Hypoxia and platelet-derived growth factor-BB synergistically upregulate the expression of vascular endothelial growth factor in vascular smooth muscle cells

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Abstract Vascular endothelial growth factor (VEGF) mRNA expression was analysed in rabbit vascular smooth muscle cells following exposure to hypoxia and platelet-derived growth factor-BB (PDGF-BB). Hypoxia potently upregulated VEGF mRNA steady-state levels in a time- and concentration-dependent manner reaching a maximum level (~30-fold increase) after 12–24 h at 0% O_2. In contrast, PDGF-BB caused a modest increase in VEGF expression. However, the combination of PDGF-BB and a threshold hypoxic stimulus (2.5% O_2 for 4 h) had a marked synergistic effect. Synergy between hypoxia and PDGF-BB was selective for VEGF expression as hypoxia had no effect on the PDGF-induced upregulation of the proto-oncogene c-*myc*. These results raise the possibility that hypoxia and PDGF-BB may act in concert to induce VEGF expression in the arterial wall during the development of atherosclerosis.

Key words: Hypoxia; Vascular endothelial growth factor; Platelet-derived growth factor; Vascular smooth muscle cell; Atherosclerosis

1. Introduction

Vascular smooth muscle cells (VSMC) are the main cellular component of the normal arterial wall and have a major role to play in atherogenesis [1]. These cells form a distinct muscular layer, the tunica media, which is largely avascular [2]. Oxygenation is by direct diffusion from the lumen and from the adventitial vasa vasorum [3,4]. Changes in the anatomy of the blood vessel wall such as intimal thickening or occlusion of the vasa vasorum may alter the distribution of oxygen sufficiently to render the tunica media hypoxic [5,6]. Hypoxia of the arterial wall has been implicated in the pathogenesis of atherosclerosis [5,6] but the molecular events induced in VSMC by reduced oxygen tension are largely unknown.

Vascular endothelial growth factor (VEGF; also known as vascular permeability factor) is a secreted angiogenic factor [7] which has been implicated in the neovascularisation of solid tumours [8,9]. VEGF is strongly induced by hypoxia in cultured cells, including tumour cell lines [9] and cardiac myocytes [10]. In addition, it can be induced in cultured fibroblasts by platelet-derived growth factor-BB (PDGF-BB) and by transforming growth factor-β [11,12]. VEGF is a highly specific mitogen for endothelial cells [13]. Also, it increases vascular permeability [14] and stimulates monocyte migration through endothelial layers [15]. These events may be important in the initiation and progression of atherosclerosis, a process which is driven by multiple growth factors and cytokines [1]. Within the vessel wall these regulatory molecules are likely to interact simultaneously with physicochemical changes of the local microenvironment such as hypoxia. We therefore hypothesised that these diverse stimuli may act in concert to induce VEGF expression. In this paper we report that a reduction in oxygen tension in rabbit VSMC leads to a rapid and marked induction of VEGF mRNA. We also demonstrate that PDGF-BB, which on its own causes a modest upregulation of VEGF mRNA expression, in combination with a threshold hypoxic stimulus potently upregulates the expression of this molecule.

2. Materials and methods

2.1. Materials

Tissue culture media and additives were from Gibco Life Technologies, Paisley, Scotland, UK and foetal calf serum (FCS) from Sigma, UK. Duralon-UV membranes and QuickHyb solution were from Stratagene. Radiolabelled nucleotides and Megaprime DNA labelling system were from Amersham Int., UK. Other items were from standard suppliers or as listed in the text.

2.2. Cell culture

Primary cultures of VSMC were grown by the explant technique [16,17]. One mm^2 explants from the tunica media of the thoracic aorta of New Zealand White rabbits were cultured in 100 mm-diameter tissue culture dishes (Costar Corp., Cambridge, MA) in the presence of Dulbecco's modified Eagle's medium (DMEM) containing 20% FCS, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 4 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Cultures were grown at 37°C under 5% CO_2/95% air in a humidified incubator. For experiments, semi-confluent explant cultures were trypsinized and the cells subcultured in 100 mm-diameter dishes at a density of 5×10^5 cells/dish. When the cultures became confluent (usually 4–5 days after plating) the medium was replaced with DMEM containing 0.5% FCS and the cells were incubated for a further 48 h to render them quiescent prior to the initiation of each experiment.

2.3. Hypoxic conditions

Experiments at low oxygen tensions were performed in a custom made, air-tight, humidified environmental chamber (Wellcome Research Laboratories, Beckenham, Kent, UK) maintained at 37°C and flushed with a mixture of 5% CO_2 and O_2 in the range of 0–5%, the

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balance made up with N_2. The oxygen concentration inside the chamber was monitored by an oxygen electrode (Russell pH Ltd., Fife, Scotland, UK) immersed in a stirred water bath. The electrode was connected via an oxygen meter (Grffin&George, Loughborough, UK) to a custom made electronic gas controller which, continuously throughout the experiment, monitored and automatically adjusted the oxygen concentration to the desired preset level.

2.4. Northern blot analysis

Cell monolayers were washed twice with ice-cold PBS and total cellular RNA extracted according to the acid guanidinium thiocyanate/phenol/chloroform method [18] using 2 ml per dish of RNAzol B (Cinna/Biotecx Laboratories Inc., Houston, TX). RNA (20 μg per lane) was electrophoresed on 1% agarose/6% formaldehyde gels and transferred to Duralon-UV membranes using a positive pressure blotter (Strategene Ltd., Cambridge, England, UK). Hybridisations were performed at 64°C for one hour in QuickHyb Solution (Strategene) containing 0.1 mg/ml denatured salmon sperm DNA and $1.5–2 \times 10^6$cpm/ml ^{32}P-labelled cDNA probes (specific activity of $0.5–1 \times 10^9$ cpm/μg) made by random priming using [α-^{32}P]dCTP. The following DNA templates were used for random priming: a 540 base pair *Bam*HI–*Hind*III fragment of human $VEGF_{121}$cDNA [19], a 850-base pair *Pst*I–*Hind*III fragment (nucleotides 540 to 1390) of human c-*myc* cDNA (kindly provided by Dr. H. Land, Imperial Cancer Research Laboratories, London, UK) and a 1100-base pair fragment of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (Clontech Laboratories, Palo Alto, CA). Following hybridisation, filters were washed in $1 \times$ SSC/0.1% SDS for 15 min at room temperature and then for 15 min at 55°C. Filters were autoradiographed and the resulting bands quantified by densitometry. To verify the relative amount of total RNA filters were stripped and hybridised with a 28S rRNA antisense oligonucleotide probe (Clontech Laboratories) (0.2×10^6 cpm/ml) labelled by phosphorylation with [γ-^{32}P]ATP using T4 polynucleotide kinase (Gibco BRL). Each experiment was performed at least three times and the results from one representative experiment are shown.

2.5. Reverse transcriptase-polymerase chain reaction analysis of RNA

Synthesis of cDNA templates for PCR analysis was carried out for one hour at 37°C in a final reaction volume of 20 μl containing 1 μg total cellular RNA sample, 10 mM DTT, 0.5 mM dNTPs, 3.6 μg random hexamer primers (Boehringer-Mannheim), 17 units RNAguard (Pharmacia), 100 units Superscript reverse transcriptase (Gibco BRL) and buffer supplied by the manufacturer of the enzyme. The cDNA contained in 1 μl aliquots of the reverse transcriptase reaction was amplified in a 50 μl reaction using a Perkin Elmer 9600 thermal cycler and Amplitaq DNA polymerase (Perkin-Elmer Cetus) as described by the manufacturer. The oligonucleotide primers used for amplification of VEGF cDNA, 5′-CTGCTCTCTTGGGTGCACTGG-3′ (5′-primer) and 5′-CACCGCCTTGGCTTGTCACAT-3′ (3′-primer), were derived from the coding region of the rat cDNA sequence [20]. The primers used for amplification of β-actin cDNA were 5′-GAAACTACCTTCAACTCCATC-3′ and 5′-CGAGGCCAGGATGGAGCCGCC-3′. The reaction was allowed to proceed for 25 cycles, each cycle consisting of 30 s at 94°C, 60 s at 55°C (for VEGF) or at 60°C (for β-actin), and 60 s at 72°C. Fifteen μl aliquots of the PCR reactions were seperated on 1.5% agarose gels. VEGF PCR products were identified by Southern blot analysis using a murine ^{32}P-labelled $VEGF_{164}$ cDNA probe [21].

2.6. Immunoprecipitations and Western blotting

Phosphotyrosyl proteins were immunoprecipitated from cellular lysates with the anti-phosphotyrosine monoclonal antibody (mAb) PY20 (Transduction Laboratories Inc.) and resolved by polyacrylamide gel electrophoresis as previously descibed [22]. For immunoblotting of PDGF-β receptor, cellular proteins were solubilised by boiling intact cells directly in SDS sample buffer without immunoprecipitation. Following protein transfer to Immobilon Membranes (Millipore Corp.), these were blocked using 5% non-fat dried milk in PBS and incubated for 2 h with 1 mg/ml of either PY20 mAb or anti-PDGF-β receptor mAb (Transduction Laboratories Inc.) in PBS containing 0.05% Tween-20. Immunoreactive bands were visualized using ^{125}I-labelled sheep anti-mouse IgG and autoradiographed.

3. Results

3.1. Hypoxia upregulates VEGF mRNA levels in vascular smooth muscle cells

Exposure of confluent and quiescent rabbit VSMC to 0% O_2 resulted in a marked time-dependent increase in the steady-state levels of a major 3.7 kb VEGF mRNA species (Fig. 1A). The rise in the expression of this transcript was detectable as early as 30 min after incubation at 0% O_2 and reached a maximum level (30-fold increase) between 12–24 h. In contrast, the basal expression of VEGF mRNA in cells incubated at 21% O_2 was barely detectable and did not change during the 24 h period of incubation (Fig. 1A). It was consistently noted that hypoxia induced the expression of additional minor VEGF-related transcripts, the most prominent having a size of 1.5 kb. Hypoxia-induced VEGF expression was also dependent on the concentration of O_2 (Fig. 1B). After 4 h at 5% O_2, steady-state levels of VEGF mRNA were not significantly different to levels detected at 21% O_2, whereas below 5% O_2 the level of expression increased markedly (>3.5-fold). Similar results were obtained with human aortic VSMC (results not shown). Hybridisation to a GAPDH probe showed that the mRNA for this enzyme

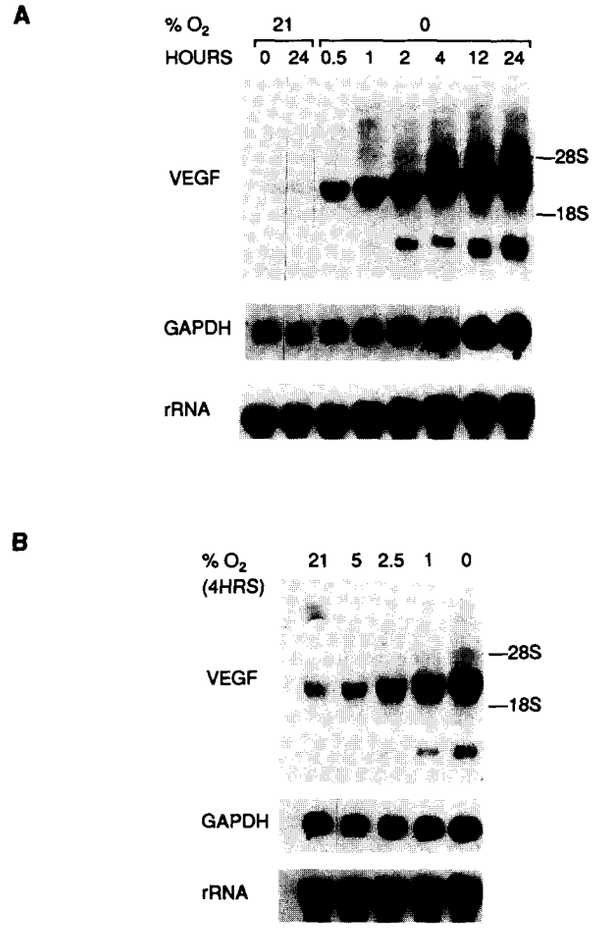

Fig. 1. Upregulation of VEGF mRNA expression by hypoxia in rabbit VSMC. Cells were exposed to 0% O_2 or 21% O_2 for various lengths of time (A) or to various O_2 tensions for 4 h (B) and then levels of mRNA expression were determined by Northern blot analysis. Filters were sequentially hybridised to VEGF, GAPDH and 28S rRNA ^{32}P-labelled probes.

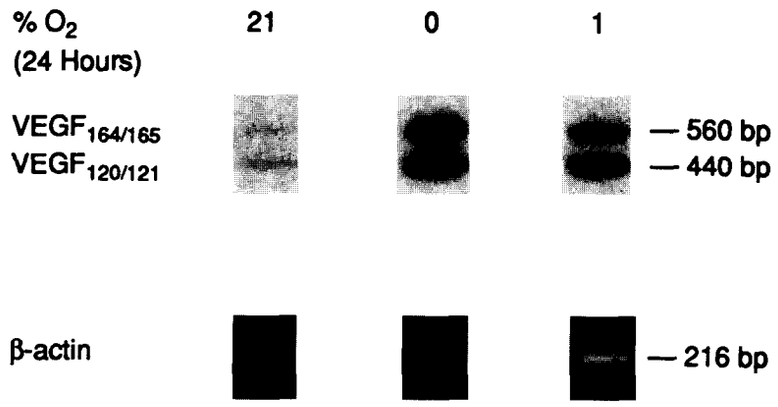

Fig. 2. Characterisation of VEGF transcripts upregulated by hypoxia in VSMC. Total cellular RNA was prepared from cells exposed to 0%, 1% or 21% O_2 for 24 h and then analysed by reverse-transcriptase polymerase chain reaction. VEGF cDNA amplification products were visualised by Southern blot analysis using a ^{32}P-labelled $VEGF_{164}$ cDNA probe. β-Actin cDNA amplification was visualised by direct staining of the agarose gel with ethidium bromide.

was also increased, but only following prolonged incubations (>4 h) at 0% O_2 and to a much lesser degree (1.5–3-fold) than VEGF (Fig. 1A). Comparable loading of total RNA in each lane was therefore confirmed by hybridisation to a 28S ribosomal RNA probe.

Different isoforms of VEGF have been characterised which are products of alternatively spliced mRNA [21,23]. To investigate which VEGF isoforms were expressed by VSMC, we performed reverse-transcriptase polymerase chain reaction analysis of the RNA samples. Consistent with results obtained by Northern blot analysis, low levels of VEGF mRNA were present in VSMC incubated at 21% O_2 (Fig. 2). In contrast, in RNA samples from VSMC incubated at 0% or 1% O_2 for 24 h two prominent transcripts of 563 bp and 431 bp, corresponding respectively to the sizes expected for $VEGF_{164/165}$ and $VEGF_{120/121}$, were detected (Fig. 2). These VEGF isoforms have been shown to be readily secreted by producer cells [21,23]. VEGF transcripts encoding the larger cell-associated isoforms were not detected.

3.2. *Hypoxia and PDGF-BB synergistically induce VEGF mRNA expression in vascular smooth muscle cells*

PDGF-BB has been reported to induce the expression of VEGF in NIH-3T3 fibroblasts [11]. This prompted us to investigate the effect of PDGF-BB on VEGF mRNA expression in VSMC. Fig. 3 (top left panel) shows that incubation with PDGF-BB for 4 h at 21% O_2 caused a dose-dependent increase in VEGF mRNA expression. This increase was lower than that seen following incubation for 4 h at 0% O_2 (Fig. 1). Strikingly, the combination of PDGF-BB and a submaximal hypoxic stimulus (2.5% O_2 for 4 h) caused an increase in the levels of VEGF mRNA to an extent that was greater than the additive effect of the two respective stimuli alone. This synergistic effect was evident at all the concentrations of PDGF-BB tested but was most marked below 25 ng/ml (~2 times greater than the expected additive effect). Furthermore, synergistic enhancement of VEGF expression by hypoxia and PDGF-BB was also observed when cells were incubated for 1 h at 1% O_2 (results not shown).

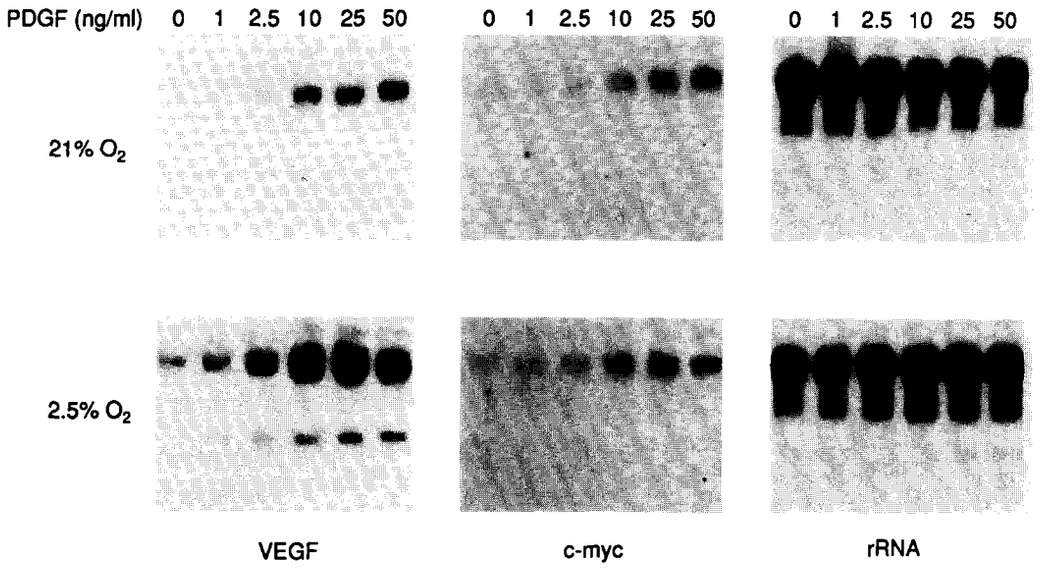

Fig. 3. Synergistic upregulation of VEGF mRNA expression by hypoxia and PDGF-BB. Cells were incubated for 4 h with different concentrations of PDGF-BB either at 2.5% O_2 or at 21% O_2 and then levels of VEGF and c-*myc* mRNA expression were determined by Northern blot analysis.

In order to investigate whether the synergy between PDGF-BB and hypoxia was selective for VEGF mRNA expression, we examined the effect of PDGF-BB and hypoxia on the expression of the proto-oncogene c-*myc*. In agreement with previous findings in a variety of cell types, PDGF-BB increased c-*myc* mRNA expression in quiescent rabbit VSMC in a concentration-dependent manner (Fig. 3, top middle panel). This response was similar to that observed for induction of VEGF. However, in contrast to the synergistic enhancement of VEGF mRNA expression, c-*myc* expression was not enhanced by incubation at 2.5% O_2 (Fig. 3, lower middle pannel) or 0% O_2 (results not shown).

The observed synergistic stimulation of VEGF mRNA expression could result from upregulation of PDGF receptors. To test this possibility we examined the effect of hypoxia on the expression of PDGF-β receptors and on receptor autophosphorylation. Incubation of VSMC in 0% O_2 for 4 h or 12 h caused no increase in the expression of the PDGF-β receptor as determined by Western blotting (results not shown). In addition, exposure of cells to 0% O_2 for 4 h followed by treatment with 25 ng/ml PDGF-BB for 10 min and Western blotting with an anti-phosphotyrosine mAb showed that hypoxia had no effect on the stimulation of PDGF receptor autophosphorylation (results not shown).

4. Discussion

The cells involved in atherosclerosis are known [1]. The molecular events induced in these cells during the initiation and progression of the disease remain unclear. Animal models of atherosclerosis have implicated the luminal endothelium as an initial mediator of this process but there has been little investigation into the initiating role of VSMC. Evidence has accumulated that hypoxia within the tunica media of the arterial wall may be one of the many microenvironmental factors that mediates the early events involved in atherogenesis [5,6,24,25].

The present study demonstrates that hypoxia induces a marked concentration-dependent and time-dependent increase in the expression of VEGF mRNA in rabbit VSMC and that hypoxia can act in synergy with PDGF-BB in the upregulation of this transcript. This effect was selective for VEGF as hypoxia had no effect on PDGF-induced expression of the growth-regulated proto-oncogene c-*myc*. We have verified that hypoxia neither altered the level of PDGF-β receptor expression nor the level of PDGF receptor autophosphorylation in rabbit VSMC. It is therefore unlikely that the observed synergy between PDGF-BB and hypoxia was indirectly due to an increase either in the number of PDGF receptors or the activity of PDGF receptor protein tyrosine kinase. The human VEGF gene promoter region contains a specific hypoxia-sensitive region with no significant homologies to other transcriptional control elements (Dr. Peter Ratcliffe, John Radcliffe Hospital, Oxford, UK; personal communication) and also several potential AP-1 and AP-2 binding sites [19]. PDGF activates c-AMP and protein kinase-C signal transduction pathways [26] and both these pathways have been directly implicated in the induction of VEGF expression [11]. Thus the synergy between PDGF-BB and hypoxia could be a manifestation of the activation and cooperation of several transcriptional control elements stimulated by multiple signal transduction mechanisms.

Brogi et al. recently reported that hypoxia regulates VEGF mRNA expression in human VSMC [27]. These workers only examined VEGF expression in cells exposed to severe hypoxia for prolonged periods of time (24–48 h) and did not investigate the combined effect of hypoxia and individual growth factors. In contrast, the present work examined synergistic interactions between hypoxia and PDGF-BB under less extreme hypoxic conditions for relatively short time periods (2.5% O_2 for 4 h). These experimental conditions may more closely represent the local microenvironment within the tunica media in vivo, thus suggesting a mechanism by which the hypoxic arterial wall could modulate endothelial cell function.

'Response to endothelial injury' theories of atherosclerosis have stressed the initiating role of the endothelium by acting as a source of regulatory molecules which modulate the function of VSMC [1]. Much less attention has been given to the response of medial VSMC to physicochemical changes in the arterial wall, such as hypoxia. Induction of VEGF by hypoxia may promote early atherogenesis both through its effect on endothelial permeability and by a direct effect on monocyte migration. VEGF may also be involved in the advanced stages of atherosclerosis as a result of its angiogenic properties. Detection of VEGF mRNA in human coronary atherosclerotic lesions by in situ hybridisation [28] supports this possibility. Furthermore, neovascularisation of complex plaque is well described in the literature [29,30] and may contribute to the clinical presentation of acute ischaemic syndromes. Our observation that VEGF expression in VSMC is exquisitely sensitive to both hypoxia and PDGF is entirely consistent with a role for VEGF in the aetiology of atherosclerosis.

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