Journal of Thrombosis and Haemostasis, **4**: 2461–2469

ORIGINAL ARTICLE

Vascular endothelial growth factor expression in monocytes from patients with primary antiphospholipid syndrome

M. J. CUADRADO,* P. BUENDÍA,† F. VELASCO,† M. A. AGUIRRE,† N. BARBARROJA,† L. A. TORRES,† M. KHAMASHTA* and C. LÓPEZ-PEDRERA†

**Lupus Research Unit, St Thomas' Hospital, London, UK; and †Research Unit and Rheumatology Department, Reina Sofía University Hospital, Cordoba, Spain*

To cite this article: Cuadrado MJ, Buendía P, Velasco F, Aguirre MA, Barbarroja N, Torres LA, Khamashta M, López-Pedrera C. Vascular endothelial growth factor expression in monocytes from patients with primary antiphospholipid syndrome. *J Thromb Haemost* 2006; **4**: 2461–9.

Summary. *Background:* One of the described mechanisms leading to thrombosis in antiphospholipid syndrome (APS) is overexpression of tissue factor (TF) in the monocytes and endothelial cells of patients with antiphospholipid antibodies (aPL). Vascular endothelial growth factor (VEGF) may stimulate monocyte TF expression through its receptor, the tyrosine kinase Flt-1. *Objectives:* This study aimed to analyze the following in monocytes of 55 primary APS patients: VEGF and Flt-1 expression levels, their potential regulation by aPL, and the association of VEGF and Flt-1 expression with the increased TF expression found in APS patients. *Results:* Purified monocytes from APS patients showed higher levels of VEGF and Flt-1 than healthy donors, which further correlated with immunoglobulin G (IgG) anticardiolipin titers and TF expression rank. Moreover, monocyte VEGF and Flt-1 levels were significantly higher in patients with than in patients without previous thrombosis. *In vitro*, IgG from APS patients increased monocyte VEGF and Flt-1 expression in a dose-dependent manner. VEGF and Flt-1 expression was significantly inhibited by the p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580; this suggests the involvement of this kinase in the aPL-induced VEGF and Flt-1 upregulation. *Conclusions:* Our data show, for the first time *in vivo*, that monocytes from primary APS patients have an increased expression of VEGF and Flt-1. Furthermore, *in vitro* results indicated that this cytokine is produced by monocytes when treated with aPL, and that the p38 MAPK signaling pathway plays an important role. Thus, VEGF might act as a regulatory factor in aPL-mediated monocyte activation and TF expression, thereby contributing to the proinflammatory–prothrombotic phenotype of APS patients.

Correspondence: Chary López-Pedrera, Unidad de Investigación, Hospital Universitario Reina Sofía, Avda. Menéndez Pidal s/n, E-14004 Córdoba, Spain.
Tel.: +34 957 010 194; fax: +34 957 010 340; e-mail: rosario.lopez.exts@juntadeandalucia.es

Received 4 April 2006, accepted 28 August 2006

Keywords: antiphospholipid syndrome, Flt-1, mitogen-activated protein kinase signaling, vascular endothelial growth factor.

Introduction

The antiphospholipid syndrome (APS) is characterized by thrombosis and/or pregnancy morbidity in the presence of autoantibodies against phospholipid or phospholipid-binding protein cofactors (aPL).

Despite the strong association between aPL and thrombosis, the pathogenic role of aPL in the development of thrombosis has not been fully elucidated. Proposed pathophysiological mechanisms may be categorized into two types. First, aPL may act *in vivo* by disrupting the kinetics of the normal procoagulant and anticoagulant reactions occurring on cell membranes. Second, aPL may interact with specific cell surface receptors (proteins and/or lipids), inducing signals that have consequences downstream, and will ultimately result in upregulation of cell surface proteins [1]. One of the mechanisms contributing to thrombosis in APS patients might be the increased expression of tissue factor (TF), the major coagulation initiator *in vivo*, by endothelial cells and monocytes [2]. Our group and others [3,4] have shown that patients with primary APS have increased expression of TF on the monocyte surface, along with increased mRNA-TF and TF antigen and activity levels in peripheral blood mononuclear cells, where the source of TF is the monocyte.

Previous reports indicate a close relationship between TF and vascular permeability factor/vascular endothelial growth factor (VPF/VEGF), a family of proteins involved in normal vascular development and in important pathologies including cancer, wound healing and inflammation. The VPF/VEGF proteins are widely regarded as the most relevant proteins involved in the development of the vasculature. VEGF-A is thought to be the single most important angiogenic factor. Its expression is regulated in different tissues and circumstances by a number of factors including hypoxia, other cytokines and growth factors, various hormones and, importantly in tumors, by oncogenes and tumor suppressor genes [5,6]. VEGF-A also alters the gene expression pattern of endothelial cells by

upregulating a number of transcription factors, matrix metalloproteinases, antiapoptotic factors, endothelial cell adhesion molecules and proteins associated with thrombosis, mainly TF [7].

Two distinct receptors for VEGF, the tyrosine kinase receptors Flt-1 and Flk-1/KDR, have been described. In contrast to endothelium, monocytes express only the VEGF receptor Flt-1, a functional receptor for VEGF in monocytes and endothelial cells that works as a mediator of monocyte recruitment and procoagulant activity [8,9]. VEGF signaling is mediated by receptor dimerization, leading to autophosphorylation of the cytosolic domains of these receptors.

A very important point to consider is the fact that TF also has the capacity to upregulate VEGF-A expression and downregulate transcription of the angiogenesis inhibitor thrombospondin. Furthermore, the upregulation of VEGF-A induced by TF has the potential to set up a self-perpetuating feedback loop, because VEGF-A expression also upregulates TF expression [10].

Protein kinases are key regulators of cellular signaling that control inflammation, cell differentiation, cell growth and cell death [11]. Among them, p38 mitogen-activated protein kinase (MAPK) is an important component of intracellular signaling cascades that initiate various inflammatory cellular responses. Moreover, recent studies have suggested that aPL antibodies induce TF expression in endothelial cells and monocytes from APS patients by activating, simultaneously and independently, the phosphorylation of MEK1/ERK proteins, and the p38 MAP kinase-dependent nuclear translocation and activation of NFκB/Rel proteins [12,13]. Enhanced phosphorylation of the p38 MAP kinase and, to a certain degree, of the Erk-1/2 MAP kinase, comprise two essential mediators of VEGF-induced endothelial TF activity [14]. However, in monocytes, the signaling events leading to VEGF-mediated TF induction are only partially understood.

In the present study, we analyzed the VEGF and Flt-1 expression levels in monocytes of APS patients, the molecular mechanisms involved in their aPL-induced expression, and their association with the elevated TF expression found in these patients. Our data primarily showed that monocytes from APS patients expressed increased levels of both VEGF and Flt-1 in comparison with monocytes from healthy donors. In addition, our results strongly suggest that circulating monocytes of APS patients are subjected to an upregulated VEGF expression as a consequence of aPL activation, and that the p38 MAP kinase pathway may be directly involved.

Subjects and methods

Subjects

Fifty-five patients (38 non-pregnant female and 17 male; mean age 43; range: 18–74 years) fulfilling the classification criteria for APS [15] were included after informed consent. Forty-six out of 55 patients (84%) had an objectively verified history of thrombosis [29 (63%) arterial, 11 (24%) venous, and six (13%) both arterial and venous] and nine (16%) had recurrent pregnancy loss only. All patients were studied at least 9 months after their latest thrombotic event or pregnancy loss. We excluded APS patients who had evidence of an underlying systemic disease or antibodies against double-stranded DNA or extractable nuclear antigen. All patients were tested for the presence of anticardiolipin autoantibodies (aCL) and lupus anticoagulant (LA) as previously described [16]. Briefly, immunoglobulin G (IgG) and IgM aCL were determined by enzyme-linked immunosorbent assay using a commercial kit (Cheshire Diagnostic Ltd, Chester, UK). For LA detection, patients were screened by activated partial thromboplastin time, kaolin clotting time and dilute Russell's viper venom time following the guidelines recommended by the Subcommittee for Standardization of the International Society on Thrombosis and Haemostasis [17]. IgG and IgM aCL were positive in 33 (60%) and 29 (53%) patients respectively, and LA in 42 (76%). As controls, we studied 12 patients with thrombosis but without antiphospholipid antibodies [seven non-pregnant women and five men, mean age 40 (range: 32–51 years), including patients with objectively verified thrombotic events: seven deep venous thrombosis and five thrombosis in intracerebral vessels], and 15 age- and sex-matched healthy controls, comprising nine non-pregnant women and six men, mean age 38 (range: 25–49 years). None of the healthy controls had a history of autoimmune disease, bleeding disorders, thrombosis or pregnancy loss. Both patients and controls had normal blood pressure.

Monocyte isolation

Peripheral venous blood samples of patients and controls were collected in sterile precooled tubes containing 0.129 M sodium citrate (1/9, v/v) (Becton Dickinson Vacutainer System Europe, Meylan, France) as the anticoagulant and centrifuged immediately at $500 \times g$ for 10 min at 4 °C to remove platelets. Isolation of monocytes from peripheral blood was performed by depletion of non-monocytes, using a commercial available kit (Miltenyi Biotech, Bergisch Gladbach, Germany). Briefly, peripheral blood mononuclear cells (PBMC) were obtained by density gradient centrifugation over Ficoll Paque. For depletion of T cells, natural killer cells, B cells, dendritic cells and basophils from PBMC, these cells were indirectly magnetically labeled using a cocktail of hapten-conjugated CD3, CD7, CD19, CD45RA, CD56 and anti-IgE antibodies and microbeads coupled to an antihapten monoclonal antibody (mAb). The magnetically labeled cells were depleted by retention on a column in a magnetic field. The purity of the isolated monocytes was evaluated by staining aliquots of the cell fraction obtained with a fluorochrome conjugated antibody against monocytes (anti-CD14-FITC), and analysis by flow cytometry.

Flow cytometry analysis

Cells were washed in phosphate-buffered saline (PBS), incubated for 30 min at 4 °C with PBS containing 20% heat-inactivated normal human serum, washed again and

incubated for 30 min at 4 °C with 15 nM of specific mAbs to human TF [clone TF9-6B4, fluorescein isothiocyanate (FITC)-conjugated; American Diagnostica, Greenwich, CT, USA] or to human CD14 [R-phycoerythrin (PE)-conjugated; Caltag Laboratories, San Francisco, CA, USA]. Flow cytometry analyses were performed on a FACScan (BD Biosciences, San Jose, CA, USA). Control cells incubated with FITC- or PE-conjugated non-specific antibodies from the same manufacturer were used to set the threshold for the fluorescence parameter, such that the fraction of cells with positive fluorescence was $< 2.5\%$ of total cells. The percentage of TF or CD14 positive cells was determined from the fraction of cells in the sample incubated with specific antibodies that exceeded in fluorescence signal intensity the threshold obtained with the control sample.

Immunocytochemistry and ELISA for VEGF and Flt-1

Cytospins monocyte preparations were fixed in Bouin solution (Sigma, St Louis, MO, USA). Fixative was removed by sequential washing with destilated water, PBS, alcohol iodide and sodium metabisulfite. Endogenous peroxidase activity was blocked by incubating slides in 3% hydrogen peroxide for 5 min, followed by washing in PBS. The slides were then incubated for 30 min with polyclonal anti-VEGF and anti-Flt-1 antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at a dilution of 1:200. This incubation was followed by sequential 15-min incubations with a biotinylated link antibody and peroxidase-labeled streptavidin (Dako, High Wycombe, UK). The color reaction was developed using 3,3′-diaminobenzidine as chromogen. Slides were mounted on mounting medium (DPX). For the negative control slides, normal rabbit immunoglobulin (Dako) was used at the same protein concentration as the antibody.

Levels of VEGF protein in plasma from APS patients were determined using the human VEGF ELISA kit (R & D Systems, Minneapolis, MN, USA). Briefly, the VEGF-ELISA kit employs a monoclonal anti-VEGF as the capturing antibody. After incubation of plasma (100 μL per well) in microtest strip wells precoated with the capturing antibody, VEGF was detected using a polyclonal, horseradish peroxidase (HRP)-conjugated anti-VEGF antibody that specifically recognizes VEGF, thus generating a colored product that was quantified using a spectrophotometer. VEGF concentration was determined by comparing the absorbance obtained from each plasma sample with that achieved from a standard curve, performed using known concentrations of human VEGF protein.

Purification of IgG and determination of antibodies

IgG from seven patients with APS and from the pooled sera of 10 healthy subjects (as controls) was purified by protein G sheparose high-performance affinity chromatography (MAbTrapTM Kit; Amersham Biosciences, Uppsala, Sweden), according to the manufacturer's recommendations. All patients had a history of thrombosis. IgG fractions were checked for the absence or presence of endotoxin by the *Limulus* amebocyte lisate assay (Amebolysate; ICN Biomedical, Costa Mesa, CA, USA).

Anti-β2-GPI antibody activity of purified IgG was confirmed by ELISA. IgG and aCL were determined as above described and titers were reported in IgG phospholipids (GPL) units.

In vitro *exposure of normal monocytes to aPL antibodies*

Purified normal monocytes (1.5×10^6 cells mL^{-1}) were incubated with different doses of purified IgG APS (200,100, 50 and 25 μg mL^{-1}) or normal IgG (IgG-NHS) for 6 h at 37 °C. In some experiments, cells were pretreated for 30 min with SB203580 (0.1–10 μM). Then, purified IgG APS was added and cells were incubated for a further 6 h.

RNA isolation and quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Total cellular RNA from monocytic cells was extracted using Tri® Reagent (Sigma) according to the manufacturer's recommendations. RNA concentration was determined spectrophotometrically at 260 and 280 nm and its integrity was verified following separation by electrophoresis into a 0.8% agarose gel containing ethidium bromide. RNA samples were stored at −80 °C until use.

The expression levels of TF, VEGF, and Flt-1 genes, and GAPDH as housekeeping gene, were measured by quantitative real-time RT-PCR using the LightCycler thermal cycler system (Roche Diagnostics, Indianapolis, IN, USA). Sequence of primers used for study and theoretic size of PCR products were: TF: (283 bp), forward primer: CTACTGTTTCAGTGTTCAAGCAGTGA; reverse primer: CAGTGCAATATAGCATTTGCAGTAGC; VEGF: (470 bp), forward primer: CGAAGTGGTGAAGTTCATGGATG; reverse primer: TTCTGTATCAGTCTTTCCTGGTGA; Flt-1: (232 bp) forward primer: CAAGTGGCCAGAGGCATGGAGTT; reverse primer: CGTCGCTCTTGGTGCTGTAGATT; GAPDH: (240 bp), forward primer: TGATGACATCAAGAAGGTGGTGAAG; reverse primer: TCCTTGGAGGCCATGTAGGCCAT.

RT-PCR was performed on one-step using the QuantiTect SYBR Green RT-PCR kit (QIAGEN GmbH, Hilden, Germany) following the manufacturer's protocol. Briefly, 100 ng total RNA was placed into a 20-μL reaction volume containing 150 nM of each primer, 10 μL of SYBR Green PCR master mix, and 0.2 μL of RT. cDNA was synthesized at 50 °C for 20 min, followed by a step of HotStarTaq DNA Polymerase activation, Omniscript and Sensiscript Reverse Transcriptases deactivation and template cDNA denaturation at 95 °C for 15 min, and 50 cycles of denaturation at 94 °C for 15 s, annealing at 60 °C for 30 s and extension at 68 °C for 40 s. The temperature transition rate was set at 20 °C s^{-1}. Detection of the fluorescent product was performed at the end of the extension at 68 °C. To confirm amplification specificity, the PCR products were subjected to a melting curve analysis from 65 to 90 °C. mRNA samples were analyzed at least in three similar RT-PCRs performed in

triplicate. Negative controls containing water instead of RNA were run in order to confirm that the samples were not cross-contaminated. Quantification of relative expression was determined by standard curve method according to the manufacturer's instructions. Target amount was normalized to *GAPDH* gene and relative expression was calculated by ratio of normalized target values and calibrator normalized target values.

Western blotting

Cytoplasmic and nuclear extracts were prepared by standard protocols [18] and used for Western blotting. Briefly, 5×10^6 monocytes isolated from each sample patient were resuspended in 400 μL of cold buffer A (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride). Samples were incubated on ice for 10 min, then vortexed for 10 s, and finally pelleted by brief centrifugation (1 min). The supernatant was saved as the cytoplasmic extract for Western blotting, and the pellet was resuspended in 100 μL of storage buffer (20 mM HEPES pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.21 mM EDTA, 0.5 mM dithiothreitol and 0.2 mM phenylmethylsulfonyl fluoride). Samples were then incubated on ice for another 20 min followed by centrifugation. Supernatants were collected for use as nuclear extracts. Protein concentrations were determined using a Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Fifty microgram of cytoplasmic extracts were resolved on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and then transferred to nitrocellulose membranes. Following standard protocols, VEGF, Flt-1, and phosphorylated and non-phosphorylated p38 MAPK protein levels were determined by Western blotting using either monoclonal or polyclonal anti-VEGF, anti-Flt-1, anti-phospho p38 MAPK, anti-p38 MAPK, and anti-actin antibodies (Santa Cruz Biotechnology Inc.). Visualization of immune complexes was performed using secondary antibodies conjugated to HRP and the Luminol Reagent detection system (Santa Cruz Biotechnology Inc.). Protein levels were quantified using the image analysis software INTELLIGENT QUANTIFIER version 2.1.1 (Bio Image, Ann Arbor, MI, USA). Results were calculated in terms of integrated optical density (IOD) and expressed in arbitrary units (AU).

Statistical analysis

All data are expressed as mean ± the SEM. Statistical analyses were carried out using the software package SIGMASTAT (Jandel Scientific GmbH, Erkrat, Germany). Before comparing two data groups, a normality test and an equal variance test were performed. If data groups passed both tests, a comparison was made by a parametric test (paired Student's *t*-test). If the normality and/or equal variance test was violated, a comparison was made by a non-parametric test (Mann–Whitney rank sum test). Correlations were assessed by Pearson's product-moment correlation. Differences were considered significant if $P < 0.05$.

Results

Gene and protein TF expression

TF mRNA levels were found to be significantly increased in preparations of total RNA of uncultured monocytes from 39 of 55 APS patients (71%: 36 of 46 with thrombosis and three of nine without thrombosis) compared with patients with thrombosis but without APS ($P < 0.001$), and to healthy controls ($P < 0.001$). Moreover, mean levels of TF mRNA were significantly higher in monocytes from patients with a history of thrombosis than in those from patients without thrombosis ($P < 0.035$) (Fig. 1A). Cell surface-associated TF was detected in samples from 39 of the 55 APS patients, and its levels were significantly increased (47.2 ± 2.9% positive cells) in patients with thrombosis compared with the levels observed in patients without thrombosis (20.2 ± 2.1%, $P < 0.001$), patients with thrombosis but without APS (18.9 ± 1.8%, $P < 0.001$), and with healthy controls (5.6 ± 1.3%, $P < 0.001$) (Fig. 1B). Fig. 1C shows typical histograms from a patient with thrombosis, a patient without thrombosis, a patient with thrombosis but without APS, and a healthy control.

VEGF and Flt-1 expression

Levels of soluble VEGF were significantly increased in plasma of patients (54.4 ± 8.1 pg mL^{-1}) compared with controls (4.0 ± 2.1) ($P < 0.001$). To elucidate the origin of this plasma VEGF, we measured VEGF expression in monocytes from APS patients by several methods, including quantitative real-time RT-PCR, Western blot and immunocytochemistry. We found significantly higher levels of both mRNA and protein VEGF compared with controls (Fig. 2; $P = 0.04$ and $P = 0.005$, respectively), suggesting that monocytes might constitute a relevant source of VEGF in primary APS patients. Subgroup analysis showed that monocyte protein VEGF in patients with previous thrombosis (3399.9 ± 311.9 AU of IOD) were significantly higher than the VEGF levels of those without thrombosis (2277.4 ± 355.1 AU of IOD; $P = 0.03$) and than those with thrombosis but without APS (1147.2 ± 566.1 AU of IOD; $P < 0.001$).

VEGF-stimulated activity in monocytes is mediated by the VEGF receptor Flt-1 [8]. We found significantly higher levels of both mRNA and protein levels of Flt-1 in monocytes from APS patients compared with controls (Fig. 3), with significant increased expression among those patients with thrombosis compared with the levels observed in patients without thrombosis ($P = 0.035$) and with those of patients with thrombosis but without APS ($P = 0.002$).

Correlations among aPL, VEGF, Flt-1 and TF expression

Pearson's relational statistic indicated positive correlation between both plasma VEGF levels and cell surface TF expression levels and the titer of IgG aCL ($P = 0.038$ and $P = 0.008$, respectively; Fig. 4A,B). There was also a signifi-

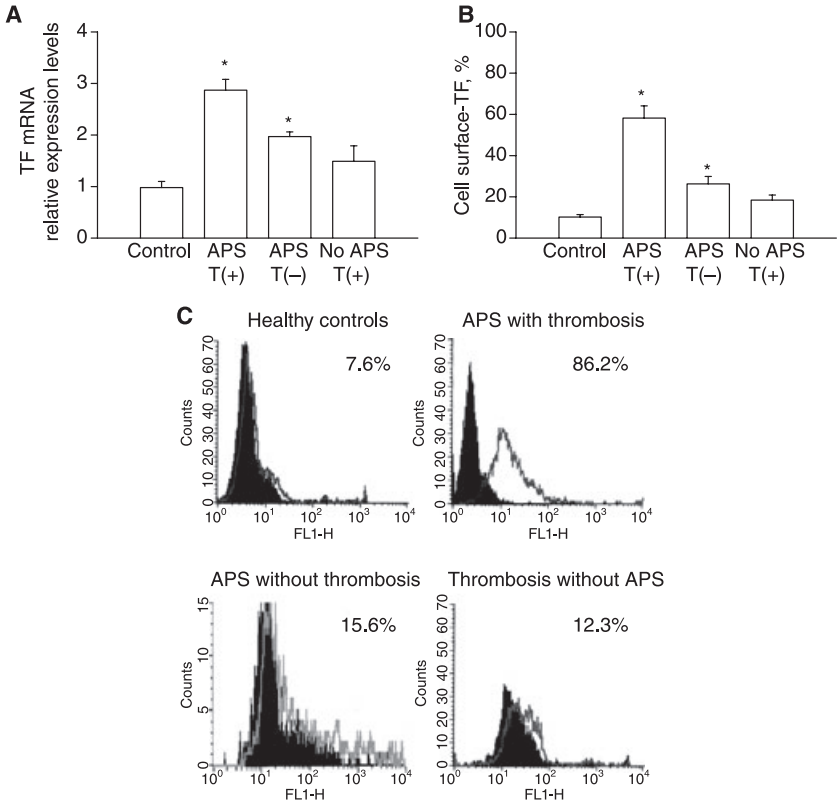

Fig. 1. Tissue factor (TF) expression on monocytes from antiphospholipid syndrome (APS) patients. (A,B) TF-mRNA relative expression levels and cell surface TF expression in monocytes of APS patients and controls. The bar graphs show mean cell surface and mRNA TF levels ± SEM of APS patients with or without previous thrombosis, patients with thrombosis but without APS, and healthy controls. Significant differences at $P < 0.05$ vs. healthy controls. (C) Detection of cell surface TF on monocytes from representative subjects from APS patients with or without thrombosis, patients with thrombosis but without APS, and healthy controls, by flow cytometry.

cant rank correlation between the levels of both plasma and protein VEGF and cell surface TF ($P < 0.001$ and $P = 0.01$, respectively; Fig. 4C,D). Moreover, titers of Flt-1 correlated positively with both protein TF ($P = 0.008$; Fig. 4E) and VEGF ($P = 0.03$; Fig. 4F).

Involvement of p38 MAPK activation in the aPL-induced upregulation of monocyte VEGF expression

Characterization of IgG aPL All of the IgG aPL samples from the seven patients were positive for aCL (> 100 GPL units) and all of the IgG samples from the healthy controls were negative for aCL (< 10 GPL units). The anti-ß2-GPI antibody activities of the IgG aPL and control IgG were a mean ± SD of 87.2 ± 19.3 GPL units and 5.3 ± 0.6 GPL units, respectively. All patient IgG selected for this study had moderate to high activity of anti-ß2GPI, which correlated with the level of IgG observed in these patients ($P = 0.0352$; $r = 0.904$). All IgG preparations tested negative for lipopolysaccharide in the *Limulus* amebocyte lysate assay.

As shown in Fig. 5, IgG from APS patients significantly increased monocyte VEGF and Flt-1 expression at both mRNA and protein levels, when compared with IgG from controls, in a dose-dependent manner. To elucidate the role of p38 MAPK in VEGF and Flt-1 expression, the expression of both proteins induced by aPL antibodies on normal monocytic cells was also examined in the presence and in the absence of the specific p38 MAPK inhibitor SB203580. As shown in Fig. 6, VEGF and Flt-1 expression, at both mRNA and protein levels, were significantly inhibited by SB203580 in a dose-dependent way. These data suggested the involvement of p38 MAPK activation in the aPL-induced upregulation of VEGF and Flt-1.

Discussion

The data obtained in this study strongly suggest that circulating monocytes of APS patients are subjected to an upregulated VEGF expression as a consequence of aPL activation. Our results primarily showed significantly higher levels of plasma VEGF in APS patients compared with controls, as previously demonstrated by Williams *et al.* [19]. In addition, our data indicated that monocytes from APS expressed increased levels of both mRNA and protein VEGF than monocytes from healthy donors, thus pointing to the monocytes as a possible source of VEGF in primary APS patients.

Originally identified on the basis of its ability to induce vascular permeability [20], VEGF is now recognized as a potent inducer of endothelial proliferation, migration, and survival. Furthermore, VEGF also acts as a proinflammatory cytokine

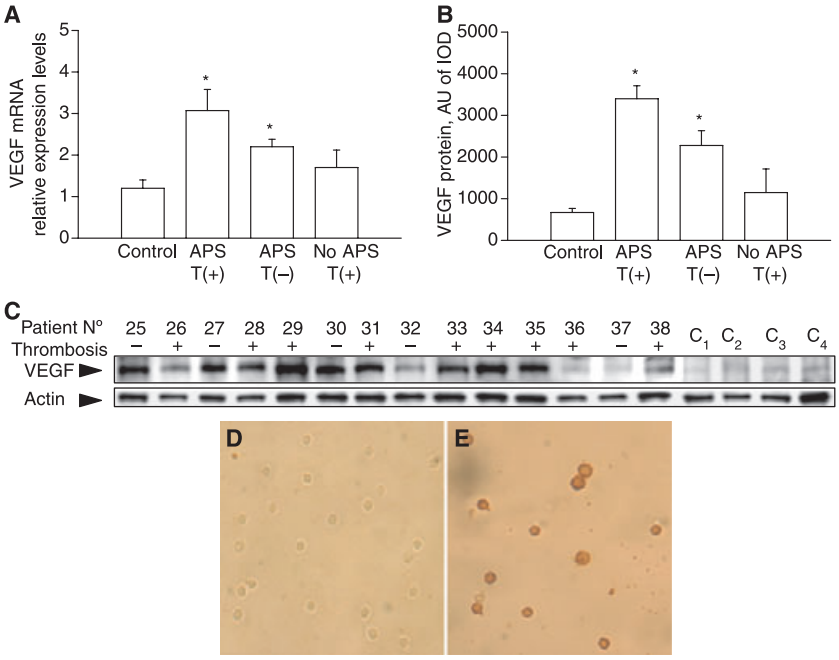

Fig. 2. Vascular endothelial growth factor (VEGF) expression on monocytes from antiphospholipid syndrome (APS) patients. (A,B) VEGF-mRNA and protein expression levels in monocytes of APS patients and controls. The bar graphs show mean protein and mRNA-VEGF levels ± SEM of APS patients with or without previous thrombosis, patients with thrombosis but without APS, and healthy controls. Significant differences at $P < 0.05$ vs. healthy controls. Panel C is representative of various samples from APS patients and healthy controls analyzed by Western blot. Panels D and E are representative photographs from monocytes of APS patients immunostained with an anti-VEGF antibody followed by peroxidase-conjugated avidin and 3,3′-diaminobenzidine staining: (D) healthy control; (E) APS patient with thrombosis. Original magnification ×120.

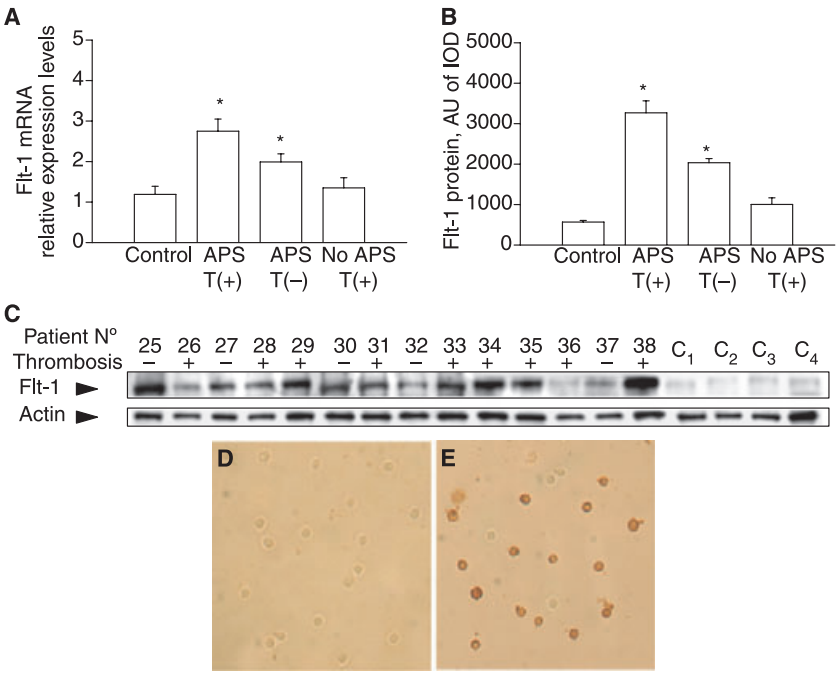

Fig. 3. Flt-1 expression on monocytes from antiphospholipid syndrome patients. See legend to Fig. 2 for further details.

that induces the expression of a number of molecules involved in the regulation of angiogenesis, such as specific enzymes (e.g. cyclooxygenase-2), adhesion molecules (e.g. E-selectin, VCAM-1) (18) and pro-coagulant factors (e.g. TF) [21]. As in other diseases [22], raised VEGF may reflect a link between abnormal endothelial damage/dysfunction, monocyte activation, and angiogenic factors activation. All of these processes may act together to alter TF expression and endothelial

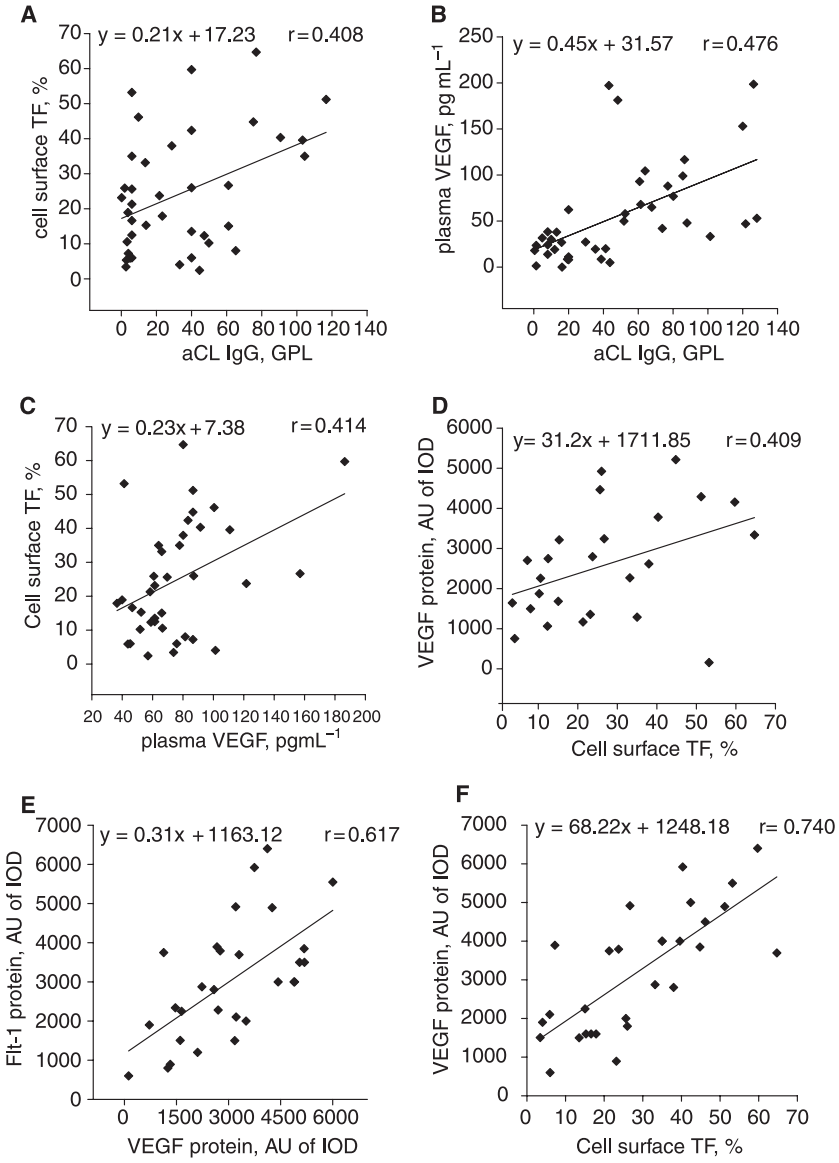

Fig. 4. Positive correlations among anticardiolipin autoantibodies, immunoglobulin G, vascular endothelial growth factor, Flt-1 and tissue factor expression. Correlations were assessed by Pearson's product-moment correlation. Differences were considered significant if $P < 0.05$.

integrity, thereby contributing to the prothrombotic state in APS. Accordingly, in our study, increased monocyte VEGF expression correlated positively with the levels of both mRNA and cell surface TF expression. Besides, we found elevated Flt-1 expression in monocytes from APS in parallel with VEGF. Moreover, a positive correlation was found between the expression of this receptor and TF expression on monocytes. Thus, our overall results indicate that increased VEGF activity might be responsible for the TF overexpression found in monocytes of APS.

On the other hand, previous studies have shown that in vascular endothelial cells and tumor cells, a cycle exists with respect to TF and VEGF, so that each can induce the other. Furthermore, it has been demonstrated that some transcription factors regulating TF also induce VEGF transcription; thus, simultaneous activation of both proteins might also occur in monocytes from APS patients [10]. Nevertheless, other possible sources of VEGF production in APS patients cannot be excluded. In that way, it has been demonstrated that platelets, smooth muscle cells, macrophages, fibroblasts and endothelial cells can express VEGF [23]. Moreover, some cytokines, including the transforming growth factor, the hepatocyte growth factor and the fibroblast growth factor-2 act, at least in part, by regulating VEGF expression [5]. However, a recent study by Dupuy *et al.* [24] has shown that thrombin is also involved in the upregulation of VEGF by endothelial cells. In addition, thrombin levels are also significantly increased in plasma of APS patients [25]. Hence, that excess of thrombin, most likely induced by TF expression, and acting through the activation of protease-activated receptors [26], might also be related to the elevated VEGF production found in these patients.

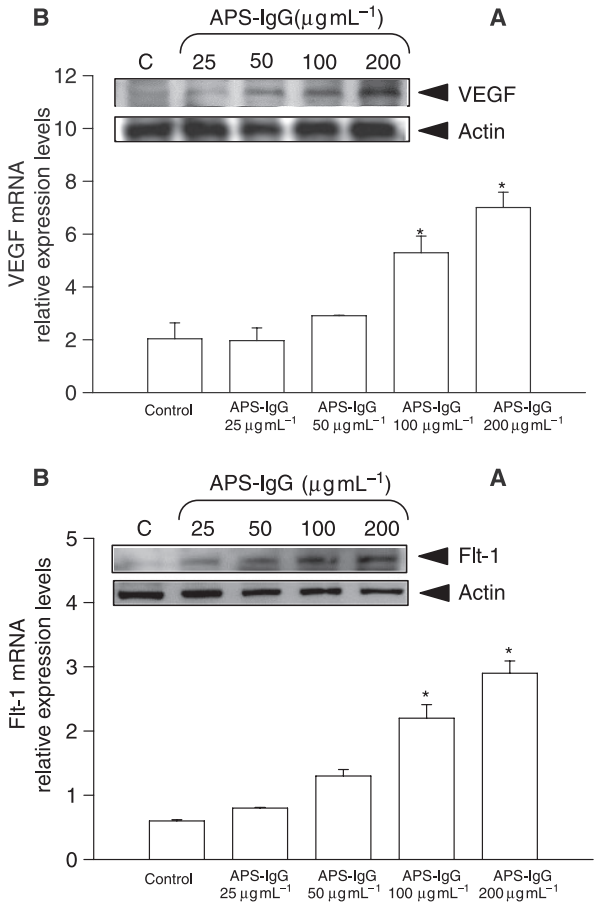

Fig. 5. Dose dependence of antiphospholipid syndrome (APS) immunoglobulin G (IgG)-induced vascular endothelial growth factor (VEGF) and Flt-1 expression. Normal monocytes were incubated with varying concentrations of APS IgG or normal IgG for 6 h. (A) Detection of protein VEGF and Flt-1 on monocytes from a representative experiment by Western blot. (B) The bar graphs show mean mRNA-VEGF and Flt-1 relative expression levels ±SEM of seven experiments. Significant differences at $P < 0.05$ vs. untreated cells.

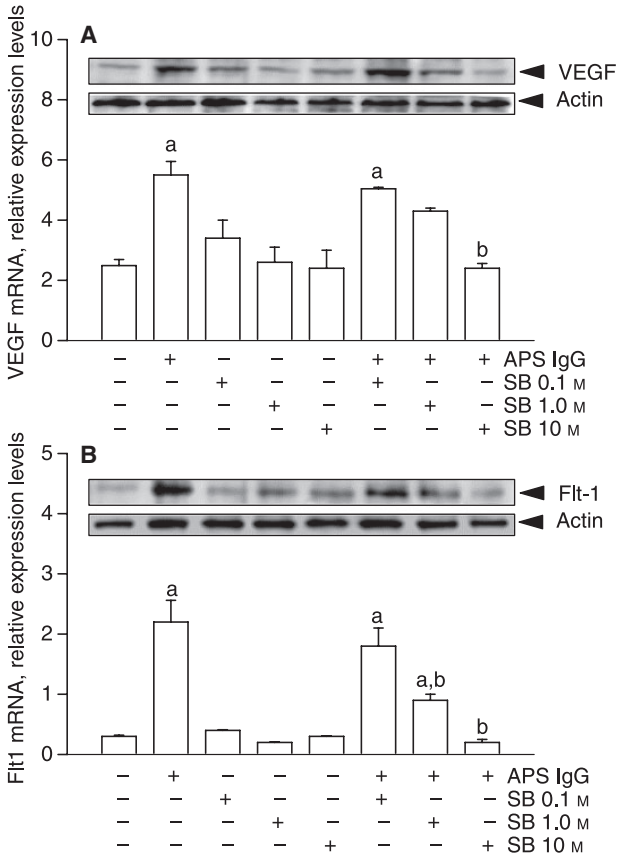

Fig. 6. Intracellular pathways involved in the induced antiphospholipid syndrome (APS)-immunoglobulin G (IgG)-monocyte vascular endothelial growth factor (VEGF) and Flt-1 expression. Normal monocytes were pretreated with SB203580 (0.1–10 μM) for 30 min, followed by APS-IgG (100 μg mL^{-1}) exposure for 6 h. VEGF and Flt-1-mRNA relative expression levels and protein VEGF and Flt-1 expression were then measured as described in Material and methods. (A) Detection of protein VEGF and Flt-1 on monocytes from a representative experiment by Western blot. (B) The bar graphs show mean mRNA-VEGF and Flt-1 levels ±SEM of three separate experiments. Significant differences at $P < 0.05$, (a) vs. untreated cells, (b) vs. cells incubated with APS-IgG alone.

In the present study, we demonstrated that the p38 MAPK-dependent signaling pathway participates in aPL-mediated VEGF expression. Activation of p38 is considered to be critical for normal immune responses. Moreover, the p38 MAPK pathway has been implicated in the regulation of TF expression in monocytes, endothelial cells and smooth muscle cells [14,27,28]. p38 MAPK has also proven to be involved in both the upregulation of VEGF, and the VEGF-induced activation of TF expression in various cell models [29,30]. Thus, a role for this kinase in the VEGF-induced activation of TF expression in monocytes from APS patients cannot be ruled out.

In conclusion, our data clearly show, for the first time *in vivo*, the increased expression of VEGF in monocytes from APS patients. Furthermore, *in vitro* results indicated that VEGF was produced by monocytes when treated with aPL. Thus, this cytokine might play a role as a regulatory factor in aPL-mediated monocyte activation and TF expression, and therefore may contribute to the proinflammatory–prothrombotic phenotype observed in monocytes of APS patients. Our results also demonstrated that, as previously showed for the regulation of aPL-induced TF expression [13], the p38 MAPK signaling pathway also plays an important role in aPL-induced VEGF expression in monocytes. This pathway, likely to be involved in maintaining active disease, might constitute a valuable target for therapy.

Addendum

M. J. Cuadrado and C. López-Pedrera originated and coordinated the study together with F. Velasco. M. J. Cuadrado wrote the manuscript. P. Buendía, N. Barbarroja and L. A. Torres performed the analyses. All other authors were involved in designing the study and in collecting patient data and reviewing the manuscript.

Acknowledgements

We thank all patients and healthy subjects for their participation in the study.

Disclosure of Conflict of Interests

C. López-Pedrera was supported by a Science and Technology postdoctoral fellowship from the Spanish government. This work was supported by grants from the 'Junta de Andalucía' (exp 171/03) and the 'Fondo de Investigación Sanitaria' (FIS 03/1033) of Spain.

References

1 Espinosa G, Cervera R, Font J, Shoenfeld Y. Antiphospholipid syndrome: pathogenic mechanisms. *Autoimmun Rev* 2003; **2**: 86–93.
2 Dobado-Berrios PM, López-Pedrera CH, Velasco F, Cuadrado MJ. The role of TF in the antiphospholipid syndrome. *Arthritis Rheum* 2001; **44**: 2467–76.
3 Cuadrado MJ, López-Pedrera CH, Khamashta MA, Camps MT, Tinahones F, Torres A, Hughes GRV, Velasco F. Thrombosis in primary antiphospholipid syndrome. A pivotal role for monocyte TF expression. *Arthritis Rheum* 1997; **40**: 834–41.
4 Amengual O, Atsumi T, Khamashta MA, Hughes GRV. The role of the TF pathway in the hypercoagulable state in patients with the antiphospholipid syndrome. *Thromb Haemost* 1998; **79**: 276–81.
5 Brown LF, Detmar M, Claffey K, Nagy JA, Feng D, Dvorak AM, Dvorak HF. Vascular permeability factor/vascular endothelial growth factor: a multifunctional angiogenic cytokine. *EXS* 1997; **79**: 233–69.
6 Dvorak HF. Vascular permeability factor/vascular endothelial growth factor: a critical cytokine in tumor angiogenesis and a potential target for diagnosis and therapy. *J Clin Oncol* 2002; **20**: 4368–80.
7 Wang H, Keiser JA. Vascular endothelial growth factor upregulates the expression of matrix metalloproteinases in vascular smooth muscle cells: role of flt-1. *Circ Res* 1998; **83**: 832–40.
8 Clauss M, Weich H, Breier G, Knies U, Rockl W, Waltenberger J, Risau W. The vascular endothelial growth factor receptor Flt-1 mediates biological activities. Implications for a functional role of placenta growth factor in monocyte activation and chemotaxis. *J Biol Chem* 1996; **271**: 17629–34.
9 Masood R, Cai J, Zheng T, Smith DL, Hinton DR, Gill PS. Vascular endothelial growth factor for VEGF receptor-positive human tumors. *Blood* 2001; **98**: 1904–13.
10 Chen J, Bierhans A, Schiekofer S, Andrassy M, Chen B, Stem DM, Nawroth PP. Tissue factor – a receptor involved in the control of cellular properties, including angiogenesis. *Thromb Haemost* 2001; **86**: 334–45.
11 Su B, Karin M. Mitogen-activated protein kinase cascades and regulation of gene expression. *Curr Opin Immunol* 1996; **8**: 402–11.
12 Vega-Ostertag M, Kasper K, Swerlik R, Ferrara D, Harris EN, Pierangeli SS. Involvement of p38 MAPK in the up-regulation of tissue factor on endothelial cells by antiphospholipid antibodies. *Arthritis Rheum* 2005; **52**: 1545–54.
13 López-Pedrera CH, Buendía P, Cuadrado MJ, Siendones E, Aguirre MA, Barbarroja N, Montiel-Duarte C, Torres A, Khamashta MA, Velasco F. Antiphospholipid antibodies from antiphospholipid syndrome patients induce monocyte expression through the simultaneous activation of both NFκB/Rel proteins via p38 MAPK pathway, and the MEK1/ERK pathway. *Arthritis Rheum* 2006; **54**: 301–11.
14 Blum S, Issbrüker K, Willuweit A, Hehlgans S, Lucerna M, Mechtcheriakova D, Walsh K, von der Ahe D, Hofer E, Clauss M. An inhibitory role of the phosphatidylinositol 3-kinase-signaling pathway in vascular endothelial growth factor-induced tissue factor expression. *J Biol Chem* 2001; **276**: 33428–434.
15 Miyakis S, Lockshin MD, Atsumi T, Branch DW, Brey RL, Cervera R, Derksen RH, De Groot PG, Koike T, Meroni PL, Reber G, Shoenfeld Y, Tincani A, Vlachoyiannopoulos PG, Krilis SA. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). *J Thromb Haemost* 2006; **4**: 295–306.
16 Dobado-Berrios PM, Lopez-Pedrera CH, Velasco F, Aguirre MA, Torres A, Cuadrado MJ. Increased levels of tissue factor mRNA in mononuclear blood cells of patients with primary antiphospholipid syndrome. *Thromb Haemost* 1999; **82**: 1578–82.
17 Brandt JT, Triplett DA, Alving B, Scharrer I. Criteria for the diagnosis of lupus anticoagulants: an update. *Thromb Haemost* 1995; **74**: 1185–90.
18 Andrews NC, Faller DV. A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucleic Acids Res* 1991; **19**: 2499.
19 Williams FMK, Parmar K, Hughes GRV, Hunt BJ. Systemic endothelial cell markers in primary antiphospholipid syndrome. *Thromb Haemost* 2000; **84**: 742–6.
20 Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF. Tumor cells secrete a vascular permeability factor that promote accumulation of ascites fluid. *Science* 1983; **219**: 983–5.
21 Kim I, Moon SO, Kim SH, Kim HJ, Koh YS, Koh GY. Intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-Selectin through Nuclear Factor-kB activation in endothelial cells. *J Biol Chem* 2001; **276**: 7614–20.
22 Freestone B, Chong AY, Lim HS, Lip GY. Angiogenic factors in atrial fibrillation: a possible role in thrombogenesis? *Ann Med* 2005; **37**: 365–72.
23 Dvorak HF. Angiogenesis: update 2005. *J Thromb Haemost* 2005; **3**: 1835–42.
24 Dupuy E, Habib A, Lebret M, Yang R, Levy-Toledano S, Tobelem G. Thrombin induces angiogenesis and vascular endothelial growth factor expression in human endothelial cells: possible relevance to HIF-1alpha. *J Thromb Haemost* 2003; **1**: 1096–102.
25 Hwang KK, Grossman JM, Visvanathan S, Chukwuocha RU, Woods Jr VL, Le DT, Hahn BH, Chen PP. Identification of thrombin-antithrombin antibodies in the antiphospholipid syndrome that interfere with the inactivation of thrombin by antithrombin. *J Immunol* 2001; **167**: 7192–8.
26 Tsopanoglou NE, Maragoudakis ME. Role of thrombin in angiogenesis and tumor progression. *Semin Thromb Haemost* 2004; **30**: 63–9.
27 Ohsawa M, Koyama T, Nara N, Hirosawa S. Induction of tissue factor expression in human monocytic cells by protease inhibitors through activating activator protein-1 (AP-1) with phosphorylation of Jun-N-terminal kinase and p38. *Thromb Res* 2003; **112**: 313–20.
28 Herkert O, Diebold I, Brandes RP, Hess J, Busse R, Garlach A. NADP oxidase mediates tissue factor-dependent surface procoagulant activity by thrombin in human vascular smooth muscle cells. *Circulation* 2002; **105**: 2030–6.
29 Itaya H, Imaizumi T, Yoshida H, Koyama M, Suzuki S, Satoh K. Expression of vascular endothelial growth factor in human monocyte/macrophages stimulated with lipopolysaccharide. *Thromb Haemost* 2001; **85**: 171–6.
30 Jung YD, Liu W, Reinmuth N, Ahmad SA, Fan F, Gallick GE, Ellis LM. Vascular endothelial growth factor is upregulated by interleukin-1 beta in human vascular smooth muscle cells via the p38 mitogen-activated protein kinase pathway. *Angiogenesis* 2001; **4**: 155–62.