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Vascular Endothelial Growth Factor ($VEGF^{165}$) Plus Basic Fibroblast Growth Factor (bFGF) Producing Cells induce a Mature and Stable Vascular Network—a Future Therapy for Ischemically Challenged Tissue

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Background. **Vascular endothelial growth factor ($VEGF)^{165}$ induces formation of immature blood vessels with increased permeability. In this study, we used a cell-based gene-transfer model of fibroblasts to investigate the effects of a combined *in vivo* treatment consisting of the VEGF165 and basic fibroblast growth factor (bFGF) proteins on ischemic and non-ischemic tissues.**

Materials and Methods. **After controlled *in vitro* adenoviral transfection we transplanted fibroblasts into either healthy tissue, or into an ischemic skin flap model at different tissue locations and at different time points. Subsequent protein expression and angiogenic effects were measured using ELISA, PCR, immunohistology, planimetry, and microangiography.**

Results. **Transfected fibroblasts temporarily produced $VEGF^{165}$ and bFGF. After transdermal implantation we found an up-regulation of genes encoding for both factors in tissue samples. The combined transplantation of $VEGF^{165}$ and bFGF modified cells increased the number of sm-actin+/CD31+ blood vessels and reduced necrosis by 25%. The number of functional blood vessels increased over a period of 168 d even in healthy tissue.**

Conclusions. **We achieved stable vessel growth in healthy tissue by inducing a temporary overexpression of $VEGF^{165}$ and bFGF and improved the survival of ischemic tissue. One possible mechanism for the latter observation is the stabilization of $VEGF^{165}$-induced hyperpermeable vessels by a bFGF-mediated pericytial recruitment of smooth muscle cells.**

Key Words: **bFGF; VEGF; arteriogenesis; cell based gene transfer.**

INTRODUCTION

All blood vessels arise from endothelial precursor cells (EPCs), which organize into a primitive vascular network of small capillaries in a process known as vasculogenesis [1]. Subsequently, "vessel tightening" occurs whereby immature vessels are covered by pericytes and smooth muscle cells (SMCs). The formation of mature vessel structures is referred to as arteriogenesis [1].

Vascular endothelial growth factor 165 ($VEGF^{165}$) has already been shown to influence angiogenic processes by binding to different tyrosine kinase receptors. These receptors transmit signals promoting endothelial cell migration and proliferation, which is followed by endothelial tube formation [2, 3]. In contrast, factors such as the platelet-derived growth factor (PDGF)-BB recruit mural cells to the immature endothelial channels. Various groups have reported that VEGF can induce the formation of irregular and unstable hyperpermeable blood vessels that are characterized as "leaking blood vessels," thus resulting in a high interstitial pressure [4, 5].

Vessel growth is a process that requires some processing time to occur, due to the time required for the protein-signaling cascade. The activation *via* the hypoxia induced factor 1 alpha (HIF-1α) pathway induced

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by tissue hypoxia [6] leads to the activated transcription of numerous genes, including VEGF [6], but appears to be only one facet of a complex system of angiogenesis and arteriogenesis. Obviously, high levels of $VEGF^{165}$ or basic fibroblast growth factor (bFGF) alone do not lead to functional arteriogenesis [7, 8]. As described by Rhodin and Fujita, the maturation of arterial vessels does not only require sprouting and budding from pre-existing vessels, but also a tightening of the vessel by pericytes clumping to the fragile vessel wall [9]. This process may lead to functional "leak-proof" vessel architecture.

Amongst other steps, the recruitment of SMCs to primitive tubular vessels is necessary for the development of arterial attributes. Kenagy *at al*. showed that SMC migration from primate aortic explants is dependent on the presence of the endogenous factors matrix-metalloprotease (MMP), platelet derived growth factor (PDGF), and bFGF, suggesting a key role of these proteins in determining the fate of blood vessels [10]. However, since most experimental approaches in the field of angiogenesis target processes in preexisting arterial vessel walls, such as the sprouting and budding processes, the field of arteriovenous fusion and vessel destiny requires more research.

While $VEGF^{165}$ has generally been used to improve the blood supply to ischemic limbs in peripheral arterial disease and in ischemic heart muscle [11, 12], functional angiogenesis plays an important role in numerous clinical applications in the field of plastic and reconstructive surgery, such as treatment of tissue within the scope of free tissue transfer or chronic wound healing [13]. There are very few studies that have analyzed the angiogenic effects of $VEGF^{165}$ and bFGF in a combined treatment, although some authors have assumed an additional effect when such a combined treatment is administered [14, 15].

Previous studies have established that a single dose treatment with $VEGF^{165}$-producing cells does not lead to a significant reduction in the necrosis of an axial pattern flap in rats [16]. We further investigated the *in vivo* effect of $VEGF^{165}$-modified fibroblasts on healthy and ischemic tissue. In a random-pattern flap model, the temporary expression of $VEGF^{165}$ induced therapeutically relevant angiogenesis and improved the blood supply if modified cells were applied one week prior to the induction of tissue ischemia [8].

In this study, we investigate the effects of a combined treatment with bFGF- and $VEGF^{165}$-modified fibroblasts in an ischemically challenged McFarlane [17] flap model, as well as in healthy panniculus carnosus tissue. The aim of this study was to investigate whether or not a temporary overexpression of bFGF and $VEGF^{165}$ can induce functional and lasting angiogenesis, and thereby reduce skin necrosis in ischemically challenged tissue.

MATERIAL AND METHODS

Adenoviral Vector Constructs

A bicistronic recombinant adenoviral vector, under the control of a CMV-promoter, was generated to encode either murine $VEGF^{165}$ or murine bFGF and coexpress GFP. pAdcos45 was constructed by isolating the Bst1107 I to Aat II fragment from ⊿E1sp1A that contained the adenovirus left hand TR. The remaining portion of the adenoviral genome was obtained by the Bst1107 I to BamH I fragment and the BamH I to BamH I fragment of pBHG11. The ligation and the *in vitro* packaging of the five DNA fragments yielded pAdcos45. From this adenovirus cosmid, which contained a correctly reconstituted viral genome, recombinant adenoviral genomes were formed upon transfection into 293 cells.

Expression cassettes consisting of the CMV_{ie}-promoter that encode for regions that are separated by the poliovirus IRES element (black box) and the SV40 polyadenylation signal were demonstrated previously [8]. Using the methods of cosmid cloning, the expression cassettes are integrated into the E1 position of the adenocosmid vector pAdcos45 (36 kb) containing the complete E1/E3 deleted AdV type 5 genome. After transfection into 293 cells, viral replication led to the excision of the recombinant adenoviral genome. Optimization of the transduction rates with an enhanced green fluorescent protein (eGFP) control vector indicated an multiplicity of infection (MOI) of 100 resulting in a 81.4% ± 5.1% transduction rate and a 87.3% ± 6.2% survival rate for transfected isogenic fibroblasts (FBs). Higher MOIs resulted in a significant decrease in cell survival although these cells possessed similar transfection rates (Fig. 1A). An MOI of 100 delivered significantly higher transfection rates compared with a MOI of 30 and 10 ($P < 0.001$).

Fibroblast Cell Culturing and Adenoviral Transfection

Isogenic fibroblasts were harvested as described before [8]. Recombinant adenoviruses were obtained from the German Research Centre for Biotechnology (Braunschweig, Germany). Adenoviruses encoded for eGFP, with either murine $VEGF^{165}$ or with murine bFGF in a co-expression manner. Tom Wickham (GenVec, Inc., Rockville, MD) kindly provided the plasmid pK7 for the production of the β-Gal control vector. Cell transfection was performed on FB cultures after the third passage by incubation with MOI-related numbers of virions for 1 h.

After transfection, 5×10^5 FBs per well were analyzed for recombinant protein using the Quantikine immunoassay system for murine $VEGF^{165}$ and bFGF (R and D Systems, Wiesbaden, Germany) according to the manufacturer's instructions. In order to assess the protein production by cells in the culture, protein measurements were performed daily during the first week after transfection and every second day during the following 2 wk. In this case, 1 mL of medium was harvested from cells in one well (12-well plates) after a 24-h incubation period and were subsequently filtered and analyzed in duplicate. The results were normalized for the number of cells and for the time of medium exposure. Three dishes of cells were assayed per cell type.

FBs were adenovirally transfected in their third passage in order to transplant cells to animals of the corresponding groups. Group nmFB (non-modified fibroblasts), group VECTOR (pAdcos45-eGFPc1 as a control-vector) and group DMEM [(Dulbecco's modified Eagle medium (DMEM only)] served as controls. The total amount of transplanted FBs was evenly distributed in all groups. FBs were suspended in 1 mL DMEM before transplantation.

Enzyme-Linked Immunosorbent Assay (ELISA)

We analysed FB cultures for recombinant protein using quantikine immunoassay systems for murine $VEGF^{165}$ and bFGF (both: R and D Systems Inc.) strictly according to the manufacturer's instructions.