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Phosphorylation of the endothelial nitric oxide synthase at Ser-1177 is required for VEGF-induced endothelial cell migration

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Abstract Vascular endothelial growth factor (VEGF) stimulates endothelial cell (EC) migration. The protein kinase Akt activates the endothelial NO synthase (eNOS) by phosphorylation of Ser-1177. Therefore, we investigated the contribution of Akt-mediated eNOS phosphorylation to VEGF-induced EC migration. Inhibition of NO synthase or overexpression of a dominant negative Akt abrogated VEGF-induced cell migration. In contrast, overexpression of constitutively active Akt was sufficient to induce cell migration. Moreover, transfection of an Akt site phospho-mimetic eNOS (S1177D) potently stimulated EC migration, whereas a non-phosphorylatable mutant (S1177A) inhibited VEGF-induced EC migration. Our data indicate that eNOS activation via phosphorylation of Ser-1177 by Akt is necessary and sufficient for VEGF-mediated EC migration.

Key words: Migration; Proliferation; Protein kinase; Nitric oxide; Vascular endothelial growth factor

1. Introduction

Vascular endothelial growth factor (VEGF) is a potent soluble growth factor, which plays a key role in regulating normal and abnormal angiogenesis [1]. VEGF acts via stimulation of the VEGF receptors 1 (Flt-1) and 2 (Flk-1/KDR), which are both expressed on endothelial cells (EC) [2]. VEGF not only promotes EC proliferation and migration [3], but also prevents apoptosis of endothelial cells thereby improving EC survival [4].

Several reports have implicated NO in the angiogenic response to VEGF. VEGF has been shown to up-regulate NO synthesis in endothelial cells [3,5,6]. In vitro, VEGF-induced EC proliferation requires NO production [7,8]. More recently, the chemotactic effect of VEGF on endothelial cells was shown to depend on endothelial NO synthesis [9]. A key role for NO as a downstream mediator of VEGF is further implied by the finding that mice lacking endothelial NO synthase (eNOS) are resistant to VEGF-induced angiogenesis [10]. The molecular mechanism underlying the requirement of NO to mediate the angiogenic effects of VEGF on EC is unknown.

Several recent studies documented the involvement of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway to contribute to the cytoprotection of EC by VEGF [4,11]. Likewise, the PI3K/Akt pathway was shown to directly activate eNOS by phosphorylation at Ser-1177 rendering enzyme activity calcium-independent [12,13]. Therefore, we investigated whether Akt-dependent phosphorylation of eNOS mediates the effects of VEGF on EC migration using a 'scratched wound assay', which resembles re-endothelialization processes.

2. Materials and methods

2.1. Cell culture

Human umbilical venous endothelial cells (HUVEC) (Clonetics, Solingen, Germany) were cultured in EGM-1 medium supplemented with hydrocortisone (1 μg/ml), bovine brain extract (3 μg/ml), gentamicin (50 μg/ml), amphotericin B (50 μg/ml), epidermal growth factor (10 μg/ml), and 10% fetal calf serum (FCS) until the third passage.

2.2. Cell migration

For detection of cell migration, in vitro 'scratch' wounds were created by scraping cell monolayers with a sterile disposable rubber policeman [14]. Therefore, HUVEC were grown on 6 cm wells, which were previously labeled with a traced line. After injury, the cells were gently washed with medium and stimulated with recombinant VEGF and the respective inhibitors. EC migration from the edge of the injured monolayer was quantified by measuring the distance between the wound edges before and after injury using a computer-assisted microscope (Zeiss, Jena, Germany) at five distinct positions (every 5 mm).

2.3. Detection of proliferating cells

HUVEC were incubated with 10 μM bromodeoxyuridine (BrdU) for 1 h, and incorporated BrdU was detected by staining with a FITC-linked anti-BrdU antibody (50 μg/ml in phosphate-buffered saline (PBS)/0.1% bovine serum albumin, Boehringer Mannheim, Germany) followed by counterstaining with 4′,6-diamidinophenylindole (DAPI) (1 μg/ml). The absolute numbers of BrdU-positive cells were counted at five different positions out of 500 cells.

2.4. Plasmids and transfection

The plasmid encoding the human eNOS was a gift from Dr. Nakane and was subcloned as described previously [12]. The putative Akt sites were changed by site-directed mutagenesis [12]. The plasmids encoding Akt were cloned in pcDNA3.1 vector as described [15]. Clones were transfected in HUVEC (3.5×10^5cells/6 cm well) with 3 μg plasmids and 25 μl Superfect (Qiagen, Hilden, Germany) as described previously [12]. After the transfection procedure, culture medium was removed and 3 ml medium was added for 3 h before 'scratch' wounds were created. Transfection efficiency was about 50% as determined using green fluorescent protein and was maximal from 18 to 36 h. Expression of the eNOS constructs was determined by Western blot analysis as described previously [12]. Immunohistochemistry against the myc tag of the constructs was performed after fixation of the cells with paraformaldehyde using anti-myc antibodies (1:70 in PBS/5% FCS, Santa Cruz) as primary and anti-mouse-antibodies linked to FITC (1:20 in PBS/5% FCS, Dako) as secondary antibodies.

2.5. Statistics

Data are expressed as mean ± S.E.M. from at least three independent experiments. Statistical analysis was performed by *t*-test for the

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comparison of two groups and for multiple groups by one-way ANOVA analysis (LSD test).

3. Results

3.1. VEGF-induced EC migration depends on PI3K/Akt and NO synthase

Incubation of HUVEC with VEGF induced a dose-dependent increase in EC migration with a maximal effect at 10 ng/ml as detected with the 'scratched wound assay' (Fig. 1A). VEGF is known to stimulate EC proliferation [2]. Therefore, we tested whether the enhanced closure of the wound might involve a proliferative effect. In general, a very low proliferation rate of about 3% was detected in re-endothelialized parts of the culture dish in time course experiments (12, 24, 36, and 48 h) (Fig. 1B) confirming previous publications demonstrating that the scratched wound assay mainly determines migration of cells [14]. The absolute number of proliferating EC in re-endothelialized areas of the wound was even lower compared to control cells or to the EC growing outside the wound (33 ± 7%) (Fig. 1C). However, although the absolute number of proliferating EC in the re-endothelialized area was very low, VEGF stimulation slightly enhanced the proliferation index of EC in this area at all time points with maximal effects at 48 h (control: 2.8 ± 0.02%; VEGF: 4.2 ± 0.06% BrdU-positive cells).

Next, we tested the involvement of PI3K/Akt and NO in EC migration. Inhibition of NOS by N^G-mono-methyl-L-arginine (LNMA) completely prevented VEGF-induced migration (Fig. 2A), demonstrating that VEGF-induced EC migration depends on endogenous NO synthesis. The pharmacological PI3K inhibitors Ly294002 and wortmannin suppressed VEGF-induced migration even below unstimulated controls (Fig. 2A). Previous studies excluded toxic effects of the inhibitors [15]. Overexpression of a dominant negative Akt mutant prevented VEGF-induced EC migration (Fig. 2B). The involvement of Akt was further demonstrated by investigating the effect of a constitutive active Akt (T308D/S473D). Overexpression of the active Akt kinase was sufficient to stimulate EC migration even in the absence of VEGF (Fig. 3A). Immunohistochemical analysis using an antibody against the myc tag of the active Akt illustrates the distribution of the Akt-expressing cells (Fig. 3B). Finally, LNMA was shown to prevent Akt-induced EC migration (Fig. 3A), demonstrating that Akt acts via NO.

3.2. Ser-1177 of eNOS is required for VEGF-mediated EC migration

Having demonstrated that VEGF-induced EC migration requires activation of Akt, we analyzed the specific role of phosphorylation of eNOS at Ser-1177, which serves as acceptor amino acid for Akt-mediated eNOS phosphorylation

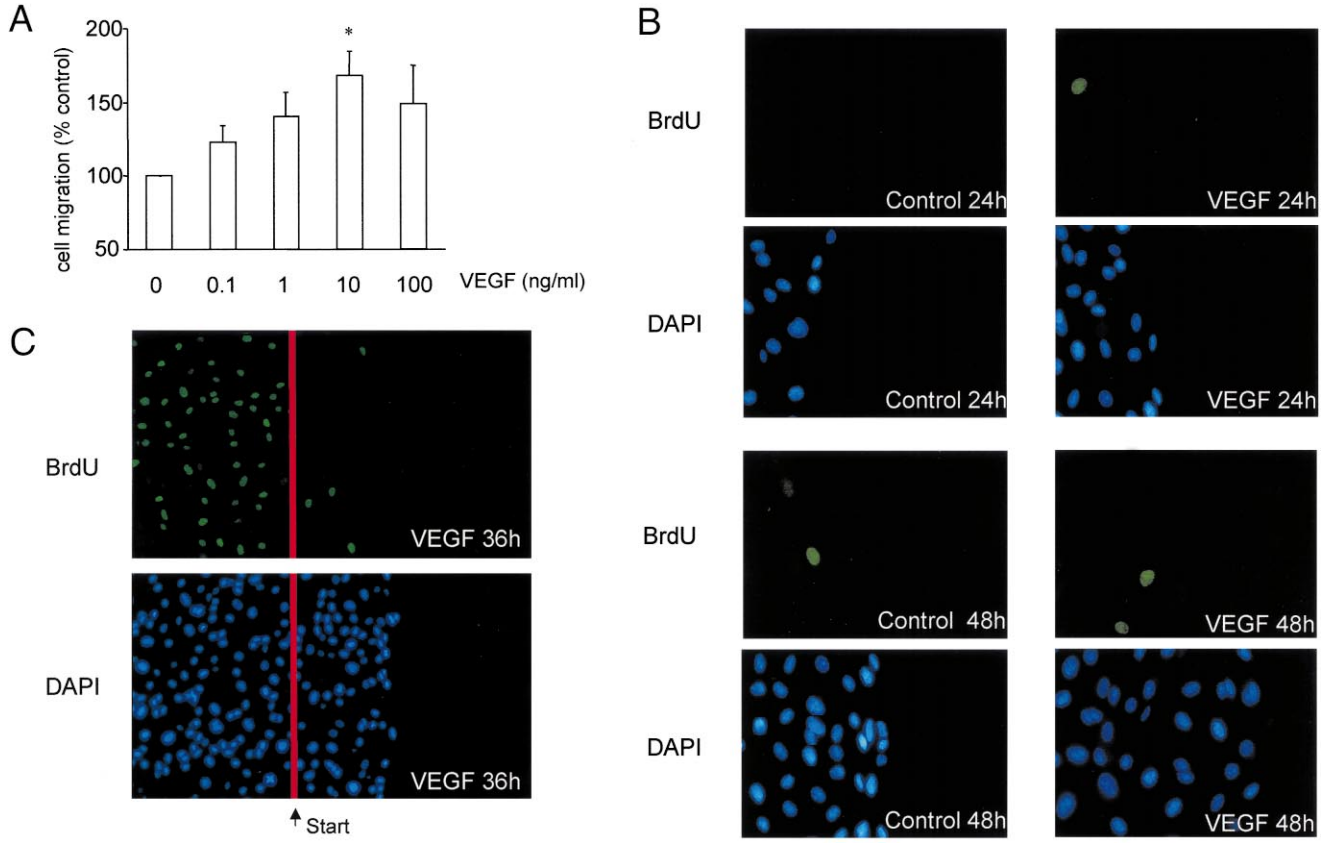

Fig. 1. Detection of VEGF-induced EC migration and proliferation. A: HUVEC were scratched and stimulated with the indicated concentrations of VEGF. After 48 h, cell migration was detected as described in Section 2. Data are mean ± S.E.M., $n = 4$ with $^*P < 0.05$ versus control. B,C: Detection of proliferating endothelial cells. HUVEC were scratched and stimulated with VEGF (10 ng/ml). At the indicated time points, proliferating EC were detected by anti-BrdU staining (see Section 2). Nuclei were stained with DAPI. B: Magnification: 11×. C: Overview with 22× magnification after treatment with VEGF for 36 h. The red line denotes the edge of the scratched wound. Representative experiments are shown ($n = 3$).

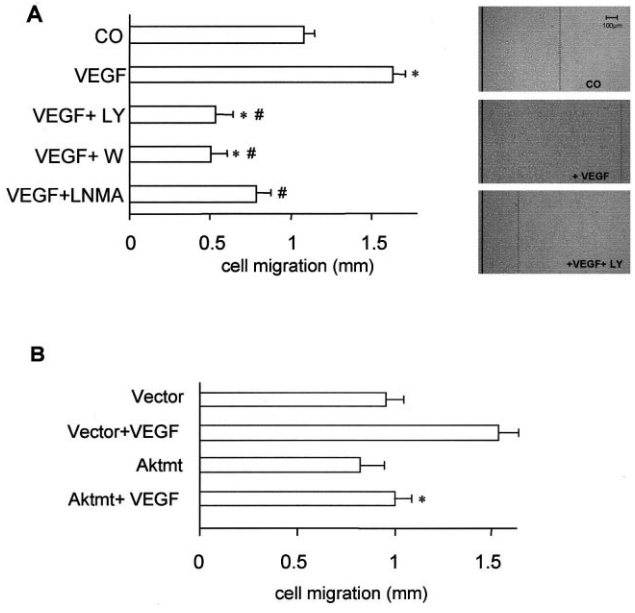

Fig. 2. VEGF-induced EC migration depends on PI3K and NO. A: HUVEC were scratched and incubated with LNMA (1 mM), Ly294002 (Ly, 10 μM), wortmannin (W, 20 nM) and VEGF (10 ng/ml). Cell migration was determined after 48 h ($n=9$, $^{*}P<0.01$ versus control, $^{\#}P<0.01$ versus VEGF). Representative pictures are shown on the right. B: HUVEC were transfected with an empty control vector (pcDNA3.1), dominant negative Akt (Aktmt) or active Akt (T308D/S473D) and cell migration was stimulated with VEGF (10 ng/ml) for 48 h. Data are mean ± S.E.M., $n=6$, $^{*}P<0.01$ versus vector+VEGF, $^{\#}P<0.05$ versus vector, $^{**}P<0.01$ versus active Akt.
←

[12,13]. For that purpose, we overexpressed a phospho-mimetic eNOS construct, where Ser-1177 was replaced by aspartate (S1177D). Expression of the phospho-mimetic eNOS construct significantly stimulated EC migration in the absence of VEGF (Fig. 4A,B). Incubation with VEGF did not further stimulate cell migration in cells expressing the phospho-mimetic S1177D eNOS mutant (Fig. 4A,B). Inhibition of NOS by LNMA completely blocked EC migration induced by eNOS S1177D, demonstrating the NO dependence of the effect (Fig. 4A). In contrast, EC migration in eNOS wild type transfected cells was stimulated by VEGF to a similar extent compared to cells transfected with the control vector (Fig. 4A). To finally test whether eNOS phosphorylation at Ser-1177 is necessary for VEGF-induced cell migration, Ser-1177 was replaced by alanine (S1177A) to prevent Akt-mediated activation of the eNOS. Overexpression of eNOS S1177A abrogated VEGF-induced EC migration (Fig. 4A,B). As mentioned above, VEGF slightly increased EC proliferation in the re-endothelialized area of the wound. Therefore, we investigated the effect of the phospho-mimetic and non-phosphorylatable eNOS constructs on the proliferation of EC. However, trans-

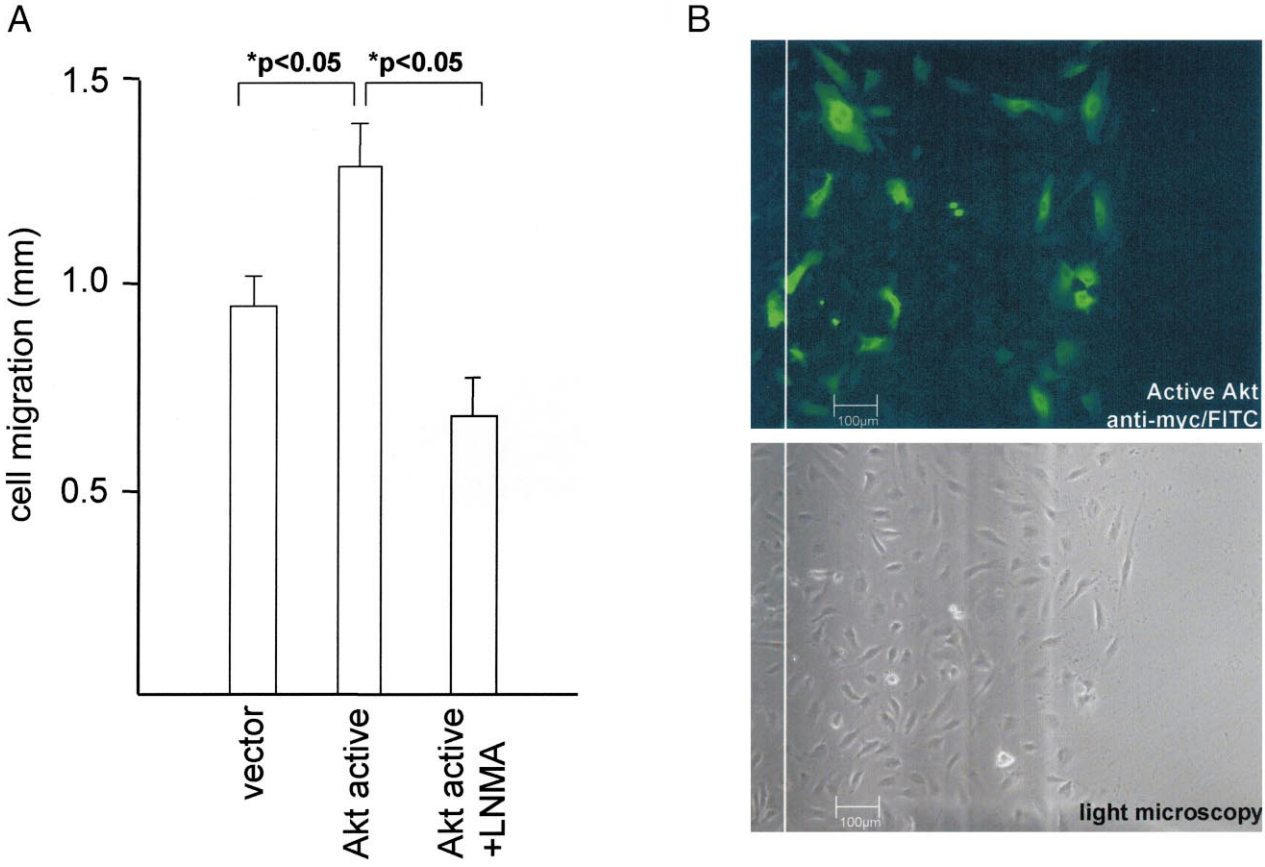

Fig. 3. Akt stimulates endothelial cell migration. A: HUVEC were transfected with vector or active Akt (T308D/S473D) and stimulated with VEGF (10 ng/ml) and LNMA (1 mM). After 48 h EC migration was determined. Data are mean ± S.D., $n=4$. B: Immunohistochemical staining against the myc tag of the active Akt (T308D/S473D) in transfected cells after stimulation with VEGF for 36 h. Magnification: 14×. A representative image is shown.

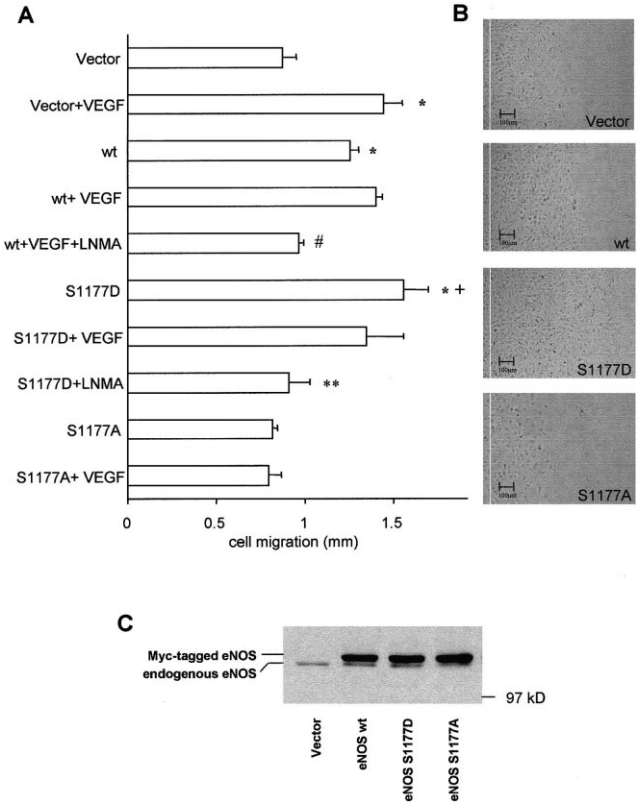

Fig. 4. Phosphorylation of Ser-1177 is essential for VEGF-induced EC migration. A,B: HUVEC were transfected with different eNOS constructs (wild type eNOS: wt; phospho-mimetic eNOS: S1177D; non-phosphorylatable eNOS: S1177A) or control vector. Cell migration was stimulated by VEGF (10 ng/ml) and determined after 48 h. NO synthase was inhibited by LNMA (1 mM). Data are mean ± S.E.M., $n=6$ with $^{*}P<0.01$ versus vector, $^{\#}P<0.05$ versus wt+VEGF, $^{+}P<0.05$ versus wt and $^{**}P<0.05$ versus S1177D. Representative pictures are shown in B. C: Expression of the eNOS constructs. After transfection protein was isolated and a Western blot against eNOS was performed. A representative blot is shown ($n=7$).

fection with the S1177D or S1177A construct revealed no effect on the number of BrdU-positive cells in the re-endothelialized areas of the wound (vector: 2.8 ± 0.03%; S1177D: 2.75 ± 0.05%, S1177A: 2.73 ± 0.1% BrdU-positive EC). Equal expression of the eNOS constructs was demonstrated by Western blot analysis (Fig. 4C).

4. Discussion

Previous studies suggested that activation of the protein kinase Akt by VEGF or shear stress inhibits EC apoptosis and, therefore, promotes EC survival [4,11,15]. The results of the present study now extend these findings by demonstrating that VEGF-induced EC migration also depends on the PI3K/Akt pathway. Thereby, Akt-mediated eNOS activation mediates VEGF-stimulated EC migration. Phosphorylation of eNOS at Ser-1177 is not only necessary, but also sufficient for EC migration.

It is well established that endothelium-derived NO plays an important role in VEGF-induced angiogenesis in vitro and in vivo [7,9,10]. Initial studies suggested that VEGF stimulation leads to a rapid increase in intracellular calcium [16], which then triggers calcium/calmodulin-dependent NO synthesis. The calcium-dependent activation of NO synthase, however, lasts only for a very short time period, approximately 5 min. Although VEGF was shown to up-regulate eNOS mRNA and protein levels [17], activation of eNOS is still required to enhance the enzymatic activity. The data of the present study provide a molecular explanation for the NO dependence of EC migration. Phosphorylation of eNOS at Ser-1177, which has been shown to serve as the acceptor amino acid of Akt-mediated phosphorylation [12,13], stimulates EC migration even in the absence of VEGF. Interestingly, the non-phosphorylatable eNOS completely abrogates the effects of VEGF on EC migration. The complete inhibition of VEGF-induced cell migration by the non-phosphorylatable eNOS construct is surprising, bearing in mind that the transfection efficiency is about 50%. However, control experiments confirmed equal expression levels of the transfected proteins and further excluded potential toxic effects of the construct (data not shown). The inhibitory effect might be explained by the fact that the S1177A construct acts as a dominant negative mutant as suggested by our previous experiments [12]. The inhibition of NO synthesis, which prevents cell migration of the transfected cells, may influence the migration of the overall cell population possibly by impairment of the cell–cell interaction required for migration. It is well established that endothelial cells are linked to each other by adherens-type junctions, called cadherins. These cadherins transfer intracellular signals and play a major role in cell migration [18]. Thus, inhibition of migration of an individual cell is always accompanied by an inhibitory signal on the neighboring cells. Taken together, these data demonstrate that stimulation of EC migration by VEGF requires the phosphorylation of eNOS at Ser-1177 via Akt.

It is well established that VEGF also promotes EC proliferation. However, assessing the proliferation rate in the re-endothelialized areas of the wound revealed a very low number of proliferating endothelial cells, which indicates that the closure of the wound is mainly due to migration of EC into the wound. Importantly, the proliferative response is not affected by transfection with the non-phosphorylatable eNOS constructs. Therefore, one may speculate that eNOS phosphorylation and activation predominantly regulates cell migration but not proliferation. This observation may closely correspond to recently published data demonstrating that angiopoietin 1, which promotes cell survival and cell migration but does not directly stimulate cell proliferation, activates the Akt pathway [19]. Therefore, stimulation of cell proliferation may need additional pathways activated by VEGF but not by angiopoietin 1, which are independent of Akt-dependent NOS phosphorylation [20].

Stimulation of angiogenesis may be a potential therapeutic strategy for angiogenesis and postnatal neovascularization in patients with ischemic heart disease [1]. Gene transfer with naked DNA encoding VEGF or recombinant VEGF has been used as a therapeutic approach [1]. However, this treatment depends on the functional integrity of the signal transduction pathways including the expression of the VEGF receptors and the activation of eNOS, which is often impaired in patients with atherosclerotic risk factors [21]. Since the phospho-mimetic eNOS construct enhances EC migration to a similar extent as VEGF but acts downstream of VEGF, gene therapy with this construct might be a novel therapeutic approach to induce angiogenesis or re-endothelialization.

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