various molecular species of VEGF. But most of the endothelial cell mitogenic activity released by the tumour cells *in vitro* was strongly retained by heparin-sepharose and was eluted in the presence of 0.9 M NaCl (unpublished observations). This chromatographic behaviour is consistent with $VEGF_{165}$ but not with the other isoforms of VEGF[16]. This molecular species of VEGF is soluble after secretion and therefore is largely free to diffuse and reach its receptors in the vasculature[6,16].

Our findings demonstrate for the first time, to our knowledge, that blocking the action of a paracrine mediator that acts on the vasculature may have a significant or even dramatic inhibitory effect on tumour growth and emphasize the significance of VEGF as an important mediator of tumour angiogenesis. Therefore, blocking VEGF action has the potential to be of therapeutic significance for several highly vascularized and aggressive malignancies. □

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Recombinant fibroblast growth factor-1 promotes intimal hyperplasia and angiogenesis in arteries *in vivo*

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THE prototype members of the heparin-binding fibroblast growth factor (FGF) family[1-6], acidic FGF (FGF-1) and basic FGF (FGF-2), are among the growth factors that act directly on vascular cells to induce endothelial cell growth and angiogenesis. *In vivo*, the role of the FGF prototypes in vascular pathology has been difficult to determine. We report here the introduction, by direct gene transfer into porcine arteries, of a eukaryotic expression vector encoding a secreted form of FGF-1. This somatic transgenic model defines gene function in the arterial wall *in vivo*. FGF-1 expression induced intimal thickening in porcine arteries 21 days after gene transfer, in contrast to control arteries transduced with an *Escherichia coli* β-galactosidase gene. Where there was substantial intimal hyperplasia, neocapillary formation was detected in the expanded intima. These findings suggest that FGF-1 induces intimal hyperplasia in the arterial wall *in vivo* and, through its ability to stimulate angiogenesis in the neointima, FGF-1 could stimulate neovascularization of atherosclerotic plaques. Potentially, gene transfer of FGF-1 could also be used as a genetic intervention to improve blood flow to ischaemic tissues in selected clinical settings.

The FGF prototypes lack a classic signal sequence[1] (ss) for secretion, making it difficult to study their biological effects as extracellular polypeptides. It is now possible, however, to deliver recombinant genes directly into vascular cells at specific sites *in vivo*[7-12] to determine their effects in the arterial wall. A secreted form of the FGF-1 gene was derived by ligation of the signal sequence from the hst/KS3 (FGF-4) gene to the 5′ end of the open reading frame of FGF-1 (ref. 13) in the pMEX neo eukaryotic expression vector[14]. The pMEX neo-ss-hsk/KS3:FGF-1 expression vector plasmid was transfected into porcine iliofemoral arteries by direct gene transfer[8,15,16], and controls were transfected with the *E. coli* β-galactosidase gene. The presence of the ss-hst/KS:FGF-1 plasmid was confirmed using polymerase chain reaction (PCR) in transfected iliofemoral arterial segments (Fig. 1*a*, lanes 1 and 2) but not in nontransfected carotid artery segments from the same pig (data not shown), and the presence of its messenger RNA was confirmed by reverse transcription PCR (Fig. 1*b*, lane 4). Expression of recombinant FGF-1 protein was confirmed by immunohistochemistry in transduced arterial segments. Porcine arteries transfected with ss-hst/KS:FGF-1 had immunoreactive protein primarily in the intima, including the endothelium, 21 days after transfection, whereas no FGF-1 protein was detected in arteries transduced with the β-galactosidase expression vector (Fig. 2*a*-*c*).

To evaluate the response of the arterial wall to expression of ss-hst/KS:FGF-1, the transfected artery segments were examined by light microscopy 21 days after gene transfer. Animals transduced with β-galactosidase showed minimal intimal thickening in iliofemoral artery segments, in contrast to the ss-hst/KS:FGF-1-transduced arteries (compare Fig. 3*a* and *b*). By quantitative morphometry, the intimal to medial ratio was more than sixfold greater in FGF-1 than β-galactosidase-transduced vessels (0.27 ± 0.06 versus 0.04 ± 0.01, $P = 0.003$). Finally, in several experimental subjects, expression of ss-hst/KS:FGF-1 induced the formation of capillaries in the neointima (Fig. 4*a*, *b*), an effect not observed with the control.

Thus expression of secreted recombinant FGF-1 induced significant intimal proliferation and angiogenesis *in vivo*. Angiogenic factors[1,2,6] have been classified previously into two categories: those that act directly on vascular endothelial cells to stimulate locomotion and mitosis and those that act indirectly to induce host cells to release growth factors that target the endothelial cell. In addition, because the FGF prototypes lack a classic signal sequence for secretion, their normal mode of release is not fully understood. They are detected after arterial

injury[17,18] and can be found in the subendothelial matrix[19]. Indeed, NIH 3T3 cells can release FGF-1 *in vitro* in response to heat shock[20]. In an injury model *in vivo*, systemically administered FGF-2 is a potent mitogen for vascular smooth muscle cells[21], but it has not previously been possible to determine the function of the FGF prototypes in the normal arteries. Analysis of tissue sections in this study suggests that mitotic activity is induced by FGF-1 in the vessel wall, although it remains possible that FGF-1 affects cell migration, as suggested for platelet-derived growth factor (PDGF)[22].

Several recombinant genes induce intimal smooth muscle hyperplasia, including PDGF[16] or TGF-β1 (E.G.N. *et al.*, unpublished results). In contrast, FGF-1 stimulates intimal hyperplasia and also induces the formation of new blood vessels in the expanded intima. These data therefore suggest that smooth muscle hyperplasia alone is not sufficient for the formation of new capillaries. Luminal endothelial cells in the iliofemoral

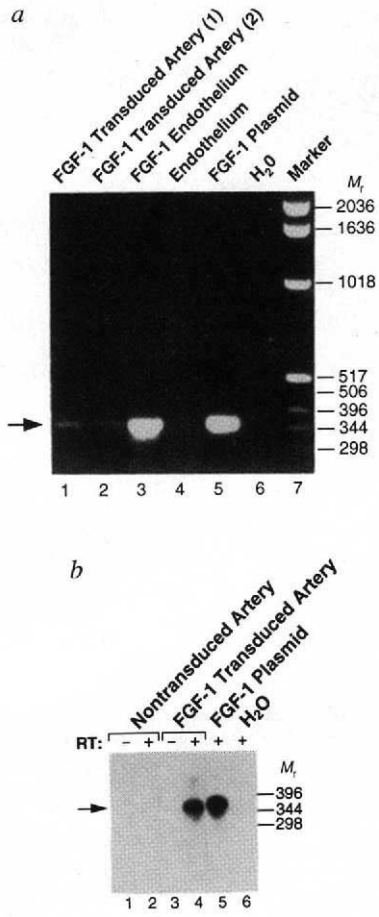


FIG. 1 Presence of ss-hst/KS:FGF-1 plasmid DNA (*a*) and mRNA (*b*) in porcine arteries after direct gene transfer *in vivo*. *a*, Recombinant ss-hst/KS:FGF-1 DNA was transfected into porcine right (lane 1) and left (lane 2) iliofemoral arteries and was detected by PCR 7 days after direct gene transfer using ethidium staining of agarose gels. *In vitro* transfected porcine endothelial cells (FGF-1 endothelium) (lane 3) compared with nontransduced porcine endothelial cells (Endothelium) (lane 4) were analysed as positive and negative controls. Additional controls included the recombinant FGF-1 plasmid (lane 5) and water (lane 6). *b*, The presence of ss-hst/KS:FGF-1 mRNA was detected using reverse transcription PCR by methods described previously[16]. The presence of recombinant FGF-1 mRNA was analysed by Southern blotting of PCR-amplified complementary DNA in nontransduced or transduced arteries treated with or without the addition of reverse transcriptase (RT) as indicated for lanes 1–4. Recombinant FGF-1 plasmid (lane 5) and water (lane 6) were included as positive and negative controls, respectively.

METHODS. Recombinant FGF-1 gene transfer in arterial segments was analysed by PCR of genomic DNA as previously described[15]. To conduct PCR analysis of recombinant FGF-1 transfected vessels, primers were synthesized from the cDNA sequence[20] which generated a 364 base pair (bp) fragment: sense (25 mer): CAA ACT CCT CTA CTG TAG CAA CGG G; antisense (25 mer): TTG CTT TCT GGC CAT AGT GAG TCC G. The sense primer was selected from a region 50 bp upstream to the transcription start site. Samples were analysed by ethidium bromide staining on a 1% agarose gel. The ss-hst/KS:FGF-1 chimaera was prepared and inserted into the pMEX neo expression vector as previously described[20]. Primary porcine endothelial muscle cell cultures were established as previously described and transfected with Lipofectin (BRL)[15] to test the expression of the ss-hst/KS:FGF-1 vector. Transduced endothelial cells were assayed for secretion of recombinant FGF-1 into culture supernatants with a colorimetric proliferation assay by standard methods[24].

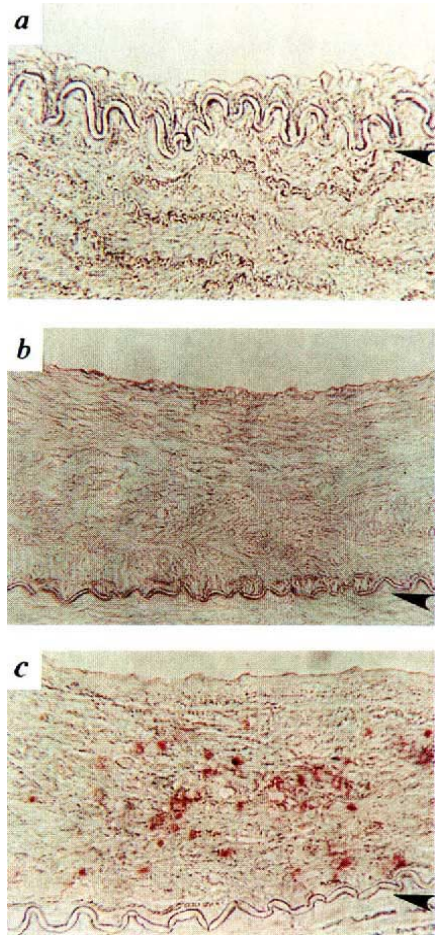
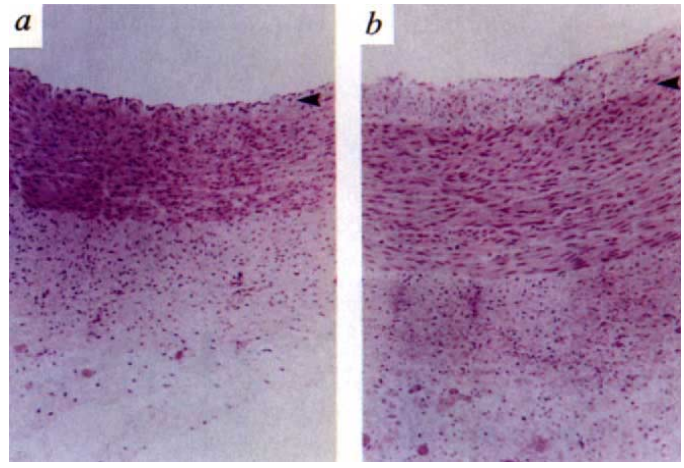


FIG. 2 Expression of recombinant ss-hst/KS:FGF-1 protein in porcine artery cells after direct gene transfer. Immunohistochemical staining of porcine arteries transduced with the *E. coli* β-galactosidase gene (*a*), or recombinant ss-hst/KS:FGF-1 gene (*b*, *c*) at 21 days using a control purified rabbit IgG antibody (*b*), or an affinity-purified rabbit antibody to FGF-1 (*a*, *c*). Arrow denotes the internal elastic lamina. (Magnification ×300).

METHODS. Recombinant FGF-1 protein expression was analysed by immunohistochemistry of artery segments transduced with the recombinant ss-hst/KS:FGF-1 or *E. coli* β-galactosidase genes. Artery segments embedded in Tissue-Tek OCT compound (Miles, Elkhart, IN) were sectioned (6 μm) and were incubated in 10 mM Tris–Cl pH 7.5, 150 mM NaCl, 1 mM EDTA (TNE), and 1% fetal bovine serum with 1:150 dilution of an affinity-purified rabbit anti-human FGF-1 antibody for 1 h at room temperature. Endogenous peroxidase activity was blocked by preincubation in TNE with 0.1% H_2O_2 for 45 min[25]. Peroxidase-conjugated goat anti-rabbit IgG (H+L) antibody (Vector Laboratories, Burlingame, CA) (1:400) was added for 30 min at room temperature and samples were stained in 50 μM sodium acetate (pH 5.0), 20 μg ml^{-1} 3-amino-9-ethyl-carbazole, and 0.015% H_2O_2 for 15 min. FGF-1-transduced arterial segments were also analysed with a control first antibody, purified rabbit IgG, and peroxidase conjugated goat anti-rabbit IgG (1:400) (Vector Laboratories).

FIG. 3 Intimal hyperplasia in porcine arteries 21 days after direct gene transfer of ss-hst/KS:FGF-1 and *E. coli* β-galactosidase. Vessels were transduced with an *E. coli* β-galactosidase (negative control) (*a*) or ss-hst/KS:FGF-1 (*b*) gene. Arrow denotes internal elastic lamina. (Magnification ×54, haematoxylin-eosin stain).

METHODS. Direct intra-arterial gene transfer was done *in vivo* in 12 pigs, 6 with the recombinant ss-hst/KS:FGF-1 gene and 6 with a control reporter gene, *E. coli* β-galactosidase. A double balloon intravascular catheter (C. R. Baid Inc., Billerica, MA) was inserted in porcine iliofemoral arteries as previously described[8]. The arterial segment isolated by the catheter was flushed with 5 ml saline and 5 ml Opti-MEM (BRL) to rinse blood from the vessel. The DNA liposome conjugates were prepared 10 min before transfection. Lipofectin (5 μl) was diluted into 0.2 ml of Opti-MEM at room temperature, and 2–5 μg plasmid DNA (stock concentration >1 mg ml^{-1}) was added. The solution remained at room temperature for 5–10 min, and 0.5 ml Opti-MEM was added. DNA liposomes were instilled into the arterial segment between the two balloons at 150 mm Hg in the left and right iliofemoral arteries and incubated for 20 min. Four of the twelve pigs were killed at one week, and eight pigs were killed at three weeks. Previous studies established that recombinant genes are stably expressed in vascular cells *in vivo* at 2–3 weeks[8,15], and intimal thickening induced by arterial manipulation is observed at this time point[26]. Morphometric measurements of intimal and medial thickness were done in a blinded manner (by C.C.H.). Intimal-to-medial ratios were determined ($n=12$, $n=6$ *E. coli* β-galactosidase gene, $n=6$ FGF-1 gene) as previously described[16]. Intima-to-media ratios are expressed as a mean ±s.e.m. Comparisons between ss-hst/KS:FGF-1 gene-transduced vessels and control vessels transduced with a reporter gene were made by two-tailed unpaired *t*-test.



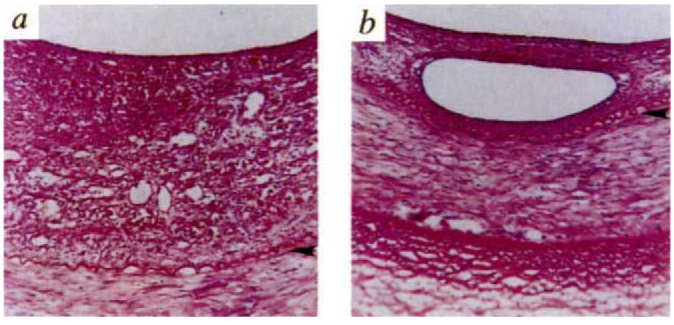


FIG. 4 Angiogenesis in the neointima of arterial segments after ss-hst/KS:FGF-1 gene transfer. Vessels were transduced with ss-hst/KS:FGF-1 and sections representing formation of multiple capillaries (*a*) or a larger intimal capillary (*b*) are shown. Arrow, Internal elastic lamina. (Magnification ×212 (*a*), ×106 (*b*), haematoxylin-eosin stain).

artery do not contain factor VIII, in contrast to those of the small capillaries in the adventitia. Endothelial cells lining the new intimal capillary beds are negative for von Willebrand factor (data not shown), suggesting that the FGF-1-induced capillaries arise from adjacent luminal endothelial cells. These findings are most consistent with a model in which FGF-1 acts locally on endothelial cells, perhaps through mitotic and locomotive effects. These angiogenic effects of FGF-1 could be mediated directly on endothelial cells and/or indirectly through the induction of other endogenous growth factors. In either case, the effects of FGF-1 are specific because they are not observed with other recombinant growth factor genes[15,16]. This model will aid the definition of such factors and the design of potential inhibitors of vascular cell proliferation.

The elaboration of FGF *in vivo* represents a potential mechanism to provide a blood supply to the hyperplastic intima. Such angiogenesis is observed in atherosclerotic plaques[23], and mechanisms to explain this observation have been lacking. Direct gene transfer of this FGF-1 or related genes *in vivo* could provide a method to stimulate collateral blood flow which would be beneficial for the treatment of ischaemic cardiovascular diseases. □

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