ICAM-2 expression in human fetal brain (Rezaoe *et al.*, 1997). ICAM-2 expression is transiently upregulated between weeks 16 and 22, at the time of microglia infiltration of the brain parenchima, and subsequently down-regulated, as shown by the very low levels of ICAM-2 in the adult brain.

TNF-α and IL-1β modulate many endothelial functions, including adhesion, migration, haemostasis and vascular permeability (reviewed in Kuno and Matsushima, 1994; Pober and Cotran, 1990; Vandenabeele *et al.*, 1995), transforming the resting anti-adhesive surface into a pro-adhesive one. TNF-α and IL-1β activate endothelial cells to express a number of proteins, including adhesion molecules E-selectin, ICAM-1 and VCAM, via a transcriptional mechanism which has been extensively investigated (Collins *et al.*, 1995; Hou *et al.*, 1994; Schindler and Baichwal, 1994). However cytokines also decrease the expression of several endothelial antigens, including the already mentioned

adhesion molecules CD31 and CD34, integrin subunits $\alpha 6$ (Defilippi *et al.*, 1991a) and $\beta 3$ (Defilippi *et al.*, 1991b), coagulation proteins TM (von der Ahe *et al.*, 1993) and protein S (Hooper *et al.*, 1994) and the VEGF receptor 2/*flk-1* (Patterson *et al.*, 1996). Several mechanisms have been implicated in this cytokine-mediated down-regulation: reduced secretion (protein S), decreased mRNA stability (CD31, $\beta 3$), or decreased transcription (TM, *flk-1*). We studied the effect of cytokines TNF-α and IL-1β on ICAM-2 promoter activity, and found that TNF-α and IL-1β inhibit the activity of the ICAM-2 promoter to levels of 25–40% of control. The down-regulation of the ICAM-2 promoter activity followed a time course similar to that observed at the protein level. Therefore we have shown that TNF-α and IL-1β negatively regulate ICAM-2 transcription in endothelial cells. That other mechanisms triggered by cytokines may also contribute to ICAM-2 down-regulation cannot be ruled out; however no soluble ICAM-2 was detected in the medium of resting or cytokine-treated cells (data not shown), suggesting that shedding does not play an important role in ICAM-2 down-regulation. The ICAM-2 promoter does not contain consensus sites for NF-κB or AP-1, the trans-activating factors most consistently involved in the cytokine-dependent upregulation of genes involved in inflammation (Collins *et al.*, 1995). Therefore cytokine-dependent negative regulatory pathways must be present in endothelial cells. Among the endothelial genes whose expression is down-regulated by TNF-α, TM is the best characterized in terms of response to cytokines. TM is a cell surface molecule constitutively expressed on endothelial cells, which maintains the anti-thrombotic properties of the endothelium by binding to thrombin and triggering the protein C-dependent anticoagulant pathway (Esmon, 1995). Inflammatory agents modify the haemostatic balance on the endothelium by upregulating the expression of pro-coagulant molecules such as tissue factor and down-regulating the expression of anti-coagulant proteins such as TM. TNF-α has been shown to decrease TM transcription (von der Ahe *et al.*, 1993); a region in the TM promoter containing binding sites for the transcription factor ETS appears to be required for the TNF-α-dependent negative regulation (von der Ahe *et al.*, 1993). Analysis of the ICAM-2 promoter sequences shows that three ETS consensus sites are present and conserved between mouse and human (Fig. 6). Studies are in progress to identify the TNF-α- and IL-1β-responsive elements in the ICAM-2 promoter.

Previous studies have failed to show an effect of inflammatory cytokines on ICAM-2 expression *in vitro* (Defougerolles *et al.*, 1991; Nortamo *et al.*, 1991; Staunton *et al.*, 1989), thus questioning its *in vivo* role in inflammation. ICAM-2 expression can be regulated both *in vitro* and *in vivo* by angiogenic factors such as basic fibroblast growth factor (Griffioen *et al.*, 1996); such factors may be present in variable concentrations in different culture conditions, and may thus in part account for the observed discrepancy. Detailed analysis of ICAM-2 expression in vessels from inflammed tissues will be required to understand the implications of our finding. Preliminary analysis of inflamed synovial tissues from patients with rheumatoid arthritis shows that not all vascular endothelium expresses ICAM-2, suggesting that some of regulation of expression may be occurring *in vivo* (Ludbrook and Randi, unpublished).

In conclusion, we have shown that ICAM-2 expression on the endothelium can be regulated by inflammatory stimuli. Prolonged activation of endothelial cells by TNF-α and IL-1β, known to induce increased adhesion and transmigration *in vitro* by upregulating ICAM-1 and VCAM (Jones *et al.*, 1994), coincided with a marked reduction of ICAM-2 expression, more pronounced at the cell junctions. This finding suggests that ICAM-2 may play a role in the inflammatory response of the endothelium. Inflammatory stimuli modulate many endothelial functions, such as maintenance of vascular tone or haemostasis, by altering the balance between promoting and inhibiting factors. Whether this is also true for the mechanisms regulating leukocyte recruitment remains to be

established. Studies are in progress to determine the role of ICAM-2 down-regulation in the context of the endothelial response to inflammation.

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References

Acheson, A. *et al.* (1997) "NCAM polysialic acid can regulate both cell–cell and cell–substrate interactions". *Journal of Cell Biology* **114**(1), 143–153.

Almenar-Queralt, Angels *et al.* (1995) "Apical topography and modulation of ICAM-1 expression on activated endothelium". *American Journal of Pathology* **147**(5), 1278–1287.

Ardman, B. *et al.* (1992) "CD43 interferes with T-lymphocyte adhesion". *Proceedings of the National Academy of Sciences of the United States of America* **89**, 5001.

Bradley, J.R. *et al.* (1995) "Hydrogen peroxide-induced endothelial retraction is accompanied by a loss of the normal spatial organisation of endothelial cell adhesion molecules". *American Journal of Pathology* **147**, 627–641.

Buckley, C.D. and Simmons, D.L. (1997) "Cell adhesion: a new target for therapy". *Molecular Medicine Today* **3**, 449–456.

Campanero, M.R. *et al.* (1993) "ICAM-3 interacts with LFA-1 and regulates the LFA-1/ICAM-1 cell adhesion pathway". *Journal of Cell Biology* **123**(4), 1007–1016.

Campanero, M.R. *et al.* (1994) "ICAM-3 regulates lymphocyte morphology and integrin-mediated T cell interaction with endothelial cell and extracellular matrix ligands". *Journal of Cell Biology* **127**(3), 867–878.

Casasnovas, J.M. *et al.* (1997) "Crystal structure of ICAM-2 reveals a distinctive integrin recognition surface". *Nature* **387**(6630), 312–315.

Collins, T. *et al.* (1995) "Transcriptional regulation of endothelial cell adhesion molecules: NF-kB and cytokine-inducible enhancers". *FASEB J* **9**(10), 899–909.

Conway, E.M. and Rosenberg, R.D. (1988) "Tumor Necrosis Factor suppresses transcription of the thrombomodulin gene in endothelial cells". *Molecular and Cellular Biology* **8**(12), 5588–5592.

Cowan, P.J. *et al.* (1996) "Targeting gene expression to endothelial cells in transgenic mice using the human intercellular adhesion molecule 2 promoter". *Transplantation* **62**(2), 155–160.

Defilippi, P. *et al.* (1991a) "$\alpha_6\beta_1$ Integrin (laminin receptor) is down-regulated by Tumor Necrosis Factor α and Interleukin-1β in human endothelial cells". *Journal of Biological Chemistry* **267**(26), 18303–18307.

Defilippi, P. *et al.* (1991b) "Tumor Necrosis Factor α and Interferon γ modulate the expression of the vitronectin receptor (Integrin β_3) in human endothelial cells". *Journal of Biological Chemistry* **266**, 7638–7645.

Defougerolles, A.R. *et al.* (1991) "Characterisation of ICAM-2 and evidence for a third counter-receptor for LFA-1". *Journal of Experimental Medicine* **174**, 253–267.

Delia, D. *et al.* (1993) "CD34 expression is regulated reciprocally with adhesion molecules in vascular endothelial cells *in vitro*". *Blood* **81**(4), 1001–1008.

Eisenberg, S.P. *et al.* (1990) "Primary structure and functional expression from complementary DNA of a human interleukin-1 receptor antagonist". *Nature* **343**, 341–346.

Esmon, C.T. (1995) "Thrombomodulin as a model of molecular mechanisms that modulate protease specificity and function at the vessel surface". *FASEB J* **9**, 946–955.

Espel, E. *et al.* (1990) "Activity and Interleukin 1 responsiveness of SV40 enhancer motifs in a rodent immature T cell line". *EMBO J* **9**(3), 929–937.

Fawcett, J. *et al.* (1992) "Molecular cloning of ICAM-3, a third ligand for LFA-1, constitutively expressed on resting leukocytes". *Nature* **360**, 481–484.

Gahmberg, C.G. *et al.* (1991) "The human leukocyte-adhesion ligand, Intercellular-Adhesion Molecule 2". *European Journal of Biochemistry* **195**, 177–182.

Granger, D.N. and Kubes, P. (1994) "The microcirculation and inflammation: modulation of leukocyte-endothelial cell adhesion". *Journal of Leukocyte Biology* **55**, 662–674.

Griffioen, A.W. *et al.* (1996) "Endothelial intercellular adhesion molecule-1 expression is suppressed in human malignancies the role of angiogenic factors". *Cancer Research* **56**(5), 1111–1117.

Hooper, W.C. *et al.* (1994) "Tumor Necrosis Factor-α down-regulates Protein S secretion in human microvascular and umbilical cells but not in the HepG-2 hepatoma cell line". *Blood* **84**(2), 483–489.

Hou, J. *et al.* (1994) "Regulatory elements and transcription factors controlling basal and cytokine induced expression of the gene encoding ICAM-1". *Proceedings of the National Academy of Sciences of the United States of America* **91**, 11641–11645.

Hynes, R.O. (1992) "Integrins: Versatility, modulation, and signaling in cell adhesion". *Cell* **69**, 11–25.

Jones, D.A. *et al.* (1994) "A two-step adhesion cascade for T cell/endothelial cell interactions under flow conditions". *Journal of Clinical Investigations* **94**, 2443–2450.

Kuno, K. and Matsushima, K. (1994) "The IL-1 receptor signalling pathway". *Journal of Leukocyte Biology* **56**, 542–547.

Li, R. *et al.* (1993a) "A leukocyte integrin binding peptide from intercellular adhesion molecule-2 stimulated T cell adhesion and natural killer cell activity". *Journal of Biological Chemistry* **268**, 21474–21477.

Li, R. *et al.* (1993b) "A peptide from ICAM-2 binds to the leukocyte integrin CD11a/CD18 and inhibits endothelial cell adhesion". *Journal of Biological Chemistry* **268**(23), 17513–17518.

Li, R. *et al.* (1995) "A peptide derived from the intercellular adhesion molecule-2 regulates the avidity of the leukocyte integrins CD11b/CD18 and CD11c/CD18". *Journal of Cell Biology* **129**(4), 1143–1153.

Menger, M.D. and Vollmar, B. (1996) "Adhesion molecules as determinants of disease: from molecular biology to surgical research". *British Journal of Surgery* **83**, 588–601.

Muller, W.A. *et al.* (1993) "PECAM-1 is required for transendothelial migration of leukocytes". *Journal of Experimental Medicine* **178**, 449–460.

Newman, P.J. (1997) "Cell adhesion in vascular biology, the biology of PECAM-1". *Journal of Clinical Investigation* **99**(1), 3–8.

Nortamo, P. *et al.* (1991) "The expression of human intercellular adhesion molecule-2 is refractory to inflammatory cytokines". *European Journal of Immunology* **21**, 2629–2632.

Patterson, C. *et al.* (1996) "Down-regulation of vascular endothelial growth factor receptors by Tumor Necrosis Factor α in cultured human vascular endothelial cells". *Journal of Clinical Investigation* **98**(2), 490–496.

Pober, J.S. and Cotran, R.S. (1990) "Cytokines and endothelial cell biology". *Physiological Reviews* **70**, 427–451.

Puri, K.D. *et al.* (1995) "Sialomucin CD34 is the major L-selectin ligand in human tonsil high endothelial venules". *Journal of Cell Biology* **131**(1), 261–270.

Rezaoe, P. *et al.* (1997) "Expression of adhesion molecules on human fetal cerebral vessels: relationship to microglial colonisation during development". *Developmental Brain Research* **104**, 175–189.

Romer, L.H. *et al.* (1995) "IFN-γ and TNF-α induce redistribution of PECAM-1 (CD31) on human endothelial cells". *Journal of Immunology* **154**, 6582–6592.

Ross, R. (1997) "The pathogenesis of atherosclerosis: a perspective for the 1990s". *Nature* **362**, 801–809.

Roth, S.J. *et al.* (1995) "Characterization of transendothelial chemotaxis of T lymphocytes". *Journal of Immunological Methods* **188**(1), 97–116.

Sambrook, J., Fritsch, E.F. and Maniatis, T. *Molecular Cloning, a laboratory manual (2nd edition)*, 1989.

Schindler, U. and Baichwal, V.R. (1994) "Three NF-κB binding sites in the human E selectin gene required for maximal Tumor Necrosis Factor α-induced expression". *Molecular and Cellular Biology* **14**, 5820–5831.

Schleiffenbaum, B. and Fehr, J. (1996) "Regulation and selectivity of leukocyte emigration". *Journal of Lab. Clin. Med.* **127**, 151–168.

Skubitz, K.M. *et al.* (1997) "CD50 monoclonal antibodies inhibit neutrophil activation". *Journal of Clinical Investigation* **159**, 820–828.

Somersalo, K. *et al.* (1995) "Activation of natural killer cell migration by leukocyte integrin-binding peptide from Intracellular Adhesion Molecule 2 (ICAM-2)". *Journal of Biological Chemistry* **270**(15), 8629–8636.

Springer, T.A. (1994) "Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm". *Cell* **76**, 301–314.

Staunton, D.E. *et al.* (1989) "Functional cloning of ICAM-2, a cell adhesion ligand for LFA-1 homologous to ICAM-1". *Nature* **339**, 61–64.

Stewart, R.J. *et al.* (1996) "Vascular endothelial platelet endothelial cell adhesion molecule-1 (PECAM-1) expression is decreased by TNF-α and IFN-γ". *Journal of Immunology* **156**, 1221–1228.

Su, S. *et al.* (1997) "High-throughput RT-PCR analysis of multiple transcripts using a microplate RNA isolation procedure". *BioTechniques* **22**(6), 1107–1113.

Vandenabeele, P. *et al.* (1995) "Two tumor necrosis factor receptors: structure and function". *Trends In Cell Biology* **5**, 392–399.

Vazeux, R. *et al.* (1992) "Cloning and characterisation of a new intercellular adhesion molecule ICAM-R". *Nature* **360**, 485–488.

von der Ahe, D. *et al.* (1993) "Ets transcription factor binding site is required for positive and TNFα-induced negative promoter regulation". *Nucleic Acids Research* **21**(24), 5636–5634.

Xie, J.L. *et al.* (1995) "Intercellular Adhesion Molecule-2 (CD102) binds to the leukocyte integrin CD11b/CD18 through the A domain". *Journal of Immunology* **155**(7), 3619–3628.

Zocchi, M.R. *et al.* (1996) "CD31/PECAM-1 driven chemokine-independent transmigration of human T lymphocytes". *European Journal of Immunology* **26**, 759–767.