



Angiogenesis and cardiovascular disease: how long will angiogenesis last and how can we stop it?

Sigrid Nikol, MD

Associated Professor of Molecular Medicine - Medical Clinic C - University Hospital - Westfälische Wilhelms University - Münster - GERMANY

The therapeutic use of proteins or of genes of naturally occurring growth factors may be potentially beneficial for controlling the growth of collaterals or the remodeling of arteries. Whether the mother vessels will regress or evolve into well-differentiated daughter vessels that continue to function indefinitely depends on the duration of therapy. A number of vector systems and sophisticated local drug delivery strategies may be of use in combination with protein or gene therapy to control the duration of therapy. Proteins and nonviral or viral gene transfer may induce transient new vessel formation. A number of anti-angiogenic chemicals and proteins may be potentially used to stop angiogenesis. In terms of gene therapy, retroviral and adeno-associated vectors allow stable gene transfer, with the potential of long-lasting therapeutic effects that can be controlled by inducible promoters.

Keywords: angiogenesis; remodeling; protein; transfection; gene integration; viral vector; nonviral gene transfer; encapsulation; inducible promoter; local drug delivery

Address for correspondence:

Sigrid Nikol, Associated Professor of Molecular Medicine, Medical Clinic C, University Hospital, Westfälische Wilhelms University, Albert Schweitzer Straße 33, D-48129 Münster, Germany (e-mail: nikol@uni-muenster.de)

Therapeutic angiogenesis is a potential method of treating chronic ischemia. It may be directed at any of the numerous growth factors and cellular components that form the complex network involved in both true new vessel formation (the in situ growth of muscular collateral arteries) and vessel remodeling.¹ On a conceptual basis, it remains unknown whether recombinant proteins for the relevant factors, or the genes for these, will provide the best and longest-lasting therapeutic effects in patients.

Using recombinant proteins may provide the advantage that the therapy will be easier to halt if necessary. If multiple angiogenic factors are required, it may be easier to administer the proteins together than combining the genes for different factors. In this connection, an early clinical trial has already been reported, using the protein vascular endothelial growth factor (VEGF). However, despite promising results from animal experiments, this trial (the VEGF in Ischemia for Vascular Angiogenesis [VIVA] trial), which was the biggest clinical angiogenesis trial to date using recombinant VEGF, comprising 178 patients, did not demonstrate the protein form of therapy to be efficient.² Direct administration of the protein may not have been effective because the protein was rapidly degraded and thus gave only a short therapeutic

response. It is also possible that the intracoronary and systemic infusions used may not have allowed sufficient uptake at the desired vessel site. One of the problems now identified is that the mother vessels induced by VEGF from preexisting microvessels after pericyte detachment and basement membrane degradation are transient structures, which do not persist for more than few days.³ Such mother vessels may either regress or evolve into well-differentiated daughter vessels that continue to function indefinitely, depending on the duration of the therapy administered. Multiple instead of single-dose therapy may be one solution. Prolonged protein delivery has been more effective at inducing angiogenesis using fibroblast growth factor (FGF) when administered via sustained-release heparin-alginate microcapsules, thus avoiding multiple applications.⁴

With the early results showing that protein therapy may be less efficient, perhaps gene therapy may provide some answers. As well as

SELECTED ABBREVIATIONS AND ACRONYMS

| | |
|-------------|--|
| FGF | fibroblast growth factor |
| VEGF | vascular endothelial growth factor |
| VIVA | VEGF in Ischemia for Vascular Angiogenesis |

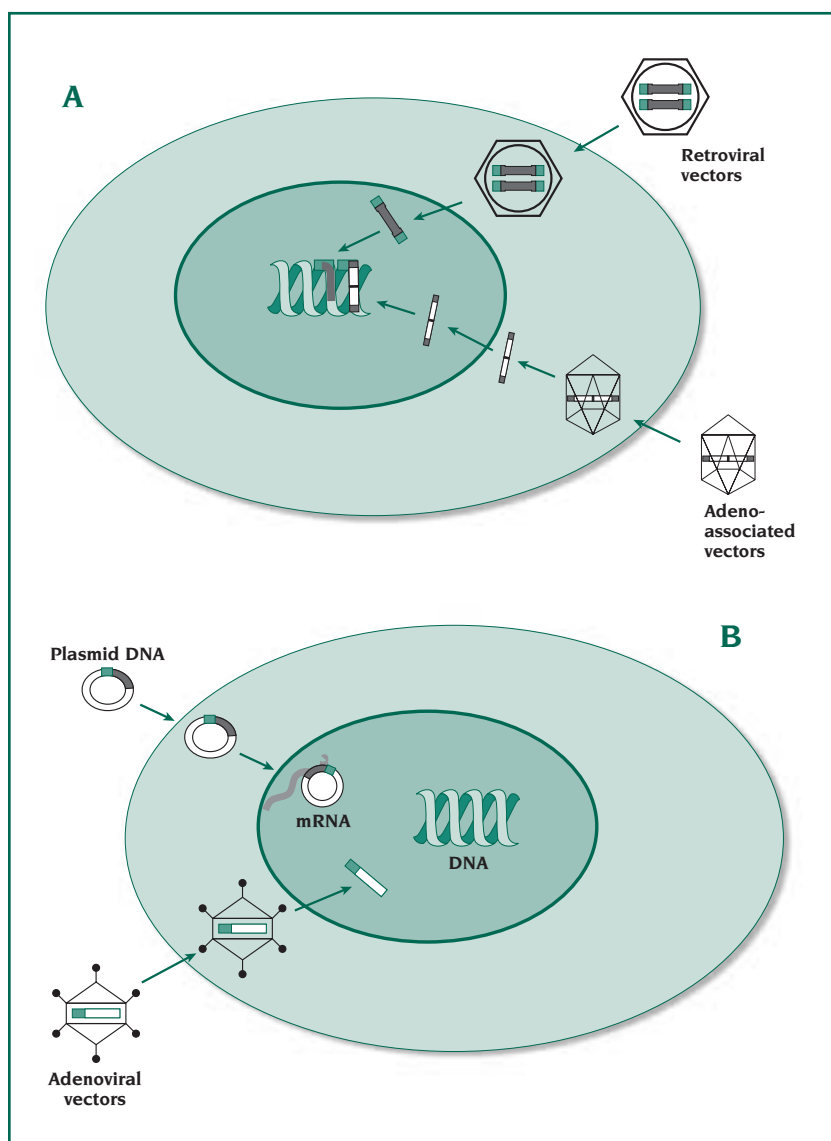


Figure 1. Representation of a cell showing two possible gene transfer approaches for the introduction of therapeutic genes.

A. Retroviral or adeno-associated viral integration of the therapeutic gene into native DNA for stable long-term gene expression. **B.** Transfection with expression plasmid vectors and adenoviral gene transfer for transient gene expression.

potential long-term effect, ideally with a minimal immune reaction. The immune and inflammatory effects of certain delivery systems, particularly adenovirus, have been highlighted by the unexpected death of a patient in a phase I study of gene therapy for the inherited disorder ornithine transcarbamylase deficiency. Angiogenic gene therapy will clearly be a more localized treatment; however, one of the concerns is naturally the possible duration of any effects following gene delivery and whether it can be halted when desired. This will depend on the vector and delivery system used. These points will also be discussed.

DURATION OF EXPRESSION OF GENE THERAPY DEPENDING ON THE VECTOR SYSTEM

Two approaches can be used to transfer genes into selected cells, transient transduction without integration of DNA, called transfection, and stable transduction by chromosomal integration (*Figure 1*). Genetic material may be delivered as nucleic acid alone, nucleic acid complexed with various chemical compounds, such as calcium phosphate, diethylaminoethyl dextran, lipospermines, or liposomes, or incorporated into a suitable virus. Transfection describes delivery of a gene predominantly into the cytoplasm and nucleus, where the gene has a relatively short-lived effect, with practically no genomic incorporation. The effect is nonselective, and DNA is introduced into cells in

being more efficient, gene therapy is potentially more controllable, through several mechanisms.

Hence, this article will discuss the potential of gene therapy in the context of inducing and controlling angiogenesis.

Protein production through gene therapy may offer a better prospect of influencing the duration of therapy than encapsulation of protein. Successful gene therapy for angiogenesis requires a combination of adequate transport into specific

cells, using a relevant vector system, together with a suitable method for introduction into the body with minimal side effects, and a valid therapeutic target. The use of new concepts, such as the use of sophisticated local drug delivery strategies may further increase the value of gene therapy. Thus, transfer efficiencies may be increased, with minimal side effects, and local deposition may result in longer-lasting therapeutic effects with minimal or no systemic distribution. One of the advantages of gene therapy over the use of proteins clearly is the

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both proliferating and quiescent states. Plasmid DNA or adenoviral vectors are mainly used for this approach. Gene expression using plasmid DNA is detectable in porcine arteries for at least 5 months following gene transfer (*Figure 2*).^{5,6} In mouse muscle tissue, expression of plasmid vectors has been observed for up to 19 months after transfer by some workers,⁷ although others in different models find that expression is limited to weeks. Gene expression in vascular cells follow-

the cell survives.¹¹ Gene integration usually takes place during DNA replication, which makes this method selective for proliferating cells. Gene transfer for integration is usually performed using vectors derived from retrovirus or adeno-associated virus. Only recently have certain retroviral vectors been developed for gene delivery and integration into quiescent cells. Most retroviruses allow gene integration only during DNA replication, which makes these a method of delivering

cells reintroduced to animals has recently been demonstrated to be more effective compared with the use of endothelial cells alone.¹² This approach mimics one of the functions of VEGF, the mobilization of endothelial progenitor cells, and may have clinical value in the future. This kind of use of angioblasts is in the early stages of research and no conclusions can yet be drawn about any potential effect on therapeutic angiogenesis. In theory, however, such cells represent another avenue of investigation, especially as the cells may be genetically modified to enhance therapeutic effects. The duration of therapeutic effect compared with the use of angiogenic genes administered directly has yet to be investigated, but could be predicted to be longer, if the treatment was successful in the first place.

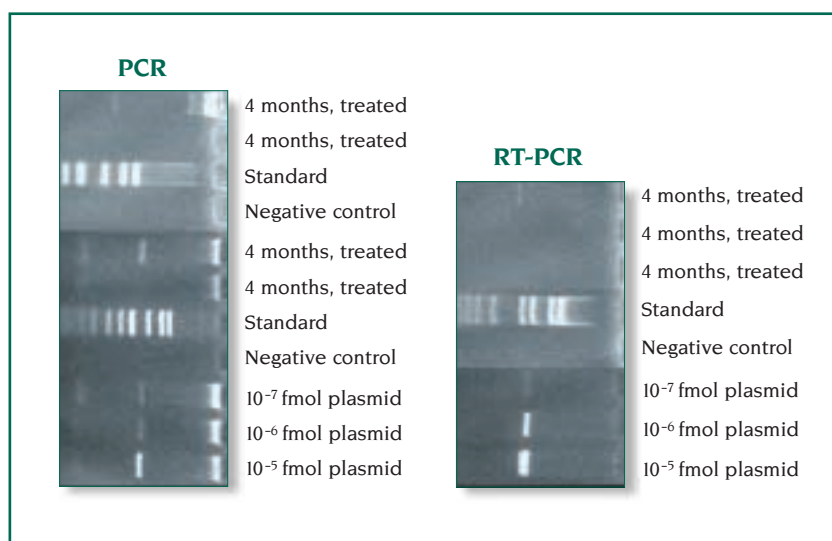


Figure 2. Polymerase chain reaction (PCR) and reverse transcriptase polymerase chain reaction (RT-PCR) demonstrating long-term gene detection and expression following nonviral gene transfer. **PCR.** Detection of plasmid DNA in treated artery tissue after gene delivery. Agarose gel, loaded as indicated (from bottom to top): a series of dilutions of plasmid DNA to document the detection limit; a negative control without any template; standard; segments following gene transfer after 4 months; a negative control without any template; standard and segments following gene transfer after 4 months. **RT-PCR.** Detection of exogenous mRNA in treated artery tissue after gene delivery. Agarose gel, loaded as indicated (from bottom to top): a series of dilutions of plasmid DNA to document the detection limit; a negative control without any template; standard and segments following gene transfer after 4 months.

ing adenoviral gene transfer is generally only for a few weeks,^{8,9} and the toxicity and immunological reactions associated with these vectors may lead to worsening of the underlying disease and therefore promote vessel occlusion.¹⁰

In contrast, chromosomal integration allows insertion into the target cell genome with more persistent gene expression, at best as long as

therapeutic genes selective to proliferating cells. Therefore, only very limited retroviral integration takes place in uninjured arterial or myocardial tissues where there is minimal cell proliferation.

As an alternative to the direct use of genes, the cells involved in angiogenesis may be used as gene delivery packages. The use of ex vivo expanded endothelial progenitor

DURATION OF ANGIOGENESIS DEPENDING ON THE DELIVERY SYSTEM

In the cardiovascular field, interventional techniques are already carried out within vessels, hence it seems logical for safety and dosage reasons to deliver therapy, and particularly gene therapy, locally in vivo using catheters. To this end, many local drug delivery catheters have been designed for transluminal or even pericardial application and are currently being evaluated in animal and clinical studies (*Figure 3*). Intraluminal infusion commonly results in high loss of agent, unless sustained-release, biodegradable polylactic-polyglycolic acid copolymer nanoparticles are used to penetrate deeper layers.¹³ Delivery to deeper layers, including media, adventitia, or pericardium, via catheters could have several advantages over intraluminal delivery, including less intraluminal loss, the ability to deposit drugs for longer periods, and

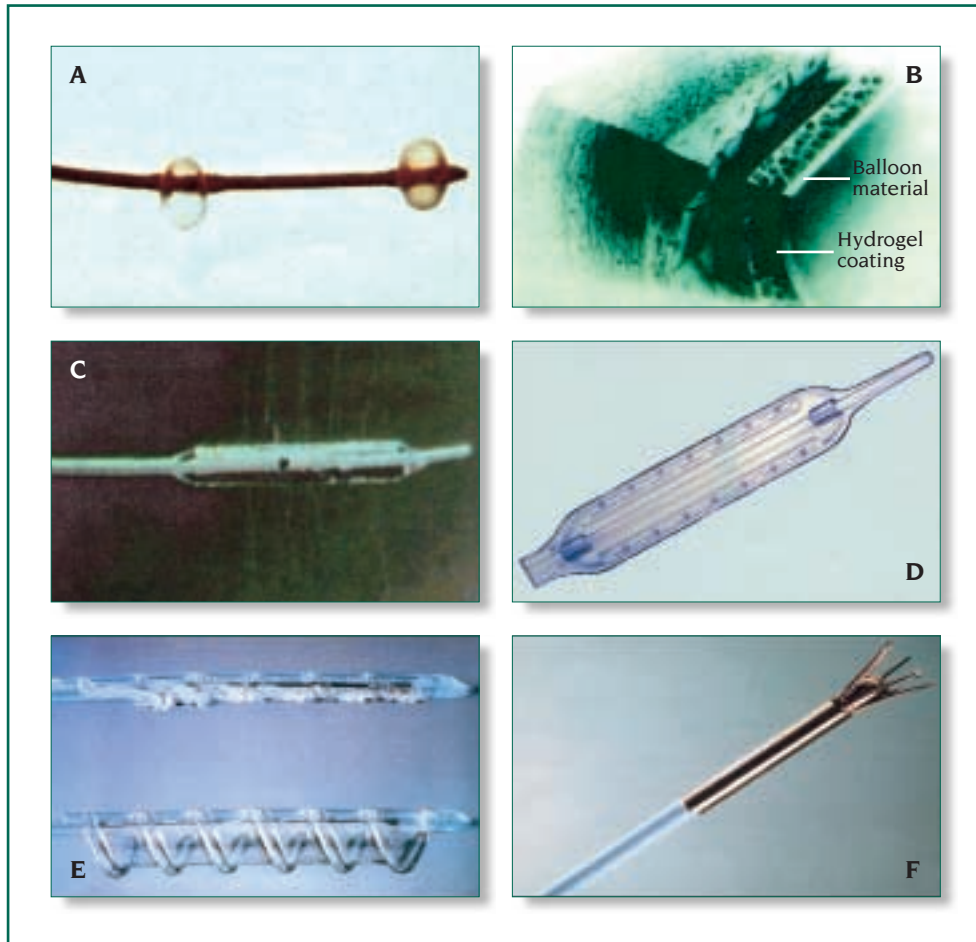


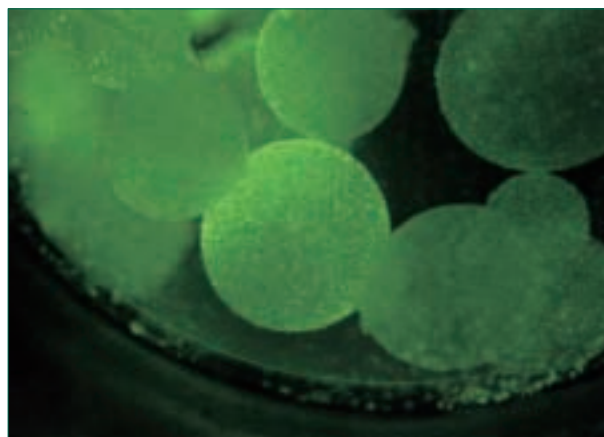
Figure 3. Various representative devices for local drug delivery: **A**, double balloon catheter; **B**, hydrogel-coated balloon catheter; **C**, porous balloon catheter; **D**, nipple catheter (Infiltrator®); **E**, chamber balloon catheter (Dispatch®); **F**, needle injection catheter.

the use of therapies that act on adventitial pathways of angiogenesis and remodelling.¹⁴ Gene expression with several genes for proteins with a potential therapeutic effect, including VEGF, has been demonstrated in all arterial layers in a pig

model up to 5 months after liposomal delivery using the needle injection catheter (Figure 3).^{5,15,16} Thus, whereas certain delivery systems lead to an immediate high concentration of drug in arterial wall, others can effectively leave a long-lasting

depot there, an effect that may further be enhanced by microencapsulation, which does not compromise viability of cells (Figure 4).^{4,17} The pharmacokinetics of any combination making up the system must be carefully evaluated.

Figure 4. Virus-producing PA317 cells encapsulated in a cellulose sulfate microcapsule were analyzed using a live/dead assay and viewed under a fluorescence microscope. Live cells fluoresce green as a result of enzymatic conversion of nonfluorescent cell-permanent calcein into fluorescent calcein (80× magnification).



HOW LONG DO WE NEED THE THERAPEUTIC EFFECT IN ANGIOGENESIS?

This is probably the question which has so far been least addressed by investigators. It is likely that angiogenic treatment would need to be long term or repeated if the effects are only of short duration, particularly as it is known that new vessels can regress in the absence of continued local activity of growth factors. In addition, the degree to which

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new vessels take up arterial blood flow—and this will clearly depend on the anatomical development in the context of extensive vessel disease in surrounding preexisting native vessels—will influence how long they stay open without repeated treatments.

Adenoviral gene transfer of VEGF demonstrated formation of mother vessels, an effect that had already resolved after 3 weeks.³ In the long term, creating an effect that avoids vessel regression and allows the formation of a robust capillary network and intact collateral circulation may be important. Whether this long-term effect should be achieved by multiple- rather than single-dose therapy, stable gene integration, the use of combinations of several growth factors instead of using one single growth factor, and what the role of flow is in maintaining patent vessels remains open.

As discussed above, gene therapy may provide more of a depot effect, particularly if combined with optimized vector and local delivery systems, but in terms of gene therapy, it is in fact not yet known how many cells need to be transduced and for how long to achieve the desired therapeutic effect. There are widely varying reports of vector efficiency; for example, arteries in patients have been locally transfected with adenoviruses with efficiencies of between 0.4% and 5%,¹⁸ compared with up to 15% achieved by transfection when combined with liposomes⁶ and only about one in a thousand cells typically infected by retroviruses.^{17,19} However, the results quoted here are only relevant when we look at the clinical effect. The therapeutic effect may also depend on the method of delivery into the artery at the injury site, on whether or not high concentrations of genes are available at the site of

therapy, with release and degradation perhaps being slowed down by gene integration, chemicals, microencapsulation, or the presence of the depot itself. The number of cells that need to be transduced is probably dependent on the particular gene, the expression efficiency achieved per cell (depending on the amount of DNA entering the nucleus), and on the duration of local levels of protein necessary for the intended biologic effect. If transfection efficiency is a limiting factor, then delivery strategies that augment the therapeutic effect by action on untransfected cells (bystander effect) will be preferable. Even low transfection efficiencies of 0.1% may lead to significant angiogenesis when the paracrine therapeutic gene VEGF₁₆₅ is injected into the adventitia in a porcine model using optimized liposomal gene transfer and long-lasting gene expression.⁵

Most approaches in angiogenesis aim to induce endothelial cell proliferation. That remodeling may also be important has been demonstrated,¹ and the factors involved may be targets for gene therapy, with the precise therapeutic goals yet to be elucidated. Possible novel approaches aiming to influence remodeling might be directed at any of the arterial layers, including the adventitia.¹⁴ If cell proliferation is proven to play a pivotal role in angiogenesis and remodeling, it is still unclear to what extent, and particularly for how long, it is preferable to induce cell replication without inducing further stenoses or benign or malignant tumors. This must be clarified in future studies using appropriate models.

The type of gene transfer that generally raises safety and ethical concerns is that which involves manipulation of the germ cell DNA, resulting in long-term effects and

affecting future generations. Germ cell gene transfer is at present restricted to producing transgenic plants or animals for economic and research purposes. In contrast, gene therapy as discussed in this article involves transfer of the gene only into specific differentiated cells. This somatic gene therapy will, at best, last as long as the treated cells survive, provided gene integration takes place. Gene therapy strategies are currently aimed at combining maximum efficacy with optimum safety, including the control of duration of therapeutic effects. Results from preclinical and clinical investigations in the field of angiogenesis suggest that transient gene expression for a minimum of several weeks and a maximum of several months may be sufficient for effective therapy. Use of nonimmunogenic vectors allows multiple applications at different time points and sites if needed, and may therefore be preferred.

HOW CAN WE STOP THE EFFECT OF THERAPEUTIC ANGIOGENESIS?

There are a number of antiangiogenic agents known to inhibit angiogenesis.²⁰ They comprise chemicals such as thalidomide or proteins that are natural inhibitors of angiogenesis.²¹ Such inhibitors of angiogenesis are predominantly investigated by oncologists who aim at inhibiting tumor angiogenesis. They may be applied directly or using microencapsulated producer cells.^{22,23} However, to what extent they may be used to halt angiogenic therapy has yet to be ascertained, and they remain an important avenue for further research.

Current lines of investigation in gene therapy include the development of more controllable promoters, as it has been recently shown that repeti-



tive angiogenic stimulation is needed to induce a stable new capillary network in animal models. Promoters to direct transcription of introduced genes can be permanently switched on or be inducible. By creating inducible promoters that allow gene transcription to be turned on within a limited time frame, there is more control over the synthesis of the therapeutic protein. Induction of the promoter can be through naturally occurring stimuli, such as hypoxia in the case of angiogenesis, or by the systemic administration of certain drugs (eg, dexamethasone, tetracycline), which may be administered repeatedly. More locally directed induction is possible by the use of radiation-induced promoters.^{24,25}

Therapeutic genes can be delivered by specific cells producing viral vectors called packaging cells. For slow release and protection against immunological reactions, such cells may be microencapsulated before implantation at the site of therapy. Packaging cells administered in vivo may contain a suicide gene to induce cell death any time desired.¹⁷ The herpes simplex virus thymidine kinase gene is a suicide gene that encodes for an enzyme that allows phosphorylation and thus incorporation of nucleoside analogues into

newly synthesized DNA, blocking DNA polymerase and premature DNA chain termination. When nucleoside analogues such as the drug ganciclovir are introduced intravenously, cell suicide occurs local to the gene therapy. Death of the packaging cells results in halting the effect of viral gene transfer.

CONCLUSION

Various aspects of protein or gene therapy are currently under investigation for clinical use to solve the problem of chronic ischemia through the creation of intact and robust new vessels by angiogenesis. Gene therapy may provide more regulation over the duration of therapy through several mechanisms, including via the selection of certain vectors, inducible promoters, encapsulation methods, local gene delivery catheters, or the use of ex vivo genetically modified cells or expanded progenitor cells. Therapy can be modified to take place over only few days or up to the life span of treated cells, and it may be halted using a number of antiangiogenic chemicals or proteins.

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