VEGF expression in human macrophages is NF-κB-dependent: studies using adenoviruses expressing the endogenous NF-κB inhibitor IκBα and a kinase-defective form of the IκB kinase 2

Serafim Kiriakidis*, Evangelos Andreakos, Claudia Monaco, Brian Foxwell, Marc Feldmann and Ewa Paleolog

Kennedy Institute of Rheumatology Division, Faculty of Medicine, Imperial College of Science, Technology and Medicine, London W6 8LH, UK
*Author for correspondence (e-mail: s.kiriakidis@ic.ac.uk)

Summary
Vascular endothelial growth factor (VEGF) is the most endothelial cell-specific angiogenic factor characterised to date, and it is produced by a variety of cell types. In macrophages, VEGF has been shown to be upregulated by the inflammatory mediator lipopolysaccharide (LPS) and by engagement of CD40 by CD40 ligand (CD40L). Because LPS and CD40L activate nuclear factor-κB (NF-κB) in monocytes, we investigated in this study whether VEGF production in macrophages, when stimulated with either LPS or CD40L, is NF-κB-dependent. We used adenoviral constructs over-expressing either IκBα (AdvIκBα), the endogenous inhibitor of NF-κB, or a kinase-defective mutant of IKK-2 (AdvIKK-2dn), an upstream activator of IκBα, to infect normal human monocyte-derived macrophages. We observed that LPS-induced production of VEGF in human macrophages was almost completely inhibited (>90%) following adenoviral transfer of IκBα. In addition, we observed significant inhibition of the CD40L-induced VEGF production in macrophages following infection with AdvIκBα. Expression of IKK-2dn in macrophages decreased VEGF production in response to LPS or CD40L by approximately 50%, suggesting that in addition to IKK-2, other kinases might be involved in NF-κB activation. These results show for the first time that VEGF production in human macrophages is NF-κB dependent. NF-κB regulates many of the genes involved in immune and inflammatory responses, and our study adds the angiogenic cytokine VEGF to the list of NF-κB-dependent cytokines.

Key words: VEGF, Macrophages, Angiogenesis, NF-κB, Signalling, CD40 ligand

Introduction
Vascular endothelial growth factor (VEGF), the most endothelial cell-specific angiogenic factor characterised to date, induces endothelial cell proliferation, promotes cell migration and inhibits apoptosis, and, as such, it is a key regulator of physiological and pathological angiogenesis (Ferrara, 2001; Neufeld et al., 1999; Robinson and Stringer, 2001).

VEGF is produced by many types of cells including fibroblasts (Pertovaara et al., 1994), macrophages (McLaren et al., 1996), neutrophils (Taichman et al., 1997; Webb et al., 1998), endothelial cells (Namiki et al., 1995) and T cells (Freeman et al., 1995). Although hypoxia is a very potent stimulus for VEGF expression (Levy et al., 1995; Shweiki et al., 1992), other factors such as cytokines, oncogenes, NO and modulators of protein kinase C have been reported to stimulate VEGF production (Brown et al., 1997; Neufeld et al., 1999). For example, tumour necrosis factor (TNF)-α upregulates VEGF production by synovial membrane cells and peripheral blood mononuclear cells from patients with rheumatoid arthritis (Bottomley et al., 2000; Paleolog et al., 1998). Although many of the signalling events downstream of VEGF receptor activation have been elucidated, the stimuli and mechanisms involved in the production of VEGF are still unclear. There is evidence that mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3-kinase) are involved in the induction of VEGF by growth factors, cytokines and hypoxia (Clarke et al., 2001; Jiang et al., 2000; Sodhi et al., 2001; Tanaka et al., 2000; Wang et al., 1999; Yamamoto et al., 2001). The downstream events of those pathways generally include activation of transcription factors such as AP-1 and nuclear factor-κB (NF-κB).

The NF-κB/Rel family includes NF-κB1 (p50), NF-κB2 (p52), p65 (RelA), RelB and c-Rel. They can all form homo- and heterodimers, with the most abundant form being p50/p65 (Tak and Firestein, 2001). These dimers are present in the cytosol in an inactive form, complexed with proteins of the IκB family, including IκBα, IκBβ, IκBε and Bcl3 (Baldwin, 1996). The best-characterised mechanism of NF-κB activation involves phosphorylation of IκBα at Ser32 and Ser36, through the IκB-kinase (IKK) complex, resulting in ubiquitination and degradation of IκBα. This enables the release and nuclear translocation of active NF-κB and its binding to promoters and enhancers, leading to gene transcription. Two IκB kinases,
IKK-1 (IKKα) and IKK-2 (IKKβ), have been well described (DiDonato et al., 1997; Mercurio et al., 1997). Although IKK-1 and IKK-2 share a high degree of amino acid sequence similarity, studies in mice indicate that they have non-equivalent functions and that activation of IKK-2, rather than IKK-1, participates in the primary pathway by which pro-inflammatory stimuli activate NF-κB (Baldwin, 2001; Hu et al., 1999; Li et al., 1999; Tak and Firestein, 2001).

NF-κB regulates the expression of many pro-inflammatory cytokines including TNF-α, interleukin-1β (IL-1β) and IL-6 (Baldwin, 2001; Bondeson et al., 1999a; Bondeson et al., 1999b; Tak and Firestein, 2001). However, the importance of NF-κB in terms of VEGF expression is controversial. For example, it was shown that induction of VEGF in UV-irradiated mouse skin cells can be blocked by treatment with NF-κB decoy-oligonucleotides (Abeyama et al., 2000). However, administration of NF-κB antisense oligonucleotides in human endothelial cells only partially abrogated VEGF expression in response to TNF-α (Yoshida et al., 1997). More recently, expression of a mutated IkBα significantly inhibited the expression of VEGF in human prostate cancer cells (Huang et al., 2001; Huang et al., 2000) but not in human head and neck squamous cell carcinomas (Bancroft et al., 2001).

In our study we investigated the role of NF-κB in the induction of VEGF in human primary macrophages, which are the main cytokine producers in chronic inflammatory diseases such as rheumatoid arthritis and a source of VEGF when stimulated with cytokines and lipopolysaccharide (LPS) (Itaya et al., 2001; Perez-Ruiz et al., 1999). However, one of the obstacles in studying biochemical signalling pathways in primary cells is the difficulty of transfecting DNA, an essential tool in this process (Stacey et al., 1993). To overcome this, we used a very efficient technique of gene transfer, based on replication-deficient adenoviruses (Bondeson et al., 1999a; Bondeson et al., 1999b; Foxwell et al., 1998), to express a kinase-negative mutant of IKK-2 (IKK-2dn), as well as the endogenous NF-κB inhibitor IκBα. We also made use of relatively specific drug inhibitors of p38 MAPK, p42/44 MAPK and PI3-kinase.

Our results show that several pathways are involved in the induction of VEGF, in that p38 MAPK and p42/44 MAPK, as well as PI3-kinase, regulate LPS-induced VEGF production in human primary macrophages. Moreover, we show for the first time that activation of NF-κB plays a key role in VEGF production by macrophages, as expression of both IκBα and IKK-2dn could significantly inhibit its production in response to either LPS or CD40 ligand (CD40L), an immune inflammatory stimulus. Our findings show that, like TNF-α and other pro-inflammatory cytokines, the potent angiogenic factor VEGF is regulated by NF-κB.

Materials and Methods

Reagents

Human recombinant macrophage colony-stimulating factor (M-CSF) was a gift from Glenn Larsen (Genetics Institute, MA). Human recombinant IL-1α and TNF-α were generous gifts from Roche and the Centre of Molecular and Macromolecular Studies (LDz, Poland), respectively. Escherichia coli LPS was obtained from Sigma (St Louis, MO). Transforming growth factor β1 (TGF-β1) and fibroblast growth factor 2 (FGF-2) were both purchased from R&D (Abingdon, UK). Recombinant human soluble CD40L (sCD40L) was obtained from Alexis Corporation (Lausen, Switzerland), whereas CD40L-transfected (CD40L+) and control (MOCK, transfected with empty plasmid) mouse fibroblast cell lines were obtained from D. Gray (University of Edinburgh, UK) and A. Schimpi (University of Wurzburg, Germany) (Wohlsch et al., 1996). The p38 MAPK inhibitor SB203580 and the p42/44 MAPK inhibitor PD98059 were purchased from Calbiochem-Novabiochem (Nottingham, UK). Wortmannin and LY294002, selective inhibitors of PI3-kinase, were purchased from Sigma (Poole, Dorset, UK). The anti-TNF-α monoclonal antibody cA2 was a gift from J. Ghayeb (Centocor, Malvern, PA). The murine anti-rat immunoglobulin (Ig)G2 isotype control was purchased from European Collection of Cell Cultures (ECACC, UK).

Cell culture

Human monocytes were obtained from single-donor plateletpheresis residues (North London Blood Transfusion Center, London, UK) and differentiated to macrophages, as described previously (Foxwell et al., 1998). Macrophages were cultured in RPMI-1640 medium containing 5% (v/v) fetal calf serum (FCS) and 100 U/ml penicillin/streptomycin (BioWhittaker, Rockland, ME) at 37°C and 5% CO2. Adherent cells were washed twice in FCS-free RPMI-1640 and removed using Cell Dissociation Medium (Sigma). The cells were washed twice more and re-plated at 1×106 cells/ml. Transfected mouse fibroblasts were maintained in RPMI-1640 with 2 mM L-glutamine containing 1% heat-inactivated FCS, 100 U/ml penicillin/streptomycin and 2 mg/ml gentamycin (G418). All reagents and media used in this study were shown to contain <0.1 U/ml LPS, measured using the Limulus amoebocyte lysate assay (BioWhittaker).

Adenoviral vectors

Recombinant, replication-deficient adenoviral vectors encoding E. coli β-galactosidase (Advβgal) or having no insert (Adv0) were generously provided by A. Byrnes and M. Wood (Oxford University, UK). Adenoviruses encoding either a dominant form of IKK-2 (single point mutation of K44→A) or porcine IκBα (AdvIKBα) with a cytomegalovirus promoter and a nuclear localisation sequence were generously provided by R. de Martin (University of Vienna, Austria). Porcine IκBα has >95% amino acid homology with the human molecule. All viruses are (E1/E3) early transcribed regions deleted, belong to the Adv5 serotype and have been previously used in other studies (Oitzinger et al., 2001; Smith et al., 2001; Wrighton et al., 1996). Viruses were propagated in the 293 human embryonic kidney cell line (American Type Culture Collection, Rockville, MD) and were purified by ultra centrifugation through two caesium chloride gradients. The titres of viral stocks were determined through a plaque assay on 293 cells, as described previously (Foxwell et al., 1998). All viruses used were plaque-purified from a master stock to prevent contamination with wild-type adenovirus.

Infection techniques

To optimise infection, purified human monocytes obtained by centrifugal elutriation were seeded at a density of 1×106 per ml in assay medium in Petri dishes. M-CSF was added to a final concentration of 100 ng/ml. Cells were cultured for 3 days at 37°C, 5% CO2. This time was necessary to allow upregulation of integrin αVβ5, which we have previously shown to be essential for adenovirus infection of monocytes (Andreakos et al., 2002; Bondeson et al., 1999a; Bondeson et al., 1999b; Ciesielski et al., 2002; Foxwell et al., 1998; Smith et al., 2001). Adherent cells were then washed twice in FCS-free RPMI-1640 and removed from plastic by Cell Dissociation Medium (Sigma). The cells were washed twice more and re-plated at 1×106 cells/ml on either 960 mm2 dishes for western blot or electrophoretic mobility shift assay (EMSA) analysis or in 30 mm2
wells for cytokine analysis. Cells were infected for 2 hours in serum-free RPMI-1640 with control adenoviruses (Adv0, Advβgal), Adv1xβBz and Adv1KK-2dn, and then incubated in RPMI-1640 containing 2 mM L-glutamine, 5% (v/v) FCS and 100 U/ml penicillin/streptomycin for 48 hours to allow for significant overexpression of proteins. A multiplicity of infection (m.o.i.) of 100:1 was used in all experiments, consistently giving >95% infection of cells as determined using Advβgal, and Fluorescein-di-β-galactopyranoside (Sigma), a fluorescent substrate of β-galactosidase, in accordance with published data (Bondeson et al., 1999a; Bondeson et al., 1999b; Foxwell et al., 1998). Use of higher m.o.i. did not result in any improvements in infectibility above 95%. This was supported by data using adenovirus encoding green fluorescent protein (AdvGFP; Quantum Biotech, Canada), which showed optimal infection at m.o.i. 100:1 (data not shown) (see Bondeson et al., 1999a).

Measurement of VEGF production in response to different stimuli

Macrophages were plated at 1x10⁵ cells per 30mm² well and stimulated for 24 hours with LPS (10 ng/ml), or sCD40L (1 μg/ml). Additionally, macrophages were seeded in 30 mm² wells at 1x10⁵ cells/well and CD40L or MOCK cells were added at 1x10⁵ cells/well (1:1 ratio). Supernatants were collected and analysed for VEGF using an in-house VEGF ELISA. Briefly, polystyrene plates (Nunc-ImmunoPlate II, BRL, Middelsex, UK) were coated with anti-human VEGF antibody (R&D; 100 ng/ml in PBS) overnight at 4°C. VEGF standard (rhVEGF, R&D) or samples were added overnight at 4°C. Biotinylated anti-human VEGF antibody (50 μg/ml in 0.5% BSA/PBS) was then added and left at room temperature for 2 hours. The plates were incubated for 1 hour with streptavidin-horseradish peroxidase (HRP) (Amersham Pharmacia Biotech, Little Chalfont). After removal of the HRP conjugate, plates were washed with PBS containing 0.05% Tween 20, and a 1:1 mixture of peroxidase solution (H₂O₂ and 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase substrate (Kirkegaard and Perry Laboratories, Gaithesburg, MD) was added for 20 minutes. After addition of 2M sulphuric acid, plates were read at 450 nm on a spectrophotometric ELISA plate reader (Labsystems Multiscan Biochromic) and analysed using Delta Soft II-4 program. In all cases, viability of the cells was not significantly affected when examined by MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay (Sigma).

Preparation of cytosolic and nuclear extracts

Two days after adenovirus infection, cells were stimulated for 45 minutes with either LPS (10 ng/ml) or sCD40L (1 μg/ml), and cytosolic and nuclear extracts were prepared as described (Whiteside et al., 1992). Adherent cells were scraped into ice-cold PBS harvested by centrifugation and washed once with ice-cold PBS. Cell-pellets were then lysed in hypotonic lysis buffer (5 mM HEPES, 1 mM MgCl₂, 0.2 mM EDTA, 0.5 M NaCl, 25% glycerol, pH 7.0). After incubation on ice for 10 minutes, lysates were centrifuged (13,000 g, 5 minutes, 4°C) to remove nuclei and cell debris. The cleared lysates were then removed to fresh tubes, frozen and stored at −20°C for subsequent estimation of protein concentration and use in western blotting. The nuclei pellets were resuspended in hypertonic extraction buffer (10 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl, pH 7.9) for 1-2 hours at 4°C under agitation. After centrifugation (13,000 g for 10 minutes at 4°C), supernatants containing the nuclear protein were removed to fresh tubes and stored at −70°C. Protein concentrations were assessed by reaction with Bradford reagent (0.1% Coomassie blue G, 5% methanol, orthophosphoric acid) (Bradford, 1976).

Western blotting

Antibodies used for western blotting (anti-human IκBα; IKK-2 antibodies and α-tubulin) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). For western analysis, equal amounts of protein extracts were separated by SDS/PAGE on a 10% (w/v) polyacrylamide gel, followed by electrotransfer onto polyvinyl difluoride membrane (Millipore, Watford, UK). Blots were blocked for 1 hour with blocking buffer (5% (w/v) fat-free milk, 0.1% (v/v) Tween 20 in PBS) followed by overnight incubation with the antibodies diluted 1:1000 in blocking buffer. Blots were then incubated in HRP-conjugated anti-mouse IgG (DAKO, Cambridge, UK) diluted 1:2000 in blocking buffer. Bound antibody was detected using the ECL kit and visualised using Hyperfilm MP (Amersham Pharmacia Biotech, Little Chalfont, UK).

Electrophoretic mobility shift assay

EMSA reactions were performed as previously described (Clarke et al., 1995). Briefly, nuclear protein extracts were incubated with the double-stranded NF-κB consensus oligonucleotide (5’-AGTTGAGGGGACTTTCCCAGGC-3’), which was end-labelled with [γ-32P]dATP (3000 Ci/mmol at 10 μCi/μl) using the Gel Shift Assay System (Promega, Southampton, UK). Formed protein/DNA complexes were separated on 5% non-denaturing polyacrylamide gels and retarded DNA:protein complexes were analysed by phosphoimaging using the Fuji FLA-2000 phosphoimager (Raytek Scientific, Sheffield, UK).

Statistical analysis

One-way ANOVA with Bonferroni post-test for multiple comparisons was used to compare the effects of different inhibitors or adenoviruses on VEGF production. All data presented are from a representative experiment, and the total number of experiments performed is indicated.

Results

p38 MAPK, p42/44 MAPK and PI3-kinase are all partly involved in LPS-simulated macrophage production of VEGF

Primary human macrophages were stimulated with selected cytokines (TNFα, IL-1α, FGF-2 and TGF-β) and LPS (all at 10 ng/ml). Fig. 1A shows that release of VEGF in response to LPS was detected after 4 hours, and continued to increase for up to 24 hours. In the same figure, comparison of the kinetics of LPS-induced VEGF and TNF-α production shows that, in contrast to VEGF, TNF-α production reached a maximum 4 hours after addition of LPS, and decreased thereafter, in agreement with published data (Denys et al., 2002). There was no significant increase in VEGF expression after the addition of TNF-α, IL-1α, FGF-2 and TGF-β (data not shown).

Subsequently, macrophages were treated for 1 hour with different MAPK inhibitors or DMSO as a vehicle control, before stimulation with LPS. The culture supernatants were harvested at 24 hours and then analysed for the presence of VEGF by ELISA. As shown in Fig. 1B,C, treatment of macrophages with the p38 MAPK inhibitor SB203580 (0.002-2 μM) and the p42/44 MAPK inhibitor PD098059 (0.01-10 μM) resulted in dose-dependent suppression of LPS-induced VEGF production, although complete inhibition of VEGF could not be achieved in any experiment. Furthermore, we used two specific inhibitors of the PI3-kinase pathway – namely,
we infected macrophages with an adenovirus overexpressing wortmannin and 88% for 10 nM wortmannin (0.1-100 nM) and LY294002 (0.01-10 μM). Addition of these inhibitors could also reduce LPS-induced VEGF production in a dose-dependent manner (Fig. 1D,E), although complete inhibition of VEGF was again not observed in any experiment. The addition of vehicle alone (DMSO) was without effect (data not shown). There was no significant effect of these inhibitors on cell viability, as determined by MTT, even at the highest concentrations used: 100% viability for 2 μM SB203580, 99% for 10 μM PD098059, 100% for 100 nM wortmannin and 88% for 10 μM LY294002.

IκBα overexpression inhibits LPS-induced VEGF release by human macrophages

To investigate whether LPS-induced VEGF requires NF-κB, we infected macrophages with an adenovirus overexpressing IκBα, the endogenous inhibitor of NF-κB. As shown in Fig. 2A, by infecting the macrophages with AdvIκBα at a m.o.i. of 100:1, we were able to induce cytosolic overexpression of IκBα as assessed by western blotting analysis of the cytosolic extracts. Stimulation with LPS induced complete degradation of IκBα, which was prevented in macrophages infected with AdvIκBα but not in cells infected with control adenovirus (Adv0, Advβgal). There was no difference between experiments using Adv0 and Advβgal as controls. In addition, LPS-induced NF-κB activation and translocation into the nucleus was also inhibited by IκBα overexpression (Fig. 2C). In parallel experiments, we stimulated macrophages for 24 hours with LPS following infection, and examined the culture supernatants for VEGF production using ELISA. In Fig. 2D a representative of ten independent experiments shows that in AdvIκBα-infected cells, LPS-induced production of VEGF was significantly inhibited. The
VEGF release in macrophages is NF-κB dependent

Expression of IKK-2dn does not inhibit LPS-induced NF-κB activation but partially abrogates VEGF production.

To examine whether macrophage NF-κB activation and VEGF production requires IKK-2, a kinase-defective (dominant negative) form of IKK-2 was adenovirally delivered into macrophages. As Fig. 3A shows, we achieved a significant expression of IKK-2dn in cells infected with AdvIKK-2dn compared with the levels in uninfected cells or cells infected with control adenovirus (Advβgal). However, we unexpectedly found that expression of dominant negative IKK-2 did not significantly inhibit LPS-induced IκBα degradation (Fig. 3B) and NF-κB activation (Fig. 3D).

Nevertheless, in all our experiments we found consistent inhibition of VEGF in LPS-stimulated macrophages infected with AdvIKK-2dn, although the extent of inhibition was less than that observed following overexpression of IκBα. The range of inhibition following overexpression of IKK-2dn was 38-74% (mean 59%). In comparison, infection with control adenovirus was without significant effect (Fig. 3E). In the same

Fig. 3. Expression of IKK-2dn in human macrophages does not inhibit LPS-induced NF-κB activation but partially decreases VEGF production. Macrophages were either left uninfected, or were infected for 2 hours in serum-free medium with control adenovirus, or adenoviruses expressing IKK-1dn and IKK-2dn. Cells were cultured in 5% FCS for 2 days and stimulated with 10 ng/ml LPS for 45 minutes. Cytosolic and nuclear extracts were then collected and examined for cytosolic levels of IKK-2 (A), IκBα (B) and α-tubulin (C) by western blot analysis or for NF-κB translocation by EMSA (D). A representative of three independent experiments is shown. For VEGF production, macrophages were left uninfected, or were infected for 2 hours in serum-free medium with control adenovirus or with an adenovirus expressing IKK-2dn. After 2 days of further culture, cells were stimulated with 10 ng/ml LPS for 24 hours (E). Data are expressed as means±s.d. of triplicates and are representative of ten different experiments: *P<0.05 versus response with control adenovirus-infected and uninfected cells.

Fig. 4. Expression of IκBα and IKK-2dn inhibits CD40L-induced VEGF release by macrophages. (A) M-CSF-differentiated macrophages were cultured for 24 hours in 30 mm2 wells with 5×10⁵ CD40L-transfected (CD40L+) or control (MOCK) cells. After 24 hours, supernatants were collected and assayed by ELISA for VEGF. In parallel experiments, macrophages were left uninfected, or infected for 2 hours in serum-free medium with Advβ-gal, AdvIKK-2dn and AdvIκBα, at m.o.i. 100:1. Cells were cultured for a further 2 days and then stimulated with 1 μg/ml sCD40L. After 45 minutes, cytosolic and nuclear extracts were obtained and examined for the presence of IKK-2dn (B) and IκBα (C) by western blotting. A representative of three independent experiments is shown. Equal amounts of protein were loaded, as determined by re-probing for α-tubulin (not shown). For measurement of VEGF production, macrophages were left uninfected, or infected as described above and after 2 days stimulated with CD40L-transfected (CD40L+) or (MOCK) cells as control. After 24 hours, supernatants were collected and assayed for VEGF (D). Mean cytokine production±s.d. of triplicate cultures is shown and is representative of three independent experiments: **P<0.01, ***P<0.001 versus response with control adenovirus-infected and uninfected cells.
experiments, infection with AdvIkBα reduced VEGF release by >90% (not shown)

Induction of VEGF by CD40 ligation is NF-κB-dependent
Two recently published reports have described that stimulation of monocytes, endothelial cells and synovial fibroblasts with sCD40L or CD40L-expressing cells resulted in VEGF production (Cho et al., 2000; Melter et al., 2000). Therefore, we were interested to examine whether CD40-CD40L engagement on macrophages induces production of VEGF. We found that IκBα degradation, which was inhibited by over-expression of both IκBα or IKK-2dn in macrophages (Fig. 4B,C) suggesting that phosphorylation and degradation of IκBα in macrophages through CD40L requires IKK-2. In addition, as shown in Fig. 4D, infection of macrophages with AdvIkBα could strongly inhibit VEGF production in response to CD40L+, suggesting that IκBα is significantly involved in the CD40L-induced production of VEGF. However, as was observed for LPS-induced VEGF release, we could observe only a partial reduction of VEGF production in response to CD40L+ using AdvIKK-2dn (Fig. 4D).

Neutralising antibody to TNF-α inhibits both LPS- and sCD40L-induced VEGF release in macrophages
In a previous study we found, using anti-TNF-α antibody cA2, that VEGF production by synovial joint cells, a heterogeneous population of T cells, macrophages and fibroblasts, is at least in part mediated by TNF-α (Paleolog et al., 1998). Macrophages respond to LPS or CD40L to produce TNF-α. To examine whether endogenous TNF-α is involved in the induction of VEGF by these stimuli, we stimulated macrophages with either LPS (10 ng/ml) or sCD40L (1 μg/ml) in the presence of anti-TNF-α antibody (10 μg/ml). We observed a potent inhibition of VEGF production in macrophages in response to both LPS (Fig. 5A) and sCD40L (Fig. 5B), suggesting that endogenously produced TNF-α plays an important role in both LPS- and CD40L-induced VEGF production by macrophages. Stimulation of macrophages with exogenous TNF-α did not significantly induce VEGF release (data not shown).

Discussion
Disregulated angiogenesis contributes to the pathology of several disease states, during which tissue proliferation outstrips the supply of nutrients and oxygen. VEGF is a relatively endothelial cell-specific angiogenic factor, and its expression is elevated in a range of angiogenesis-associated disease states, such as malignancies, retinal neovascularisation and psoriasis. In the present study, we investigated the involvement of the transcription factor NF-κB in the upregulation of VEGF in human macrophages. We chose primary human macrophages, which are a major source of pro-inflammatory cytokines and VEGF in chronic inflammatory diseases such as rheumatoid arthritis (Itaya et al., 2001; Perez-Ruiz et al., 1999). We used a very efficient technique of gene transfer based on replication-deficient adenoviruses, which provides a reliable, reproducible and convenient method of studying intracellular signalling pathways (Bondeson et al., 1999a; Bondeson et al., 1999b; Foxwell et al., 1998; Smith et al., 2001). Among the different stimuli examined, LPS induced significant production of VEGF by macrophages. By contrast, TNF-α, IL-1, FGF-2 and TGF-β were all without significant effect on VEGF production.

LPS is known to bind to CD14 on the surface of macrophages and to signal through toll-like receptor 4 (TLR4) to activate MAPK and NF-κB (Anderson, 2000; Guha and Mackman, 2001). To study whether MAPKs are involved in LPS-induced VEGF production by macrophages, we used specific MAPK inhibitors. SB203580, a specific p38 MAPK inhibitor, only partially inhibited the LPS-induced VEGF production. Additionally, we showed that other inhibitors, such as the p42/44 MAPK inhibitor PD09859 and two specific inhibitors for the PI3-kinase pathway – wortmannin and LY294002 – could also partially inhibit the production of VEGF. These data suggest that signals through those proteins are involved in the upregulation of VEGF. The inhibitors used
in this study might exert effects on other kinases at higher concentrations; however, we have previously shown that at the concentrations employed in the present study, these inhibitors have been shown by in vitro kinase assays to be specific (Crawley et al., 1996; Foey et al., 1998; Lali et al., 2000; Williams et al., 2000).

Virtually complete inhibition of VEGF production in response to LPS was achieved only by overexpression of the endogenous NF-\(\kappa B\) inhibitor, I\(\kappa B\alpha\). Ubiquitination and degradation of I\(\kappa B\alpha\) enables the release and nuclear translocation of NF-\(\kappa B\) and binding to promoters and enhancers. Overexpression of I\(\kappa B\alpha\) inhibited LPS-induced degradation of endogenous I\(\kappa B\alpha\) and NF-\(\kappa B\) nuclear translocation, and resulted in more than 90% inhibition of VEGF expression. Expression of IKK-2dn resulted in a significant inhibition of VEGF release, although complete inhibition of VEGF could not be achieved as it was in the case of I\(\kappa B\alpha\).

Previous studies on monocytic cell lines showed that IKK-2 rather than IKK-1 is required for LPS-induced NF-\(\kappa B\) activation (Hawiger et al., 1999). Expression of dominant negative forms of IKK-2 could inhibit LPS-induced \(\kappa B\)-dependent transcription (Fischer et al., 1999; O’Connell et al., 1998), whereas expression of dominant negative forms of IKK-1 had no effect on LPS-induced k\(\kappa B\)-dependent transcription in one study (O’Connell et al., 1998) but partly inhibited it in another (Fischer et al., 1999). However, we found that IKK-2dn could not prevent LPS-induced I\(\kappa B\alpha\) degradation and NF-\(\kappa B\) nuclear translocation in human primary macrophages. In addition, in a parallel study we found that IKK-2dn did not block the production of NF-\(\kappa B\)-dependent cytokines – namely, TNF-\(\alpha\), IL-6 or IL-8 – in response to LPS, suggesting that LPS-induced NF-\(\kappa B\) activation by macrophages does not require IKK-2 (E.A., C. Smith, S.K. et al., unpublished).

The selective partial inhibition of VEGF release in response to IKK-2dn suggested that, in macrophages, IKK-2 might be required in the activation of other signalling pathways contributing to the induction of VEGF. In a previous study we showed using anti-TNF-\(\alpha\) antibody that production of VEGF by synovial joint cells was in part induced by TNF-\(\alpha\) (Paleolog et al., 1998). To address the possible role of TNF-\(\alpha\) in the induction of VEGF, macrophages were stimulated with LPS in the presence of neutralising antibody to TNF-\(\alpha\). We found that blockade of endogenous TNF-\(\alpha\) activity resulted in a significant inhibition of VEGF release, although addition of exogenous TNF-\(\alpha\) did not induce VEGF production. As discussed previously, we have also observed that IKK-2 is essential for TNF-\(\alpha\)-induced NF-\(\kappa B\) activation and cytokine production in macrophages, but not for LPS-induced production of TNF-\(\alpha\) (E.A., C. Smith, S.K. et al., unpublished). A possible explanation of our data is that LPS activates macrophages through an IKK-2-independent pathway to induce degradation of I\(\kappa B\alpha\), nuclear translocation of NF-\(\kappa B\) and stimulation of TNF-\(\alpha\) transcription. Endogenously released TNF-\(\alpha\) might, in turn augment VEGF expression through an IKK-2-dependent mechanism. Support for this hypothesis comes from time-course experiments that showed much slower induction of VEGF expression, peaking after 24 hours. By contrast, TNF-\(\alpha\) expression peaks after 4 hours. However, in addition to TNF-\(\alpha\) induction, other genes induced by LPS might code for proteins that are essential for the TNF signalling or other events associated with VEGF regulation – such as the stabilisation of VEGF mRNA – and they might also contribute to the end result – VEGF induction – via the NF-\(\kappa B\) pathway.

The essential role of NF-\(\kappa B\) in LPS-induced VEGF production prompted us to investigate its role in CD40L-induced production – another major inflammatory stimulus. CD40-LCD40L signalling has been found to have multiple functions in inflammation, predominantly in the effector phase of the immune response (Van Kooten and Banchereau, 1996). In monocytes it was previously shown that CD40L activates NF-\(\kappa B\) and induces the expression of pro-inflammatory cytokines such as TNF-\(\alpha\), IL-6 and IL-8 (Alderson et al., 1993; Kiener et al., 1995). Recently, it was shown that ligation of CD40 induces the expression of VEGF by rheumatoid synovial fibroblasts (Cho et al., 2000), endothelial cells and monocytes (Melter et al., 2000). In addition, studies in non-haematopoietic cells showed that CD40 mediates NF-\(\kappa B\)-mobilisation and IL-6 production (Hess et al., 1995). Studies on human hepatic stellate cells have shown that ligation of CD40 on those cells induces the activation of important signalling pathways such as IKK/NF-\(\kappa B\) and \(c-Jun\) N-terminal kinase following secretion of IL-8 and MCP-1 (Schwabe et al., 2001).

We found that stimulation of human macrophages with a mouse fibroblast cell line transfected with a plasmid expressing CD40L (CD40L+) resulted in strong VEGF induction compared with macrophages stimulated with MOCK-transfected cells. This is, to our knowledge, the first evidence linking CD40-LCD40L interactions on normal human macrophages with VEGF production. We also found that CD40L stimulation of macrophages induced I\(\kappa B\alpha\) degradation, which was inhibited after adenovirus-mediated delivery into the cells of I\(\kappa B\alpha\) or IKK-2dn, showing that IKK-2 is essential for NF-\(\kappa B\) activation in response to CD40L. In addition, CD40L-induced VEGF production was also inhibited following I\(\kappa B\alpha\) overexpression, and we observed a 50% inhibition of VEGF after expression of IKK-2dn. This is in contrast to the response of macrophages to LPS, in which VEGF production, but not NF-\(\kappa B\) activation, was inhibited by IKK-2dn, suggesting that the involvement of components of the NF-\(\kappa B\) (I\(\kappa B\alpha\), IKK-2) pathway is stimulus dependent. The partial inhibitory effect of AdvI\(\kappa B\alpha\) and AdvIKK-2dn on CD40L-mediated VEGF secretion suggests that additional pathways are involved in the induction of VEGF release by this stimulus.

The involvement of NF-\(\kappa B\) in VEGF production has been investigated in other studies, with variable and conflicting results. NF-\(\kappa B\) has been shown to be partly responsible for the upregulation of VEGF mRNA and development of vessel-like structures in human microvascular endothelial cells in response to TNF-\(\alpha\) (Yoshida et al., 1997). In recent studies, expression of mutated I\(\kappa B\alpha\) could not inhibit VEGF production in human head and neck squamous cell carcinomas (Bancroft et al., 2001), but significantly inhibited the expression of VEGF in human prostate cancer cells (Huang et al., 2001; Huang et al., 2000). Our results suggest that the NF-\(\kappa B\) pathway is involved in the induction of VEGF release from LPS- and CD40L-stimulated human macrophages. However, the role of IKK-2 is not fully understood and needs further study.

Although the human VEGF promoter contains multiple binding sites for different transcriptional factors such SP-1, AP-1 and hypoxia-regulated elements, there is no direct
NF-κB-like binding sites within the VEGF 5′-promoter region. Sequences that differ at the level of one or two positions from the decameric κB-like binding sites (5′-HGGARNYYCC-3′, where H indicates A, C, or T; R indicates A or G; Y indicates C or T; N indicates any nucleotide) and NF-κB-like consensus sequence (5′-GGGRNNYYCC-3′, where R indicates A or G, Y indicates C or T, and N indicates any nucleotide) in the VEGF promoter (Tischer et al., 1991) are highlighted (bold and underlined).

### References

- Abeyama, K., Eng, W., Jester, J. V., Vink, A. A., Edelbaum, D., Cockerell, C. J., Bergstresser, P. R. and Takashima, A. (2000). A role for NF-κB-dependent gene transactivation in sunburn. *J. Clin. Invest.* 105, 1751-1759.
- Alderson, M. R., Armitage, R. J., Toug, T. W., Stockb hedge, L., Fanslow, W. C. and Spriggs, M. K. (1993). CD40 expression by human monocytes: regulation by cytokines and activation of monocytes by the ligand for CD40. *J. Exp. Med.* 178, 669-674.
- Anderson, K. V. (2000). Toll signaling pathways in the innate immune response. *Curr. Opin. Immunol.* 12, 13-19.
- Andreakos, E., Smith, C., Monaco, C., Brennan, F. M., Foxwell, B. M. and Feldmann, M. (2002). IkB kinase 2 but not NFκB-inducing kinase is essential for effective DC antigen presentation in the allogeneic mixed lymphocyte reaction. *Blood* Sep 19 [epub ahead of print).
- Baldwin, A. S., Jr (1996). The NF-kappa B and kappa B proteins: new discoveries and insights. *Ann. Rev. Immunol.* 14, 649-683.
- Baldwin, A. S., Jr (2001). Series introduction: the transcription factor NF-kappaB and human disease. *J. Clin. Invest.* 107, 3-6.
- Bancroft, C. C., Chen, Z., Dong, G., Sunwoo, J. B., Yeh, N., Park, C. and van Vaes, C. (2001). Coexpression of proangiogenic factors IL-8 and VEGF by human head and neck squamous cell carcinoma involves coactivation by MEK-MAPK and IKK-NF-kappaB signal pathways. *Clin. Cancer Res.* 7, 435-442.
- Bondeson, J., Browne, K., Brennan, F., Foxwell, B. and Feldmann, M. (1999a). Selective regulation of cytokine induction by adenoviral gene transfer of IkappaBalpha into human macrophages: lipopolysaccharide-induced, but not zymosan-induced, proinflammatory cytokines are inhibited, but IL-10 is nuclear factor-kappaB independent. *J. Immunol.* 162, 2939-2949.
- Bondeson, J., Foxwell, B., Brennan, F. and Feldmann, M. (1999b). Defining therapeutic targets by using adenovirus: blocking NF-kappab inhibits both inflammatory and destructive mechanisms in rheumatoid synovium but spares anti-inflammatory mediators. *Proc. Natl. Acad. Sci. USA* 96, 5668-5673.
- Bottomley, M. J., Webb, N. J., Watson, C. J., Holt, L., Bukhari, M., Denton, J., Freemont, A. J. and Brenchley, P. E. (2000). Placenta growth factor (PIGF) induces vascular endothelial growth factor (VEGF) secretion from mononuclear cells and is co-expressed with VEGF in synovial fluid. *Clin. Exp. Immunol.* 119, 182-188.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248-254.
- Brown, L. F., Detmar, M., Claffey, K., Nagy, J. A., Feng, D., Dvorak, A. M. and Dvorak, H. F. (1997). Vascular permeability factor/vascular endothelial growth factor: a multifunctional angiogenic cytokine. *EXS* 79, 233-269.
- Cho, C. S., Cho, M. L., Min, S. Y., Kim, W. U., Min, D. J., Lee, S. S., Park, S. H., Choe, J. and Kim, H. Y. (2000). CD40 engagement on synovial fibroblast up-regulates production of vascular endothelial growth factor. *J. Immunol.* 164, 5055-5061.
- Ciesielski, C. J., Andreakos, E., Foxwell, B. M. and Feldmann, M. (2002). TNFalpha-induced macrophage chemokine secretion is more dependent on NF-kappaB expression than lipopolysaccharides-induced macrophage chemokine secretion. *Eur. J. Immunol.* 32, 2037-2045.
- Clarke, C. J., Taylor-Fishwick, D. A., Hales, A., Chernajovsky, Y., Sugamura, K., Feldmann, M. and Foxwell, B. M. (1995). Interleukin-4 inhibits kappa light chain expression and NF kappa B activation but not I kappa B-alpha degradation in 70Z/3 murine pre-B cells. *Eur. J. Immunol.* 25, 2961-2966.
- Clarke, K., Smith, K., Gullick, W. J. and Harris, A. L. (2001). Mutant epidermal growth factor receptor enhances induction of vascular endothelial growth factor by hypoxia and insulin-like growth factor-1 via a PI3 kinase dependent pathway. *Br. J. Cancer* 84, 1322-1329.
- Crawley, J. B., Williams, L. M., Mander, T., Brennan, F. M. and Foxwell, B. M. (1996). Interleukin-10 stimulation of phosphatidylinositol 3-kinase and p70 S6 kinase is required for the proliferative but not the antiapoptotic effects of the cytokine. *J. Biol. Chem.* 271, 16357-16362.
- Denys, A., Udalova, I. A., Smith, C., Williams, L. M., Ciesielski, C. J., Campbell, J., Andrews, C., Kwaitkowski, D. and Foxwell, B. M. (2002). Evidence for a dual mechanism for IL-10 suppression of TNF-alpha production that does not involve Inhibition of p38 mitogen-activated protein kinase or NF-kappaB in primary human macrophages. *J. Immunol.* 168, 4837-4845.
- DiDonato, J. A., Hayakawa, M., Rothwarf, D. M., Zandi, E. and Karin, M. (1997). A cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB. *Nature* 388, 548-554.
- Ferrara, N. (2001). Role of vascular endothelial growth factor in regulation of physiological angiogenesis. *Am. J. Physiol. Cell Physiol.* 280, C1358-C1366.
- Fischer, C., Page, S., Weber, M., Eisele, T., Neumeier, D. and Brand, K. (1999). Differential effects of lipopolysaccharide and tumor necrosis factor on monocyctic IkappaB kinase signalase activation and IkappaB proteolysis. *J. Biol. Chem.* 274, 24625-24632.
- Foey, A. D., Parry, S. L., Williams, L. M., Feldmann, M., Foxwell, B. M. and Brennan, F. M. (1998). Regulation of monocyte IL-10 synthesis by
endogenous IL-1 and TNF-alpha: role of the p38 and p42/44 mitogen-activated protein kinases. J. Immunol. 160, 920-928.

Foxwell, B., Bont-Jason, J., Clark, C., de Martin, R., Brennan, E. and Feldmann, M. (1998). Efficient adenosiv infection with IkkpB alpha reveals that macrophage tumor necrosis factor alpha production in rheumatoid arthritis is NF-kappB dependent. Proc. Natl. Acad. Sci. USA 95, 8211-8215.

Freeman, M. R., Schneck, F. X., Gagnon, M. L., Corless, C., Soker, S., Niknejad, K., Peoples, G. E. and Klagsbrun, M. (1995). Peripheral blood T lymphocytes and lymphocytes infiltrating human cancers express vascular endothelial growth factor: a potential role for T cells in angiogenesis. Cancer Res. 55, 4140-4145.

Guha, M. and Mackman, N. (2001). LPS induction of gene expression in human monocytes. Cell Signal. 13, 85-94.

Hawiger, J., Veach, R. A., Liu, X. Y., Timmons, S. and Ballard, D. W. (1999). IkkpB kinase complex is an intracellular target for endotoxin lipopolysaccharide in human monocytes cells. Blood 94, 1711-1716.

Hess, S., Rensing-Ehl, A., Schwabe, R., Buffer, P. and Engelmann, H. (1995). CD40 function in nonhematopoietic cells. Nuclear factor kappa B mobilization and induction of IL-6 production. J. Immunol. 155, 4588-4595.

Hu, Y., Baud, V., Delhase, M., Zhang, P., Deerinck, T., Ellisman, M., Johnson, R. and Karin, M. (1999). Abnormal morphogenesis but intact IKK activtiton in mice lacking the IKKalpha subunit of IkkpB kinase. Science 284, 316-320.

Huang, S., Pettaway, C. A., Uehara, H., Bucana, C. D. and Fidler, I. J. (2001). Blockade of NF-kappB activity in human prostate cancer cells is associated with suppression of angiogenesis, invasion, and metastasis. Oncogene 20, 4188-4197.

Huang, S., Robinson, J. B., Deguzman, A., Bucana, C. D. and Fidler, I. J. (2000). Blockade of nuclear factor-kappB signaling inhibits angiogenesis and tumorigenesis of human ovarian cancer cells by suppressing expression of vascular endothelial growth factor and interleukin 8. Cancer Res. 60, 5334-5339.

Itaya, H., Imaizumi, T., Yoshiida, H., Koyama, M., Suzuki, S. and Satoh, K. (2001). Expression of vascular endothelial growth factor in human moonocytes/macrophages stimulated with lipopolysaccharide. Thromb. Haemost. 85, 171-176.

Jiang, B. H., Zheng, J. Z., Aoki, M. and Vogt, P. K. (2000). Phosphatidylinositol 3-kinase signaling mediates angiogenesis and expression of vascular endothelial growth factor in endothelial cells. Proc. Natl. Acad. Sci. USA 97, 1749-1753.

Kiener, P. A., Moran-Davis, P., Rankin, B. M., Wahl, A. F., Aruffo, A. and Hollenberg, D. (1995). Stimulation of CD40 with purified soluble gp39 induces proinflammatory responses in human monocytes. J. Immunol. 155, 4917-4925.

Lali, F. V., Hunt, A. E., Turner, S. J. and Foxwell, B. M. (2000). The pyridyl imidazole inhibitor SB203580 blocks phosphoinositide-dependent protein kinase activity, protein kinase B phosphorylation, and retinoic acid hyperphosphorylation in interleukin-2-stimulated T cells independently of p38 mitogen-activated protein kinase. J. Biol. Chem. 275, 7395-7402.

Levy, A. P., Levy, N. S., Wegner, S. and Goldberg, M. A. (1995). Transcriptional regulation of the rat vascular endothelial growth factor gene by hypoxia. J. Biol. Chem. 270, 13333-13340.

Li, Z. W., Chu, W., Hu, Y., Delhase, M., Deerinck, T., Ellisman, M., Johnson, R. and Karin, M. (1999). The IKKbeta subunit of IkkpB kinase (IKK) is essential for nuclear factor kappB activation and prevention of apoptosis. J. Exp. Med. 189, 1839-1845.

McLaren, J., Prentice, A., Charnock-Jones, D. S., Milligan, S. A., Muller, K. H., Sharkey, A. M. and Smith, S. K. (1996). Vascular endothelial growth factor is produced by peritoneal fluid macrophages in endometriosis and is regulated by ovarian steroids. J. Clin. Invest. 98, 482-489.

Melter, M., Reinders, M. E., Sho, M., Pal, S., Geehan, C., Denton, M. D., Mukhopadhyay, D. and Briscoe, D. M. (2000). Ligation of CD40 induces the expression of vascular endothelial growth factor by endothelial cells and monocytes and promotes angiogenesis in vivo. Blood 96, 3801-3808.

Mercurio, F., Zhu, H., Murray, B. W., Shevchenko, A., Bennett, B. L., Li, J., Young, D. B., Barbosa, M., Mann, M., Manning, A. et al. (1997). IKK-1 and IKK-2: cytosolic IkkpB kinases essential for NF-kappB activation. Science 278, 860-866.

Namiki, A., Brogi, E., Kearney, M., Kim, E. A., Wu, T., Couffinhal, T., Varticovski, L. and Isner, J. M. (1995). Hypoxia induces vascular endothelial growth factor in cultured human endothelial cells. J. Biol. Chem. 270, 31189-31195.
Interleukin 10 modulation of tumour necrosis factor receptors requires tyrosine kinases but not the PI 3-kinase/p70 S6 kinase pathway. Cytokine 12, 934-943.

Wohlleben, G., Gray, D. and Schimpl, A. (1996). In vitro immunization of naive mouse B cells: establishment of IgM secreting hybridomas specific for soluble protein or hapten from B cells cultured on CD40 ligand transfected mouse fibroblasts. Int. Immunol. 8, 343-349.

Wrighton, C. J., Hofer-Warbinek, R., Moll, T., Eytner, R., Bach, F. H. and de Martin, R. (1996). Inhibition of endothelial cell activation by adenovirus-mediated expression of I kappa B alpha, an inhibitor of the transcription factor NF-kappa B. J. Exp. Med. 183, 1013-1022.

Yamamoto, T., Kozawa, O., Tanabe, K., Akamatsu, S., Matsuno, H., Dohi, S. and Uematsu, T. (2001). Involvement of p38 MAP kinase in TGF-beta-stimulated VEGF synthesis in aortic smooth muscle cells. J. Cell Biochem. 82, 591-598.

Yoshida, S., Ono, M., Shono, T., Izumi, H., Ishibashi, T., Suzuki, H. and Kuwano, M. (1997). Involvement of interleukin-8, vascular endothelial growth factor, and basic fibroblast growth factor in tumor necrosis factor alpha-dependent angiogenesis. Mol. Cell. Biol. 17, 4015-4023.