

Angiogenesis

Summaries of Ten Seminal Papers

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Therapeutic angiogenesis.
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S. Takeshita and others. *Lab Invest.* 1996

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I. Baumgartner and others. *Circulation.* 1998

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C. Kalka and others. *Proc Natl Acad Sci USA.* 2000

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C. Kalka and others. *Circ Res.* 2000

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P. R. Vale and others. *Circulation (Online).* 2000

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Favorable effect of VEGF gene transfer on ischemic peripheral neuropathy

P. Schratzberger and others. *Nat Med.* 2000

Selection of seminal papers by **Jeffrey M. Isner, MD**
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Therapeutic angiogenesis. A single intra-arterial bolus of vascular endothelial growth factor augments revascularization in a rabbit ischemic hind limb model

S. Takeshita, L. P. Zheng, E. Brogi, M. Kearney, L. Q. Pu, S. Bunting, N. Ferrara, J. F. Symes, J. M. Isner

J Clin Invest. 1994;93:662-670

One of the first scientific papers to clearly demonstrate the therapeutic potential of angiogenic growth factor therapy was that published by Isner's group in 1994. These authors used a rabbit model of hindlimb ischemia that paved the way for the therapeutic strategies employed in recent clinical trials.

The ideal angiogenic cytokine should be easily diffusible to its target receptors and these should ideally only be expressed in the ischemic territory to avoid potential deleterious neovascularization at unwanted sites. Furthermore, such an agent should be expressed early in the angiogenic process so that it may act as a molecular trigger to initiate the highly orchestrated cascade of events required to generate new blood vessels. Isner's group recognized the therapeutic potential of vascular endothelial growth factor (VEGF) isoform 165 for a number of reasons. Firstly, VEGF itself is secreted by intact cells and the 165-amino-acid isoform is more diffusible than the 189 and 206 isoforms. This reduces binding and fixation in the extracellular matrix. Both VEGF and its receptors colocalize at sites of neovascularization, eg, the female reproductive tract and highly vascularized tumors. VEGF receptors are only expressed by endothelial cells, which are key effectors in the angiogenic process. This temporal and spatial pattern of expression in conjunction with proven specific angiogenic activity of VEGF by in vivo assays such as the rat cornea and chorioallantoic membrane provided a strong foundation to the hypothesis that VEGF may have therapeutic potential. The targeted action of such a peptide introduced early during an ischemic stress could act as a key modulator in the angiogenic response.

This study was carefully conducted to evaluate the effect of an intra-arterial bolus of VEGF₁₆₅ peptide upon collateral vessel formation in the ischemic hindlimb of the rabbit. The model involves the ligation and resection of the entire femoral artery. After 10 days, each animal received an injection of 500 or 1000 µg of VEGF₁₆₅ delivered selectively into the internal iliac artery of the ischemic limb. Sham-treated animals received carrier solution only. Both

angiography and calf blood pressure ratio (ischemic calf/normal calf) were used to assess the anatomical and functional development of the collateral vessels in the hindlimb. Statistically significant increases in the angiographic score of collateral vessel development were apparent at days 20 and 40 post ligation versus control. This was accompanied by the blood pressure ratio being double that of control in the VEGF-treated animals at day 20. Even by day 40, the blood pressure ratio was 50% higher than control animals in the VEGF-treated group. The higher dose of VEGF had no additional advantages.

This elegant study was the first to demonstrate that VEGF peptide can induce the growth of physiologically important collateral vessels that have the capacity to minimize rest ischemia. It allowed the first opportunity to manipulate angiogenesis in a clinically relevant situation where both anatomical and physiological correlates of angiogenesis could be quantitatively examined in a blinded manner. This provided the basis for later studies employing plasmid VEGF transfection and the critical safety data for the first phase I trials of angiogenic gene therapy in cardiovascular disease.

1994

American comedian William "Bill" M. Hicks dies from lung cancer, aged 32;
Edvard Munch's painting "The Cry" is stolen from a gallery in Oslo;
and US President Bill Clinton ends the economic embargo against Vietnam in effect since the end of hostilities between the two countries



Intracoronary gene transfer of fibroblast growth factor-5 increases blood flow and contractile function in an ischemic region of the heart

F. J. Giordano, P. Ping, M. D. McKirnan, S. Nozaki, A. N. DeMaria, W. H. Dillmann, O. Mathieu-Costello, H. K. Hammond

Nat Med. 1996;2:534-539

This paper was the first to describe enhanced myocardial collateral support following in vivo delivery of an adenoviral vector. The impact of the paper was considerably enhanced by the physiological relevance of the model and the clinical nature of the investigations used to document improvements in blood flow and function in collateral-dependent myocardium.

Giordano and colleagues placed an aneroid constrictor around the proximal portion of the left anterior descending coronary artery (LAD) of the pig. In this model, over the subsequent 10 days, the constriction of the LAD slowly progresses to complete occlusion. However, since collateral vessels grow during this time there is minimal infarction (<1% of left ventricle). Nonetheless, 38 days after placement of the constrictor, the collateral-dependent myocardium had a markedly diminished flow reserve with impaired systolic thickening developing during electrical pacing. This impaired thickening was accompanied by a myocardial opacification deficit following left atrial injection of an echo contrast agent. Thus, at baseline, there was evidence of a matched flow: function deficit during increased cardiac workload. At this time, pigs were either injected with an adenoviral vector encoding the reporter gene β -galactosidase (β -gal) (n=7) or the secreted growth factor, fibroblast growth factor-5 (FGF-5) (n=16). Both viruses were given by deep intracoronary injection, into the right and left coronary arteries, after confirming occlusion of the LAD. All animals received 2×10^{11} viral particles. In those receiving the β -gal-encoding virus, this resulted in almost 100% gene transfer efficiency. When pigs were reexamined 2 weeks later, only those receiving the FGF-5 virus showed any improvement. Compared with baseline, these animals showed a 2.7-fold increase in systolic wall thickening during pacing in the dysfunctional myocardial segment. Furthermore, the myocardial perfusion deficits were no longer visible since myocardial opacification with echo contrast was homogenous. This advantage following intracoronary injection of an FGF-5-encoding adenovirus was still apparent when the pacing and echo protocols were repeated at 12 weeks. These advantages in echocar-

diographic measures of myocardial ischemia were also accompanied by changes at a tissue level with evidence of enhanced capillary density and endothelial cell mitoses in FGF-5 injected hearts. Rather surprisingly, only 1.3% of the virus injected into the coronary arteries traversed the myocardial bed to appear in the coronary sinus and therefore pulmonary artery. Moreover, this virus was only able to infect permissive cells after 200-fold dilution, presumably because of the presence of neutralizing factors. These observations were in keeping with an inability to detect viral DNA in any organ other than the heart despite amplification by polymerase chain reaction (PCR). Thus, intracoronary infusion of an adenoviral vector increased collateral myocardial blood without the apparent risk of systemic exposure triggering angiogenesis in sites other than the target organ.

When this paper was published, it created a great deal of excitement, paving the way for a new therapeutic modality. Since then the gene therapy community has realized that a number of potential pitfalls exist, and, in most species other than the pig, a single intracoronary injection of adenovirus does not result in anywhere near 100% efficiency of gene transfer. Only time will tell if the human heart is like that of a pig!

1996

Muhammad Ali receives an honorary Olympic gold medal to replace the one he won in Rome in 1960 and had thrown away after a racist incident in the US; NASA announces evidence of primitive life forms in a Martian meteorite found in Antarctica; and alarm grows in Britain over a deadly form of cow disease

Clinical evidence of angiogenesis after arterial gene transfer of phVEGF₁₆₅ in patient with ischaemic limb

J. M. Isner, A. Pieczek, R. Schainfeld, R. Blair, L. Haley, T. Asahara, K. Rosenfield, S. Razvi, K. Walsh, J. F. Symes

Lancet. 1996;348:370-374

An elegant demonstration of the translation of preclinical research into a clinically applied technique is given by this paper in which Isner describes the response of a patient receiving a plasmid vector expressing the 165-amino-acid isoform of vascular endothelial growth factor (VEGF₁₆₅).

The patient was participating in a dose-ranging study. She was a 70-year-old diabetic lady with severe peripheral vascular disease affecting her right leg. She had presented with ischemic rest pain and developed progressive gangrene of the right great toe. The ankle:brachial index of this limb was 0.26, and angiography demonstrated complete occlusions of peroneal and anterior and posterior tibial arteries below the knee. There was no suitable site for bypass grafting. Therefore, she was enrolled into this phase I trial. Plasmid VEGF₁₆₅ was introduced in exactly the same way as in the animal model. A hydrogel-coated balloon angioplasty catheter was employed to deliver the 2000 µg of the plasmid into the popliteal artery. Ultrasound was employed to establish that the segment where the balloon was inflated had no stenoses. This would ensure that any improvement in perfusion could not be ascribed to dilation of remaining feeding arteries.

There was evidence of clinical and angiographic improvement in blood supply to the leg. Digital subtraction angiography demonstrated an increased number of collateral vessels in the right leg at the knee, midtibial, and ankle levels. These vessels persisted on a 12-week angiogram. Doppler-derived blood flow at rest was increased by 82%, and maximal flow was 72% greater than pretreatment. These results parallel those from the preclinical animal studies.

The principal side effects of treatment were three spider angiomas that regressed by 8 weeks post gene transfer. There was a period of transient edema, which increased the ankle width by 1cm and resolved after bumetanide therapy. This reflects the effect of VEGF in increasing capillary leakage. However, despite these promising clinical results, the patient still required below-knee amputation 5 months after gene therapy.

This study certainly highlights the therapeutic potential of intravascular gene transfer in this condition. It is interesting that despite the low transfection efficiency of plasmid DNA, enhanced collateralization occurred beyond the site of gene delivery. The poor vascular supply to the ischemic territory would be expected to have severely reduced the efficiency of gene delivery. This is probably one of the factors that resulted in the first published phase I trial employing direct intramuscular gene transfer. The fact that the patient required later amputation suggests that repeated dosing of plasmid may be required to ensure a sustained collateral vascular supply. Herein lies one of the paradoxes of angiogenic gene therapy, since one of the main stimuli for angiogenesis is hypoxia. Therefore, a negative feedback loop may thus result in collateral vessel regression as the hypoxic stimulus declines and the patients return to their pretreatment state. The strategy employed in therapeutic angiogenesis protocols to date assumes that the main reason for impaired collateral development in the first place is actually angiogenic growth factor deficiency as opposed to a resistance of the vascular bed to angiogenic stimuli. Certainly, diabetic patients are recognized to have a degree of resistance to angiogens, and thus replacing individual growth factors may not be adequate to generate a sustained and mature collateral network. Isner has clearly recognized this, hence his interest in employing endothelial progenitor cells as potential effectors in a therapeutic angiogenesis program.

1996

Mike Leigh's film "Secrets and Lies" wins the Palme d'or at the Cannes Film Festival;
Timothy Leary, the prophet of the 1960s' psychedelic revolution, dies, aged 75;
and South Africa adopts a postapartheid constitution



Gene transfer of naked DNA encoding for three isoforms of vascular endothelial growth factor stimulates collateral development in vivo

S. Takeshita, Y. Tsurumi, T. Couffinhal, T. Asahara, C. Bauters, J. Symes, N. Ferrara, J. M. Isner

Lab Invest. 1996;75:487-501

Collateral vessel development in chronic limb ischemia is promoted by naked plasmid vascular endothelial growth factor (VEGF) transfection: this is the groundbreaking point illustrated in this comprehensive article assessing the efficiency of gene transfection as well as histological and physiological evidence of angiogenesis in the rabbit hindlimb model.

The study utilized reverse transcriptase polymerase chain reaction (RT-PCR) to detect human VEGF messenger RNA (mRNA) in rabbit tissue, which was unique to the plasmid vectors employed. This not only allowed the assessment of transfection efficiency in the rabbit hindlimb, but also the detailed analysis of tissue from remote sites. Therefore, the concerns regarding unwanted gene expression at non-target sites could be addressed for the first time in this context. Plasmid transfection offers a number of advantages, which include: (i) low toxicity to the transfected tissue, unlike adenoviral vectors; (ii) low risk of insertion into host DNA causing mutagenesis or uncontrolled modifications of the gene pool; and (iii) lower probability of transfecting tissue at remote sites. However, this comes at the cost of poor transfection efficiencies of <1% compared to levels of 20% to 30% seen with viral vectors.

Isner introduced plasmid DNA expressing the VEGF isoforms 165, 189, and 121 into the ischemic hindlimbs of 3 groups of rabbits. In each case, this was achieved by inflating a angioplasty balloon catheter coated with plasmid DNA dissolved in hydrogel in the internal iliac artery of the ischemic limb. The control group of rabbits received plasmid encoding an inert *lacZ* reporter gene only. RT-PCR demonstrated VEGF mRNA unique to the plasmids employed only from treated ischemic hindlimbs. There was no evidence of gene expression in brain, heart, liver, lung, spleen, or testis. Gene expression for each isoform was consistently observed until 14 days post transfection for each isoform, but was undetectable at 30 days with a transfection efficiency of <0.5%. Angiographic assessments demonstrated significant improvements in collateral vessel formation, which were matched by highly significant

increases in both calf blood pressure ratios and Doppler-derived blood flow. The resting flow was increased by 35% when compared with control. As in the VEGF₁₆₅ peptide study, the capillary-to-myocyte ratio was also significantly increased in the treated limbs.

A careful assessment was made to investigate potential deleterious effects. Histological analysis of remote tissue sites failed to demonstrate neoangiogenesis or inflammatory infiltrates. Indeed, the sites of gene transfer in the arterial wall had minimal neointimal thickening. This is important, since a potential hazard of growth factor therapy is plaque rupture due to neovascularization or proliferation of cellular elements secreting metalloproteinases within the plaque.

The study certainly demonstrated that despite a very low transfection efficiency, plasmid VEGF was capable of inducing collateral formation to a similar degree to that generated by VEGF peptide. Each isoform of VEGF appeared to be equivalent in efficacy, which may represent cleavage to a common VEGF₁₂₁ sequence that targets the Flk-1 receptor in this tissue. The use of RT PCR allowed a detailed search for nontarget tissue gene expression and also illustrated the transient nature of plasmid transcription, minimizing the risks of long-term exposure to gene product. Both these are important characteristics if targeted gene expression at ischemic sites is to be realized.

1996

The New York Yankees win their 23rd World Series, their first for 18 years; Islamabad is brought to a standstill as Islamic militants demonstrate against the government of Benazir Bhutto; and Boris Yeltsin publicly fires his security chief General Alexander Lebed during a television broadcast

Isolation of putative progenitor endothelial cells for angiogenesis

T. Asahara, T. Murohara, A. Sullivan, M. Silver, R. van der Zee, T. Li, B. Witzenbichler, G. Schatteman, J. M. Isner

Science. 1997;275:964-967

With this exhaustive and seminal piece of work, Isner's group paved the way for a new approach to therapeutic angiogenesis. The authors demonstrate that within the population of circulating leukocytes, cells exist that have phenotypic characteristics of angioblasts and are able to contribute to angiogenesis.

Circulating hemopoietic stem cells (HSCs) with the capability of repopulating ablated bone marrow had already been demonstrated. However, the authors had the intuitive insight to realize that this observation may impact on the vasculature, since in the early embryo vasculogenesis begins with clusters comprising central HSCs and peripheral angioblasts. Both these cell populations are thought to derive from a common lineage originating from the putative hemangioblast.

Antibody-coated magnetic beads were used to select cells from human peripheral blood expressing certain antigens. The antigens chosen were CD34, expressed by all HSCs, but lost on differentiation, or Flk-1, one of the receptors for vascular endothelial growth factor (VEGF) present on HSCs and primitive endothelial cells. Most of the results presented within the paper were from cells that had originally been isolated on the basis of CD34.

Following magnetic bead isolation, analysis by fluorescence-activated cell sorting (FACS) was used for further characterization. Initially, of the CD34 selected cells, 16% expressed CD34; however, this proportion increased dramatically to 60% after cells were plated out and maintained in culture for 7 days. This enrichment coincided with phenotypic changes such as the appearance of spindle-shaped cells and the ability of the cells to form vascular structures in vitro when grown on extracellular matrix proteins. These macroscopic changes were accompanied by other features that suggested the cells had become "endothelial-like." These changes included the ability to take up low-density lipoprotein, express nitric oxide synthase, and make nitric oxide in response to VEGF and acetylcholine. In keeping with this observation was the

fact the cells expressed Flk-1 even though they had been selected on the basis of CD34 expression.

The in vitro observation of endothelial-like behavior was reinforced by further detailed and elegant in vivo studies. The CD34+ cells were labeled with a fluorescent dye and then intravenously injected into athymic nude (β -galactosidase-negative) mice. Two days previously, one femoral artery had been excised to cause unilateral limb ischemia. One to 6 weeks after injection, histological examination revealed fluorescently labeled cells integrated into capillaries together with endogenously derived endothelial cells. The exogenous origin of the labeled cells was conclusively demonstrated by repeating the procedure with peripheral blood from a mouse line containing the bacterial gene encoding the protein β -galactosidase. Within the ischemic limb, capillaries comprised β -galactosidase-negative and -positive endothelial cells.

In yet another series of experiments, the presence of circulating endothelial progenitor cells was confirmed in the rabbit by ex vivo sorting, fluorescent labeling, and appearance within capillaries in the ischemic hindlimb following reinjection.

This article is remarkable for the wealth of techniques used to address and conclusively prove a hypothesis. The observations mean that it is potentially possible to genetically manipulate the entire vascular tree using ex vivo techniques. Moreover, as a result of this paper, circulating endothelial progenitors have been sought, found, and shown to incorporate into blood vessels in animals and patients.

1997

Deng Xiaoping, head of the Chinese Communist Party, dies, aged 92; comet Shoemaker-Holt 2 makes its closest approach to Earth (1.9245 AU); and three Swiss banks create a \$70 million Holocaust fund



Constitutive expression of phVEGF₁₆₅ after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia

I. Baumgartner, A. Pieczek, O. Manor, R. Blair, M. Kearney, K. Walsh, J. M. Isner

Circulation. 1998;97:1114-1123

This landmark study was the first to describe the effects of gene transfection in patients with peripheral vascular disease. It was a phase I nonrandomized safety study to assess the safety and feasibility of intramuscular gene transfer using the 165-amino-acid isoform of human vascular endothelial growth factor (phVEGF₁₆₅) plasmid vector.

The patients had severe peripheral vascular disease with ischemic rest pain and/or nonhealing ischemic ulcers. In the majority (7 patients), disease was so advanced amputation had been recommended. Efficacy and safety of the treatment were assessed by a combination of clinical endpoints, as well as contrast and magnetic resonance angiography. Tissue specimens from treated limbs underwent immunohistological and molecular biological analysis.

Each patient received a total dose of 4000 µg of naked plasmid DNA encoding VEGF₁₆₅ injected directly into the muscles of the ischemic limb. There was a statistically significant increase in ankle:brachial index from 0.33 at baseline to 0.48 at 12 weeks. This matches the degree of improvement that would be expected from conventional revascularization where an increase of 0.1 would be considered indicative of successful percutaneous or surgical intervention. Such a degree of improvement has not been reported to occur spontaneously or with medical therapy in this population. Digital subtraction angiography showed newly visible collateral vessels at knee, calf, and ankle levels in 7 out of 10 treated ischemic limbs.

Gene expression was assessed by enzyme-linked immunosorbent assay (ELISA), which showed a transient peak in systemic levels of VEGF at 1-3 weeks after gene transfer in 7 patients. The therapeutic effect of plasmid VEGF transfection was illustrated by the resolution of rest pain in all 3 patients presenting with this symptom alone and complete/partial resolution of limb ulcers in 4 out of 7 cases. Furthermore, all the patients experienced a significant increase in pain-free walking time (3.8±1.5 min vs 2.5±1.1 min before gene therapy, at an average of 13 weeks post treatment). Limb salvage was achieved in 3 patients in whom

amputation had been recommended prior to the trial. The tissue specimens from 1 amputee 10 weeks post therapy showed foci of endothelial cell proliferation, which were nearly absent in normal arteries. Amplification of DNA fragments was identified in several muscle samples remote from the injection site. The principal side effect of therapy was transient lower-limb edema in 6 patients.

These results are quite remarkable considering the low transfection efficiency and limited temporal expression of plasmid DNA in previous studies. This suggests that transfection efficiency is not the sole determinant of a successful clinical outcome. VEGF is secreted by the transfected cells and thus itself may exert paracrine effects. These may be further enhanced by the binding of VEGF₁₆₅ in the extracellular matrix, allowing a reservoir of active peptide to generate a sustained biological effect. Endothelial cells upregulate VEGF (KDR) receptor expression in the presence of VEGF, generating an amplifying mechanism coupled with the antiapoptotic properties of VEGF. Even the hypoxic environment of the ischemic limb may improve uptake of plasmid DNA, since transfection efficiency in ischemic skeletal muscle is improved. Although these results are encouraging, they should be interpreted cautiously given the fact that this was an unblinded nonrandomized trial and the study population too small and follow-up too short to exclude the possibility of enhanced retinopathy in diabetic patients or the development of neoplasm. However, it does suggest that serious assessment of this strategy should be undertaken in this debilitating progressive condition.

1998

The film "Titanic" cleans up at the Oscars; President Suharto of Indonesia is reelected for the seventh time despite rumors of corruption and an economic crisis; and Dr Benjamin Spock, parenting guru, dies, aged 92

Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization

C. Kalka, H. Masuda, T. Takahashi, W. M. Kalka-Moll, M. Silver, M. Kearney, T. Li, J. M. Isner, T. Asahara

Proc Natl Acad Sci U S A. 2000;97:3422-3427

In this paper, Isner's group shows that an inherently impaired angiogenic potential can be overcome by delivering heterologous endothelial cell precursors. This is of direct therapeutic relevance since collateral formation is thought to be impaired in elderly, hypercholesterolemic or diabetic patients.

Peripheral blood mononuclear cells from healthy adult humans were isolated by centrifugation and then expanded under specific tissue culture conditions involving attachment and growth on extracellular matrix protein in the presence of endothelial cell mitogens. After 7 days, these expanded cultures consisted of spindle-shaped cells that endocytosed low-density lipoprotein (LDL). These cells were subjected to further analysis by fluorescence-activated cell sorting (FACS). Using this technique, approximately 60% of the cells expressed endothelial-cell-specific antigens.

From the expanded cultures of human endothelial progenitors, 5×10^5 cells were then injected into the systemic circulation of nude athymic mice, which 1 day previously underwent unilateral femoral artery excision. These mice are known to have a relatively deficient angiogenic response. Control groups consisted of mice similarly treated to produce hindlimb ischemia, injected with human microvascular endothelial cells (HMVECs) or the culture medium used to expand the human peripheral mononuclear cells. The ability of these three treatments to benefit the ischemic limb was then measured both qualitatively and quantitatively by laser Doppler perfusion imaging using the nonischemic limb as a within-group, within-individual, control. Three days after surgery, limb perfusion was similarly reduced in all three groups. However, by day 7, those mice injected with human endothelial progenitor cells (hEPCs) showed a trend toward better perfusion that became significant at 14 days. By 4 weeks after surgery, those mice receiving the endothelial-like cells had limb blood flows of approximately 70% normal, while in the HMVEC and culture medium groups, it was similar, at 20% of normal. This increased flow was functionally significant and associated with reduced limb autoamputation and foot necrosis and enhanced limb salvage (60% vs 8%,

hEPCs vs other groups, respectively). Prior to injection the human-derived cells were fluorescently labeled. These tagged cells were visible in foci of neovascular activity. Moreover, the fluorescently labeled cells, also staining positive for human CD31, were present in 30% to 80% of vessels in the ischemic limb, but in none of the vessels in the contralateral, nonischemic limb. At day 7, this histological evidence of incorporation within the ischemic hindlimb was associated with a highly significant 4-fold increase in capillary density compared with culture medium-injected mice. Although the ratio diminished, the enhanced capillary density persisted to at least 4 weeks. The hEPCs did not give rise to cells in any other organ examined, apart from the spleen.

This study demonstrates the concept of ex vivo cell therapy for angiogenesis. Furthermore, it demonstrates that an impaired angiogenic response is not simply due to growth factor deficiency, but under certain circumstances growth factor resistance may also be a contributory factor. By implication, this resistance can be overcome by delivering healthy endothelial progenitors. Thus, this paper indicates a clear path where the angiogenic response may be optimized by the potentially synergistic therapies of enhancing growth factor levels and the growth factor-responsive cells.

2000

A massive iceberg roughly the size of the US state of Connecticut breaks free from the Antarctic's Ross Ice Shelf;

Florida jury orders tobacco industry to pay \$144.8 billion in punitive damages to some 500 000 Florida smokers, the largest award in US history; and 470 members of an obscure Ugandan doomsday cult die after apparently setting themselves on fire in a church



Vascular endothelial growth factor (165) gene transfer augments circulating endothelial progenitor cells in human subjects

C. Kalka, H. Masuda, T. Takahashi, R. Gordon, O. Tepper, E. Gravereaux, A. Pieczek, H. Iwaguro, S. I. Hayashi, J. M. Isner, T. Asahara

Circ Res. 2000;86:1198-1202

In this fascinating study, the authors demonstrate that local delivery of cDNA encoding the 165-amino-acid isoform of vascular endothelial growth factor (VEGF₁₆₅) can have systemic consequences that may augment the local angiogenic response.

The authors examined the characteristics of the circulating endothelial progenitor cells before and after intramuscular injection of VEGF₁₆₅ or empty vector in patients with critical lower-limb ischemia. Healthy controls following intramuscular injection of saline were also examined. Peripheral blood was collected from 20 patients receiving 4 mg of intramuscular VEGF₁₆₅ cDNA, 5 patients receiving similar amounts of empty vector, and 4 healthy volunteers receiving saline vehicle alone. Before and after intramuscular injection, mononuclear cells within peripheral blood were isolated by density gradient centrifugation and then enriched for endothelial progenitors by 4 days of culture under specific growth conditions. These cells were characterized while attached to plates by their ability to bind a specific protein and to endocytose fluorescently-labeled low-density lipoprotein. Using this assay to compare cell density in blood collected before treatment to blood collected at various time points after treatment showed that VEGF₁₆₅ cDNA, but no other treatment, increased the number of endothelial precursors cell per mm² of culture dish by 80% at day 7, 150% at day 14, and 80% at day 21. Moreover, this rise was related to the elevations in plasma concentrations of VEGF₁₆₅ with a correlation coefficient of 0.83. This culture-based assay was verified by fluorescence-activated cell sorting (FACS) using antibodies to detect surface expression of markers of endothelial cells and endothelial activation (KDR, VE-cadherin, CD34, E-selectin, and $\alpha_v\beta_3$). Mononuclear cells from the equivalent of 1 mL of peripheral blood were subjected to FACS. Compared with before treatment, blood taken after VEGF₁₆₅ treatment had on average a 22-fold increase in KDR-, 26-fold increase in VE-cadherin-, and 8-fold increase in CD34 positive-cell number. The temporal pattern of change in the appearance of these cells was similar to that observed in the culture-based assay and also tracked the changes in systemic VEGF₁₆₅ with a correlation coefficient of 0.83. No increase

in cells expressing these antigens was seen in patients with limb ischemia treated with empty vector or when vehicle was injected into healthy controls. Similarly, the expression of the adhesion molecules $\alpha_v\beta_3$ and E-selectin increased on average 5- and 25-fold, respectively, with no change in the other two groups. These increases, following VEGF₁₆₅ cDNA injection, in the proportion of circulating mononuclear cells expressing endothelial cell-specific antigens or markers of endothelial activation became significant after 7 days, and persisted to at least 28 days, post treatment.

The results, using two different assay techniques, conclusively show that local gene delivery mobilizes a circulating endothelial-like response. Taken together, with other information summarized in this series, it is likely that these cells are recruited to angiogenic foci within the ischemic limb and promote the angiogenic response. Thus, from a semantic point of view, the process known as vasculogenesis, involving angioblasts, that was thought to be confined to embryonic development, probably also occurs in the adult. More importantly, for an angiogenic therapy to have the greatest likelihood of success, it probably needs to comprise a vasculogenic component and thus recruit endothelial-like cells from the bone marrow.

2000

The first survey of the entire
human genome is completed;
Australia Prime Minister John Howard requests
that the remains of 3000 Aborigines held
in British museums are returned;
and President Fernando de la Rúa apologizes
for the protection given to Nazi war criminals by
Argentina after the Second World War



Left ventricular electromechanical mapping to assess efficacy of phVEGF(165) gene transfer for therapeutic angiogenesis in chronic myocardial ischemia

P. R. Vale, D. W. Losordo, C. E. Milliken, M. Maysky, D. D. Esakof, J. F. Symes, J. M. Isner

Circulation (Online). 2000;102:965-974

Building on observations made previously concerning direct intramyocardial injection of the 165-amino-acid isoform of vascular endothelial growth factor (VEGF₁₆₅) cDNA, this remarkable study looks at a contemporary technique capable of providing "real-time" information regarding myocardial electrical and mechanical activity.

Hibernation is the term used to describe reversibly injured myocardium that contracts poorly due to reduced myocardial blood flow. By definition, function is restored when myocardial flow is normalized. In this study, a percutaneous technique known as endocardial electromechanical mapping (EMM) was used to characterize the hearts of patients with angina not amenable to conventional revascularization. This characterization was performed up to 1 month before and 60 days after intramyocardial injection of VEGF₁₆₅ cDNA delivered as part of a phase I dose escalation study; therefore there was no control group.

Thirteen patients underwent a minithoracotomy to allow the epicardial injection of 250 µg or 500 µg of VEGF₁₆₅ into lateral, anterior, and/or septal myocardium. The sites of injection were targeted on the basis of the preoperative electromechanical map. Sites with poor local linear shortening (4% to 12%), but normal or near-normal electrical activity (unipolar voltage or bipolar voltage ≥ 5 or ≥ 2 mV, respectively) were preferred. Patients also underwent pre- and postoperative myocardial perfusion imaging by single photon emission computed tomography (SPECT)-sestamibi as well as assessments of angina severity during normal activity and treadmill exercise.

Gene expression was documented by enzyme-linked immunosorbent assay (ELISA), which showed a 4-fold increase in circulating levels of VEGF after a mean of 12 days post injection. At 60 days, this was accompanied by remarkable and highly significant changes in objective measures of contractility compared with before injection. Based on EMM, LV ejection fraction increased from 31% to 37%, mean linear shortening from 10% to 15%, and area of ischemic myocardium decreased from 6.45 cm² to 0.95 cm².

This was accompanied by highly significant changes in SPECT-sestamibi perfusion scores from 7.4 to 4.5 at rest and from 12.8 to 8.5 after dipyridamole stress. These changes in electrical, contractile, and perfusion parameters are beautifully depicted in the article by individual examples. Accompanying these objective changes were more clinically relevant benefits such as a reduction in anginal episodes per week from 48 to 2, weekly glyceryl trinitrate tablet usage from 55 to 2, and treadmill exercise duration from 272 to 453 seconds, comparing status before and after VEGF₁₆₅ injection.

The salutatory effects of intramyocardial injection of VEGF₁₆₅ had been reported previously by this group. The importance of this paper is that it enables a clinically relevant therapeutic strategy. There is no doubt that injection catheters allowing percutaneous intramyocardial gene delivery will be the future method of choice. Thus, by combining these with EMM it may be possible to guide delivery to those myocardial areas that will benefit most from increased blood flow. This will allow a rational approach to angiogenic therapy within a single procedure.

2000

The Japanese cabinet resigns en masse,
clearing the way for a new prime minister to replace
Keizo Obuchi after he suffers a stroke;
a 15-year-old computer hacker, "Mafiaboy,"
is arrested in Montreal after shutting down some
of America's internet powerhouses, from Amazon
to Yahoo; and Zimbabwe's ruling party
Zanu-PF amends the national constitution,
giving the government the right to seize
white-owned farms without compensation



Favorable effect of VEGF gene transfer on ischemic peripheral neuropathy

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Nat Med. 2000;6:405-413

This remarkable study provides a fascinating insight into the effects of the 165-amino-acid isoform of vascular endothelial growth factor (VEGF₁₆₅) on ischemic peripheral neuropathy. Clinical neurological deficits have been described in up to 88% of patients with this debilitating condition. This has major implications for symptomatic treatment and rehabilitation in this common condition. The study is remarkable both in the range of techniques employed to address the issue and in that it potentially opens the door to another role for angiogenic therapies.

Initially, Isner's group examined the effect of plasmid VEGF₁₆₅ (phVEGF₁₆₅) upon the rate of recovery of nerve conduction following an ischemic insult in the rabbit hind-limb model. phVEGF₁₆₅ was injected at the time of femoral artery resection. Neurophysiological testing was employed to assess the recovery of motor and sensory nerve function. This was achieved by measuring motor nerve amplitudes as compound muscle action potentials (CMAPs) and sensory nerve action potentials (SNAPs), respectively. There was an early recovery of CMAPs and SNAPs beginning by 2 weeks. Recovery reached a maximum for CMAPs at a peak of 84% of the normal level by 12 weeks. SNAPs achieved full recovery to normal levels by 4 weeks.

Laser Doppler perfusion imaging was employed to assess the effect of phVEGF₁₆₅ on peroneal nerve perfusion by the vasa nervorum. This was coupled with fluorescent BS-1 lectin staining to visualize these vessels. There was evidence of increased vasa nervorum perfusion and density of vessels following plasmid transfection. The nerve sections in treated limbs had a near-normal pattern of myelination in both small and large fibers with almost none of the myelin debris that is apparent following cell death. The effect of phVEGF₁₆₅ on established ischemic peripheral neuropathy was illustrated by a significant recovery of CMAPs to 70% of normal and full recovery of SNAPs over a period of 4.5 weeks after gene transfer.

The hypothesis that VEGF₁₆₅ may not only enhance perfusion via the vasa nervorum, but directly affect the neural

elements, was also addressed. A number of in vitro techniques were employed. Schwann cell migration, measured in a modified Boyden chamber, was enhanced by VEGF in a dose-dependent manner. Indeed, this effect was equivalent to that exerted by nerve growth factor. This was inhibited by genistein, indicating that chemotaxis was enhanced by VEGF binding to tyrosine kinase receptors as opposed to neuropilin-1. VEGF was also protective against hypoxia-induced apoptosis in the Schwann cells, which was confirmed by a TdT-mediated dUTP-biotin nick end-labeling (TUNEL) assay.

This comprehensive paper highlights Isner's group's approach employing a well-characterized animal model to assess this therapeutic strategy coupled with a diverse range of complementary molecular and cellular biological techniques to examine the mechanism of action in considerable detail. This study suggests that VEGF enhances neural activity in the ischemic limb by simply promoting vasa nervorum growth, optimizing nerve cell recovery by reversing hypoxia, and improving tissue nutrient levels. Furthermore, a direct action of VEGF upon neural cell migration and cell survival is suggested by the preliminary cell culture data. This has significant implications beyond the arena of ischemic peripheral neuropathy.

2000

A Hungarian WWII veteran is found in a Russian psychiatric hospital 55 years after being taken prisoner by the Red Army; the Russian nuclear-powered submarine "Kursk" sinks in the Barents Sea with the loss of all 116 crew; and the French Interior Minister Jean-Pierre Chevènement resigns in protest over plans for granting limited autonomy to Corsica