Akt signaling mediates VEGF/VPF vascular permeability in vivo

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Abstract VEGF is an endothelial cell cytokine that promotes angiogenesis and enhances microvascular permeability. Recently, it has been shown that the protein kinase Akt functions in a key intercellular signaling pathway downstream of VEGF. Here, we employed adenovirus-mediated gene transfer in conjunction with the Miles assay in hairless albino guinea pigs to assess the role of Akt signaling in vascular permeability. VEGF-induced vascular permeability was blocked by the transduction of a dominant negative mutant of Akt. Conversely, transduction of a constitutively active form of Akt promoted vascular permeability in a manner similar to VEGF protein administration. This Akt-mediated increase in vascular permeability was inhibited by the eNOS inhibitor L-NAME. These data show that Akt signaling is both necessary and sufficient for vascular permeability in an in vivo model.

Key words: Akt; Cytokine; Angiogenesis; Endothelial cells; Gene transfer; Signaling

1. Introduction

Vascular endothelial growth factor (VEGF) is an endothelial cell cytokine that promotes angiogenesis [1]. The protein kinase Akt, also referred to as PKB, functions downstream of VEGF to promote survival in endothelial cells [2] and mediate NO production through the direct phosphorylation of endothelial cell nitric oxide synthase (eNOS) [3]. Akt signaling is also essential for VEGF-mediated actin reorganization and directed endothelial cell migration toward VEGF [4], and for VEGF-mediated differentiation of endothelial cells into tube-like structures in vitro [5]. Because Akt has a role in diverse cellular processes that contribute to the angiogenic process, the status of Akt signaling within endothelial cells may function as an important modulator of blood vessel growth [5,6].

VEGF was first described as vascular permeability factor (VPF), a tumor-secreted factor that increases vascular permeability in guinea pig skin [7]. This property of VEGF is of significance because angiogenesis is accompanied by an increase in vascular permeability and may be required for vessel sprouting [8–10]. Moreover, this property of VEGF may contribute to tissue edema [11]. Although there is evidence to suggest that VEGF-mediated NO production is important for vascular permeability [11–16], this process is associated with endothelial cell fenestration [17–19]. The signaling mechanisms that regulate this process are largely unknown. One exception is the in vitro study performed by Lal et al. [20], where it was shown that VEGF-stimulated FITC-dextran flux across an endothelial cell monolayer was blocked by chemical inhibitors of MAP kinase and phosphatidylinositol 3-kinase (PI 3-kinase). Because Akt is one of many downstream effectors for PI 3-kinase, we reasoned that this signaling molecule might be involved in vascular permeability. Therefore, we investigated the role of Akt signaling in vascular permeability using molecular modulations of this protein kinase. For this purpose, the Miles assay of vascular permeability in guinea pig skin was modified to accommodate adenovirus-mediated transfer. Next we evaluated the activities of constitutively active and dominant negative *Akt1* gene constructs on vascular permeability in this system.

2. Materials and methods

Female, hairless albino guinea pigs that are euthymic and immunocompetent (weight: 300 to 500 g, $n = 30$; Charles River Laboratories) were used for these assays. Animals were lightly anesthetized with ketamine (10 mg/kg) and xylazine (50 mg/kg). A solution of Evans blue dye (Sigma; 0.5% in saline) was filtered through a 0.2-μm micropore filter (Corning) before use. The animals were given 0.5 to 1.0 ml of 0.5% Evans blue dye through the heart, and hyperpermeable dermal sites (1–3 mm) were assessed based upon the intensity of the blue bleb. Adenoviral constructs were injected intradermally in volumes of 0.1 ml 24 h before the injection of Evans blue dye. Adenoviral constructs encoding myristoylated, constitutively active Akt (myrAkt), dominant negative Akt (dnAkt) and β-galactosidase (βgal) were described previously [2]. Viral titer was determined by plaque assays on 293 cells and is expressed as plaque forming units (PFU) per ml. VEGF protein (R&D Systems), histamine (Sigma) and platelet activating factor (PAF, Sigma) in 0.1 ml were injected 30 min after the injection of Evans blue dye. The N_W-nitro-$_L$-arginine methyl ester (L-NAME) or D-NAME (20 mg/kg) were administrated through the femoral vein immediately before the injection of Evans blue dye. All protocols were approved by the Institutional Animal Care and Use Committee.

3. Results

3.1. Constitutive Akt signaling promotes vascular permeability

Adenoviral constructs encoding myristoylated Akt (Adeno-

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Abbreviations: VEGF, vascular endothelial growth factor; VPF, vascular permeability factor; eNOS, endothelial cell nitric oxide synthase; PI 3-kinase, phosphatidylinositol 3-kinase

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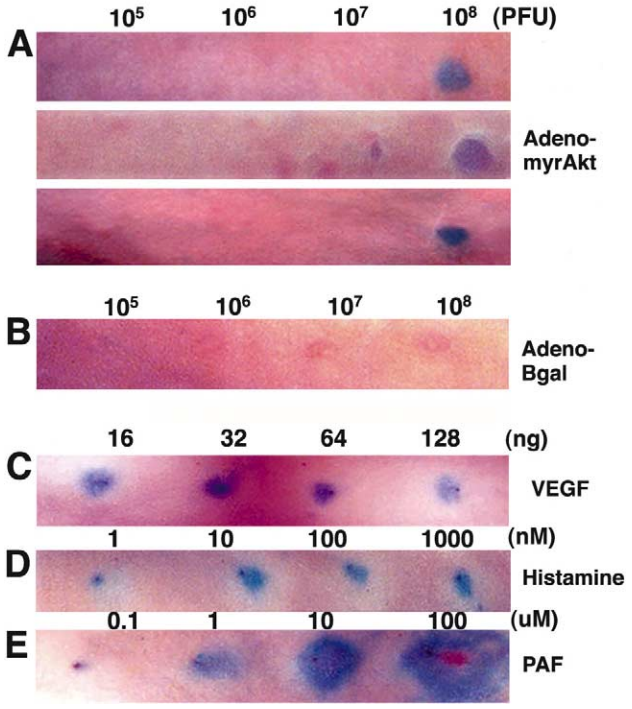

Fig. 1. Constitutive activation of Akt signaling is sufficient for vascular permeability. Representative photographs of guinea pig skin patches after Evans blue dye injection in the heart. Intradermal injections of Adeno-myrAkt (a) or Adeno-βgal (b) were performed 24 h prior to Evans blue dye administration. Intradermal injections of VEGF (c), histamine (d) or platelet activating factor (PAF) (e) were performed 30 min after the administration of Evans blue dye. a: Three examples of the dose effect of Adeno-myrAkt (1×10^5 to 1×10^8 PFU) on vascular permeability are shown. Adeno-βgal (1×10^5 to 1×10^8 PFU) was used as negative control (b), and VEGF protein (16, 32, 64, 128 ng) (c), histamine (1, 10, 100, 1000 nm) (d), and PAF (0.1, 1, 10, 100 μm) (e) were used as positive controls of vascular permeability.

myrAkt) or the control protein β-galactosidase (Adeno-βgal) were injected intradermally in the backs of hairless guinea pigs 24 h prior to the injection of Evans blue dye in the heart. Enhanced permeability was observed at the highest dose of Adeno-myrAkt (10^8 PFU), but not at lower doses (10^5-10^7 PFU). This dose–response relationship was highly reproducible and three representative experiments are shown in Fig. 1A. In contrast, the control vector Adeno-βgal did not shown any evidence of enhanced vascular permeability at any dose tested ($n=4$, representative data is shown in Fig. 1B).

Administration of VEGF protein (16–128 ng) 30 min after the administration of Evans blue dye produced notable bluing at the center of the bleb at all doses tested (Fig. 1C), similar to previously reported data [9]. The other positive controls, histamine and PAF also induced notable bluing (Fig. 1D,E). Repeated intradermal injection of the vehicle control (saline) did not produce vascular permeability (not shown).

3.2. Akt signaling is essential for VEGF/VPF-induced vascular permeability

To assess whether Akt signaling is essential for VEGF-induced vascular permeability, a replication-defective adenovirus encoding dominant negative Akt (Adeno-dnAkt, 10^8 PFU) was injected intradermally 24 h prior to the injection of Evans blue dye in the heart and the delivery of VEGF protein to the adenovirus injection site. Pretreatment with Adeno-dnAkt abolished VEGF-induced vascular permeability at all doses of VEGF examined (Fig. 2A). In contrast, pretreatment with Adeno-dnAkt had no effect on PAF-induced vascular permeability (Fig. 2B).

3.3. Role of NO in Akt-induced vascular permeability

The NO synthase inhibitor, L-NAME (20 mg/kg), was injected through the femoral vein immediately before administration of Evans blue dye in the heart to investigate the role of NO in Akt-mediated vascular permeability. Administration of L-NAME eliminated vascular permeability in response to VEGF (Fig. 3A), consistent with previous observations in this model [9]. Administration of L-NAME also inhibited Akt-induced vascular permeability (Fig. 3B). In contrast, treatment with L-NAME did not alter vascular permeability induced by PAF (data not shown). The stereoisomer D-NAME (20 mg/kg), which does not inhibit endothelial NO synthesis, also failed to inhibit Akt-induced vascular permeability (Fig. 3B), suggesting that the Akt-mediated increase in permeability is dependent on local NO production by eNOS.

4. Discussion

The present study demonstrates for the first time that Akt functions as a mediator of VEGF-induced vascular permeability in vivo. A key finding was that the VEGF-induced vascular permeability in the Miles assay was effectively blocked when tissue was transduced with a dominant negative form of Akt1. Since VEGF receptors are largely confined to the endothelial cell surface, it is reasonable to hypothesize that dominant negative Akt interferes with functions in the endothelium that are essential for vascular permeability. Presum-

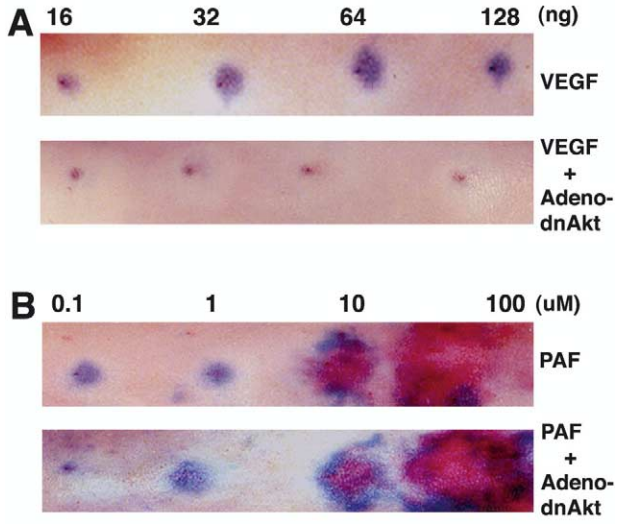

Fig. 2. Akt signaling is essential for VEGF- but not PAF-induced vascular permeability. a: Representative photographs of guinea pig skin patches after Evans blue dye injection in the heart and subdermal injection of the indicated amount of VEGF protein in the presence or absence of Adeno-dnAkt (1×10^8 PFU). VEGF was injected 30 min after and Adeno-dnAkt was injected 24 h prior to Evans blue dye administration. b: Platelet activating factor (PAF) was also tested at the indicated amounts in skin patches that were previously infected with Adeno-dnAkt (1×10^8 PFU).

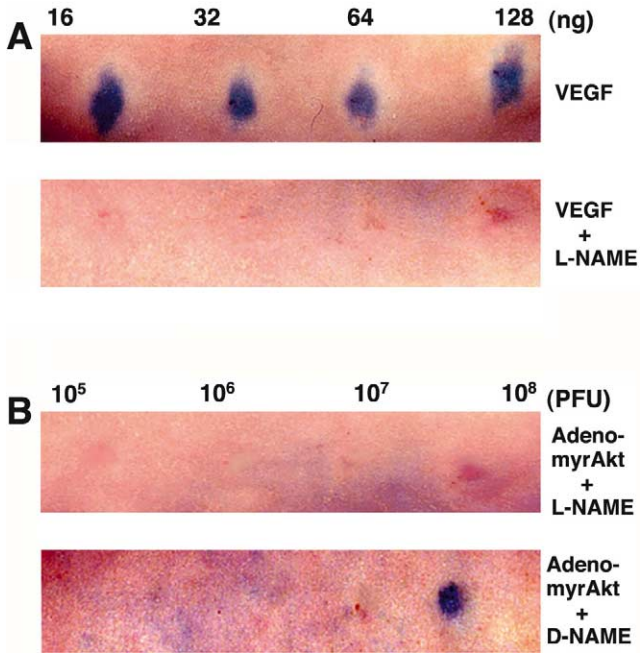

Fig. 3. The nitric oxide synthase inhibitor L-NAME blocks Akt-mediated vascular permeability. a: Representative photographs of guinea pig skin after Evans blue dye administration. Skin patches received intradermal injections of VEGF 30 min after Evans blue dye administration. Where indicated, L-NAME was administered intravenously immediately prior to Evans blue injection in the heart. b: Representative photographs of blebs following the injection of the indicated amounts of Adeno-myrAkt in animals that received systemic injections of L-NAME or D-NAME (20 mg/kg).

ably, Akt-mediated phosphorylation of eNOS in response to VEGF stimulation [3] plays an important role in permeability through its ability to modulate vasodilation and intraluminal surface area [21]. In support of this hypothesis, Fukumura et al. [16] reported that targeted disruption of eNOS, a downstream target of Akt, impaired VEGF-induced vascular permeability. In contrast, disruption of inducible NOS, which is not a substrate of Akt, had no effect on permeability. Also consistent with this hypothesis is the current observation that dominant negative Akt had no effect on PAF-induced permeability, which operates through an eNOS-independent mechanism.

A secondary finding of this study is that transduction with constitutively active Akt was sufficient to induce vascular permeability. Constitutively active Akt could be functioning through the direct activation of the microvascular endothelium. Alternatively, because adenovirus is an amphitrophic vector, it is likely that this treatment also promotes Akt-mediated VEGF synthesis in fibroblasts and vascular smooth muscle at the injection site [22]. The localized production of VEGF would, in turn, lead to an activation of the Akt–eNOS regulatory axis in the vascular endothelium. In this regard, vascular permeability induced by constitutively active Akt was blocked by L-NAME, indicating that eNOS activation is an essential mediator of this effect.

It is becoming increasingly clear that Akt signaling is essential for many of the endothelial cell responses to VEGF. In addition to eNOS regulation, Akt controls both VEGF- and matrix attachment-mediated signaling pathways that promote endothelial cell survival [2], and Akt signaling is essential for directed endothelial cell migration toward VEGF [4]. Since enhanced vascular permeability is a feature of sprout formation, the data presented here provide further support for the hypothesis that Akt signaling with the endothelium is an important regulator of blood vessel growth. Consistent with this hypothesis are the observations that Akt signaling is necessary for endothelial cell differentiation to vascular structures in vitro and reports that enhanced PI 3-kinase/Akt signaling can promote blood vessel growth in a number of animal models [5,22,23]. Collectively, these findings reinforce the notion that pharmacological modulation of Akt signaling could have utility for pro- or anti-angiogenic therapies in patient populations.

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