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The endothelial receptor tyrosine kinase *tie-1* is upregulated by hypoxia and vascular endothelial growth factor

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Abstract The receptor tyrosine kinase *tie-1* is essential for angiogenesis where it appears to have a role in vessel maturation. Here we have examined the effects of hypoxia and vascular endothelial growth factor (VEGF) on the level of *tie-1* protein expressed in bovine aortic endothelial cells. Both hypoxia (2% O_2) and VEGF were found to increase *tie-1* in a time-dependent manner. Hypoxic induction was direct and effects of hypoxia and VEGF were not additive. Experiments with actinomycin D indicate that these activators regulate *tie-1* at the transcriptional level.

Key words: Angiogenesis; Growth factor; Hypoxia; Receptor

1. Introduction

The *tie* family of receptors consist of two members, *tie-1* and *tie-2* or *tek* [1]. *Tie-1* is a transmembrane glycoprotein of approximately 120 kDa comprising an extracellular domain of two immunoglobulin-like repeats separated by three EGF-homology domains and followed by three fibronectin III-like repeats [2]. The intracellular portion of *tie-1* contains a tyrosine kinase domain with a kinase insert sequence. *Tie-2* shares the same structural features as *tie-1* and has an overall identity of 44% at the amino acid level, 76% identity in the intracellular domain [3,4]. Two ligands, angiopoietin-1 and -2, have recently been identified for *tie-2* [5,6]. Angiopoietin-2 is antagonistic to angiopoietin-1, preventing binding of the activating ligand and blocking its ability to stimulate *tie-2* kinase activity and autophosphorylation. Angiopoietin-1 and -2 do not bind *tie-1* and ligands for this receptor await identification. *Tie-1* is expressed predominantly on vascular endothelial cells and some haematopoietic cells [3] and is essential for vascular development [7]. Targeted disruption of the *tie-1* gene by mutagenesis in mice results in a lethal phenotype. Mice deficient in *tie-1* die between midgestation and around the time of birth and exhibit extensive haemorrhage and oedema [7,8]. This appears to result from a lack of endothelial cell integrity and hyperpermeability of blood vessels, particularly smaller vessels. These data are consistent with a critical role for *tie-1* in the later stages of angiogenesis promoting vessel maturation.

Physiological control of vessel formation requires the coordinated expression of angiogenic ligands and receptors at appropriate times in the developmental programme. In addition to the *tie* family, members of the family of vascular endothelial growth factor (VEGF) receptors are essential for normal vascular development [1]. The VEGF receptor family members VEGF-receptor-1 (VEGFR-1), also called *Flt-1*, and in VEGF receptor-2 (VEGFR-2), also called *Flk-1* in mice and KDR in humans, are critical in initiating the angiogenic process [9]. Expression of VEGFR-2 precedes *tie* and is seen in angioblasts at E7.0 [10]. *Tie-1* expression is first evident at embryonic day 8.5 in angioblasts of the head, endothelium of the dorsal aortae and yolk sac blood islands in the developing mouse [10]. Although *tie-1* is present in adult endothelium its expression is generally increased in developing capillaries [10,11]. *Tie-1* expression is increased in endothelial cells involved in angiogenesis during wound healing and tumour vascularisation [12,13]. Despite the importance of this receptor, the factors which control its expression are not known. Neovascularisation is recognised to occur in situations of tissue hypoxia, for example within growing tumours and ischaemic tissues [14]. In this study we have tested the hypothesis that hypoxia and VEGF control expression of *tie-1* protein. We demonstrate upregulation of *tie-1* by both hypoxia and VEGF through a transcription-dependent mechanism. These data suggest that hypoxia and VEGF could be responsible for increased expression of *tie-1* seen in vessel formation during development and under pathological conditions. These stimuli thus act to prime the newly developed vessels for later stages of vessel maturation.

2. Materials and methods

2.1. Materials

An affinity-purified polyclonal antibody raised against a peptide corresponding to amino acids 1121–1138 of human *tie* was obtained from Santa Cruz Biotechnology Inc. (supplied by Autogen Bioclear, Wilts, UK). Recombinant human $VEGF_{165}$ was obtained from Genzyme Diagnostics (Kent, UK). All other reagents were obtained from Sigma Chemical Company (Dorset, UK).

2.2. Cell culture

Bovine aortic endothelial cells (a kind gift from Dr M. Boarder, University of Leicester) were maintained in DMEM containing 10% foetal calf serum, 100 μg/ml streptomycin and 100 U/ml penicillin under 5% CO_2/95% air in a humidified incubator at 37°C. Before challenge with agonists confluent monolayers were washed three times with phosphate-buffered saline (PBS: 80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, pH 7.5) and incubated for 24 h in DMEM containing 10% foetal calf serum.

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; VEGF, vascular endothelial growth factor; VEGF-R2, vascular endothelial growth factor receptor-2

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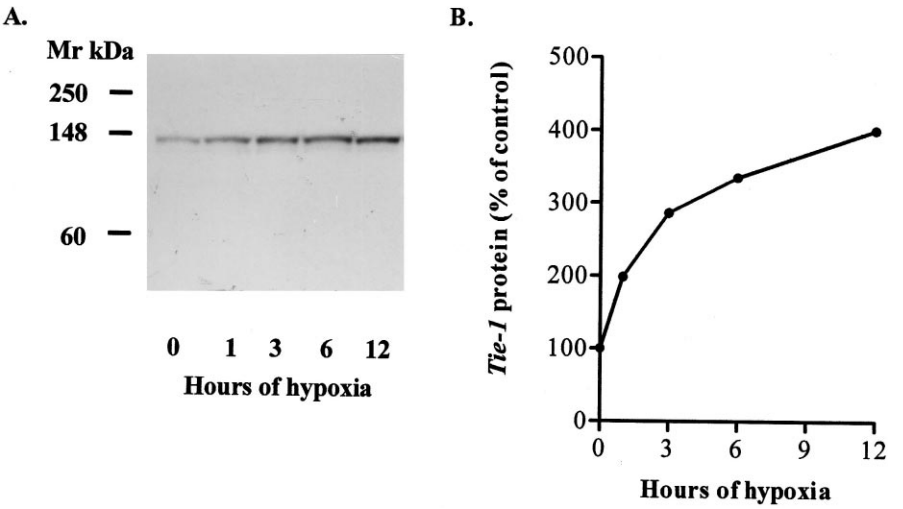

Fig. 1. Time course of the effect of hypoxia on expression of *tie-1* protein in endothelial cells. Confluent monolayers of endothelial cells were cultured under hypoxic (2% O_2) conditions for the times indicated followed by cell lysis. Equal amounts of cellular protein were resolved by SDS-PAGE (10%) and *tie-1* protein determined by Western blotting (A). Similar results were obtained in four independent experiments. The positions of molecular mass markers are indicated in kDa. Densitometric scanning of blots was used to determine the level of *tie-1* protein in cells treated with hypoxia relative to control cells (B).

2.3. Hypoxia

For experiments under low oxygen conditions, medium was pre-gassed and flasks were flushed with 2% oxygen, 5% CO_2, the balance made up with N_2 (BOC Ltd, London, UK). Oxygen concentration was measured using an electronic oxygen meter (Model 9071, Jenway Ltd, Essex, UK) as per the manufacturer's instructions. Flasks were sealed and oxygen concentration monitored periodically. Where the effects of conditioned medium was tested, cells were made hypoxic or maintained under normoxic conditions as above for a period of 18 h. Media from flasks were collected, centrifuged at $1000 \times g$ for 10 min and passed through a 0.2 μm filter. After gassing to restore normoxia the conditioned medium was added to fresh cells that had been washed in PBS as above.

2.4. Assessment of tie-1 protein expression

Following exposure to control or experimental conditions cells were washed twice with PBS and lysed by addition of 0.75% (w/v) sodium dodecyl sulphate (SDS). Lysates and cell debris were scraped from flasks and sonicated and an aliquot removed for determination of protein content, using the Micro BCA protein assay (Pierce and Warriner (UK), Cheshire, UK). The remainder was diluted with sample buffer containing 100 mM dithiothreitol and heated to 105°C for 5 min. Equal amounts of protein were loaded onto each lane of a 10% polyacrylamide gel and subjected to electrophoresis in the presence of sodium dodecyl sulphate. Following electrophoresis, proteins were transferred to nitrocellulose membranes and probed with an affinity-purified antibody to *tie-1*. Immunoreactive bands were detected with a peroxidase-conjugated secondary antibody and the ECL chemiluminescent detection system (Amersham International Plc, Bucks, UK). Bands on films were quantitated by scanning with an LKB Ultrascan densitometer.

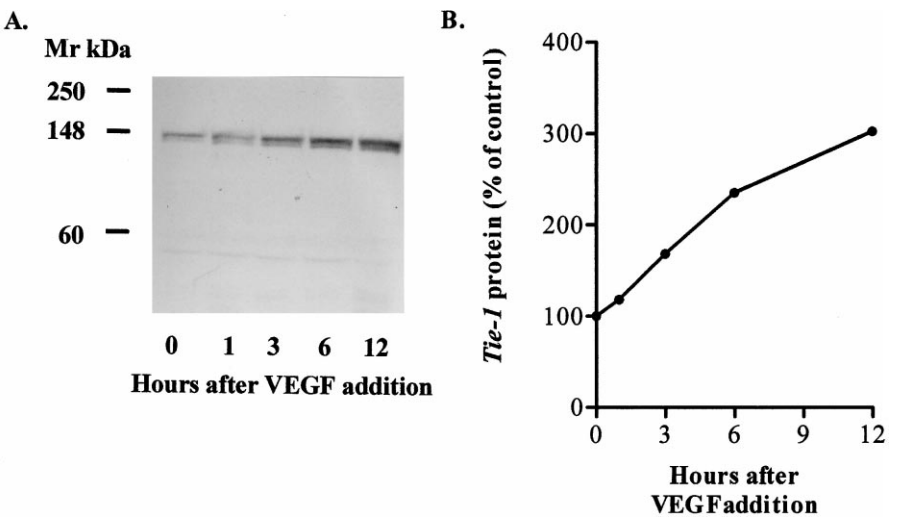

Fig. 2. Time course of the effect of VEGF on expression of *tie-1* protein in endothelial cells. Confluent monolayers of cells were cultured under normoxic (21% O_2) conditions in the presence of 100 ng/ml VEGF for the times indicated followed by cell lysis. Equal amounts of cellular protein were resolved by SDS-PAGE (10%) and *tie-1* protein determined by Western blotting (A). Similar results were obtained in four independent experiments. The positions of molecular mass markers are indicated in kDa. Densitometric scanning of blots was used to determine the level of *tie-1* protein in cells treated with VEGF relative to control cells (B).

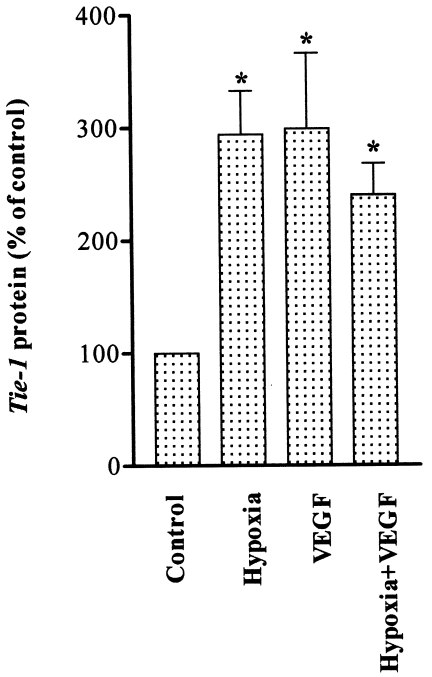

Fig. 3. The effect of hypoxia and VEGF on *tie-1* protein expression in endothelial cells. Confluent endothelial cells were incubated under hypoxic (2% O_2) or normoxic (21% O_2) conditions alone or in the presence of 100 ng/ml VEGF for 16 h as indicated. Cells were lysed and equal amounts of cellular protein were resolved by SDS-PAGE (10%) and *tie-1* protein determined by Western blotting. Levels of *tie-1* protein relative to control were determined by densitometric scanning of blots. Results are presented for four independent experiments as mean+SEM. $*P < 0.05$.

3. Results

Endothelial cells were maintained under normoxic (21% O_2) or hypoxic (2% O_2) conditions for various times then lysed and expression of *tie-1* protein determined by immunoblotting. Aortic endothelial cells express *tie-1* and under the conditions of this study the receptor migrates in SDS-PAGE as a doublet of approximately 130 and 125 kDa, the larger form predominating (Fig. 1A, Fig. 2A). Expression of *tie-1* protein is increased in response to hypoxia in a time-dependent manner (Fig. 1A,B), with the increase apparent as early as 1 h after initiation of hypoxia. In addition to hypoxia newly forming vessels are exposed to the angiogenic growth factor VEGF. We reasoned, therefore, that VEGF may also modulate *tie-1*. To test this endothelial cells were challenged with recombinant VEGF and *tie-1* protein determined by immunoblotting. VEGF increased expression of *tie-1* protein in a time-dependent manner (Fig. 2A,B), again with increases being observed 1 h after addition of the growth factor. In a number of experiments hypoxia induced a 2.9-fold increase ($P < 0.01$) and VEGF a 3-fold increase ($P < 0.05$) in *tie-1* protein expression (Fig. 3). When hypoxia and VEGF were present together, *tie-1* increased to 2.4-fold of control cells ($P < 0.02$), the effects of the two stimuli were not additive.

Hypoxia has been shown to stimulate expression of VEGF in human umbilical vein and human dermal microvascular endothelial cells [15]. It is possible, therefore, that the effects of hypoxia on *tie-1* could be mediated by induction of VEGF, or indeed another autocrine factor. Consistent with this is the failure of the combination of VEGF and hypoxia to induce *tie-1* beyond the level seen with each stimulus alone. To examine this possibility medium conditioned by endothelial cells maintained under hypoxic conditions was collected and added to fresh cells. *Tie-1* expression was not increased by treating cells with medium conditioned by hypoxic endothelial cells indicating that the effects of hypoxia on *tie-1* are not mediated by a secreted autocrine factor (Fig. 4).

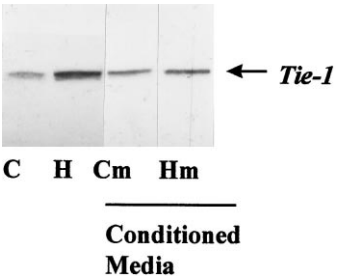

Fig. 4. Effect of medium conditioned by hypoxic endothelial cells on *tie-1*. Medium was conditioned by cells incubated under normoxic (21% O_2) or hypoxic (2% O_2) conditions for 16 h. Medium was removed, cleared, filtered and made normoxic before addition to fresh endothelial cells. Cells were treated for 7 h with medium conditioned by normoxic (Cm) or hypoxic (Hm) cells. After treatment cells were washed, lysed and *tie-1* protein measured by Western blotting. *Tie-1* expression in the normoxic (C) and hypoxic (H) cells used for conditioning media is also presented.

Hypoxic induction of another angiogenic receptor tyrosine kinase, VEGFR-2, in endothelial cells does not require increased transcription [16]. This has led to the suggestion that regulation of receptor tyrosine kinases by hypoxia may be exerted at the post-transcriptional level [16]. We were interested, therefore, to determine whether the effects of hypoxia or VEGF on *tie-1* involved a transcriptional component. Actinomycin D at a concentration of 20 ng/ml, or control vehicle, was added to cells 30 min before activation and cells were challenged with hypoxia and VEGF as before. *Tie-1* protein was not increased by either hypoxia or VEGF in cells treated with actinomycin D, whereas both stimuli increased *tie-1* in control treated cells (Fig. 5).

4. Discussion

The present study is the first to identify factors that control *tie-1* protein expression in vascular endothelial cells. These

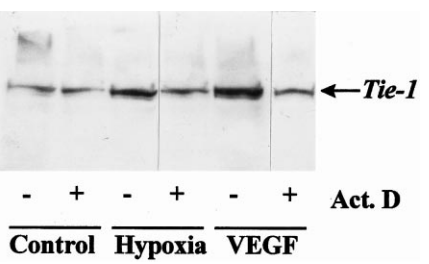

Fig. 5. Effect of actinomycin D on hypoxia- and VEGF-induced *tie-1* in endothelial cells. Confluent monolayers of endothelial cells were treated for 15 min with 20 ng/ml actinomycin D or control vehicle. Cells were then maintained with actinomycin D or control vehicle under normoxic (21% O_2) or hypoxic (2% O_2) conditions or treated with 100 ng/ml VEGF, as indicated. Cells were washed, lysed and *tie-1* protein analysed by Western blotting.

factors, hypoxia and VEGF, are closely associated with active angiogenic sites in pathological conditions and developmental vascularisation [14] and are therefore likely to mediate the increased expression of *tie-1* seen in these situations. Induction of *tie-1* protein by either stimulus is time-dependent with levels reaching up to 3-fold those of control cells. Hypoxia has been shown to upregulate expression of another angiogenic receptor tyrosine kinase in vitro, VEGFR-2, either due to the direct effects of hypoxia on endothelial cells [16,17] or via a factor released from smooth muscle cells in response to decreased oxygen tension [18]. Where hypoxia acts directly, VEGFR-2 levels are increased to approximately 1.5-fold of control cells [17]. Previous observations that hypoxia can increase VEGF expression in endothelial cells [15], and the effects of VEGF seen in the present study, suggested that hypoxia could act to increase *tie-1* by increasing production of VEGF or another autocrine factor. However, the inability of conditioned medium from hypoxic cells to increase *tie-1* expression argues against involvement of a secreted intermediate factor. Our observation that actinomycin D inhibits induction of *tie-1* protein by hypoxia and VEGF is consistent with the idea that these stimuli affect expression of this receptor tyrosine kinase at the level of transcription. This is in contrast to the direct effects of hypoxia on VEGFR-2 which appear to be independent of transcription [16].

VEGF is a direct potent stimulator of endothelial cell proliferation and migration in vitro [19–23], and is angiogenic in vivo [19,24]. In addition it induces expression of proteases which have a role in degradation of the vascular basement membrane early in angiogenesis [25]. Expression of VEGF is increased under conditions of hypoxia in vivo, including wound healing [26], in the ischaemic myocardium [27] and tumour growth [28,29]. Exposure of several cell types to low oxygen tension in vitro, including vascular smooth muscle and endothelial cells, induces VEGF expression [15,30]. Conversely, in the retina, high oxygen tension inhibits VEGF expression leading to apoptosis of retinal endothelial cells and vessel regression [31]. Together these observations support a model whereby vessel growth is matched to the oxygen requirements of the tissue. The ability of hypoxia and VEGF to modulate *tie-1* refines this model further to include a mechanism whereby new vessels are primed to respond to *tie* ligands which may have a role in later stages of the angiogenic programme to promote vessel integrity.

The effects of hypoxia on several genes, including VEGF, are mediated largely by hypoxia-inducible factor-1 (HIF-1), or a related HIF [32]. Examination of the 5′ flanking region of the *tie-1* gene [33] did not reveal any *cis*-acting elements closely similar to those known to interact with HIF-1 in VEGF or other hypoxia-sensitive genes. It is possible that consensus HIF binding sites exist further upstream than the current published *tie-1* 5′ untranslated region. Alternatively, hypoxia could be acting indirectly or by an HIF or HIF-like transcription factor interacting with hypoxia-responsive elements distinct from those known to bind HIF-1. In this regard, it is noteworthy that several groups have recently reported cloning HIF-1-related sequences that are preferentially expressed in endothelial cells and the developing vascular system, and that mediate hypoxia-induced transcriptional activation [34–36]. These sequences have been designated endothelial PAS-1 (EPAS-1), HIF1α-like factor (HLF) and HIF-related factor (HRF), and appear to be present at much higher levels than HIF-1α in the vasculature [34–36]. The close similarity between sequences suggest they may encode the same or very closely related factors. Over-expression of EPAS-1 induces expression of a reporter construct containing 10.3 kbp of regulatory sequence from the *tie-2* gene [36]. Importantly this reporter gene was not stimulated by HIF-1α, suggesting that EPAS-1 could interact with specific response elements not recognised by HIF-1α. It would be of considerable interest to determine whether the effects of hypoxia on *tie-1* are mediated by EPAS-1.

In conclusion, this study demonstrates that hypoxia and VEGF increase expression of *tie-1* protein in vascular endothelial cells. These observations provide a potential mechanism for the increased expression of this receptor seen in situations of active angiogenesis, including wound healing, tumour growth and development. The ability of VEGF to modulate *tie-1* demonstrates this growth factor has a function in co-ordinating the angiogenic programme in endothelial cells, being able to initiate early stages in vessel growth as well as upregulate factors involved in the later stages of vessel maturation.

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