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Simultaneous surgical revascularization and angiogenic gene therapy in diffuse coronary artery disease

H. Huwer[a,*], C. Welter[b], C. Özbek[c], M. Seifert[b], U. Straub[a], P. Greilach[a], G. Kalweit[a], H. Isringhaus[a]

[a]*Department of Cardiothoracic Surgery, Völklingen Heart Centre, Richardstrasse D-66333, Volklingen/Saar, Germany*
[b]*Institute of Human Genetics, University of Saarland, Homburg/Saar, Germany*
[c]*Department of Cardiology, Völklingen Heart Centre, Richardstrasse D-66333, Volklingen/Saar, Germany*

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Abstract

Objective: The cytokine vascular endothelial growth factor (VEGF) is capable of triggering angiogenesis and at higher concentrations vasculogenesis. We report on a pilot study where VEGF-DNA as an additional therapy to coronary artery bypass grafting was injected into the myocardium in 24 patients (pts) with proximal coronary artery stenosis and diffuse peripheral disease. One region of the myocardium with proven ischemia remained unsupplied after surgery because the respective epicardial coronary artery was not graftable. **Methods and results**: Plasmid DNA encoding for the 165- and 167-amino acid isoform of the human VEGF genes was injected directly into the myocardium, not amenable to surgical revascularization at a dosage of 1000 μg each, using a standardized protocol. A ^{99m}Tc-sestamibi-SPECT at rest performed 7 days prior to the operation, had shown decreased marker activity in the region of interest. Controls were made 1 week and 80–100 days postoperatively. Transmural scaring was ruled out intraoperatively. Coronary and left ventricular angiographies were performed preoperatively and 3 months postsurgery, respectively. One or more of the following angiographic items were found in 16/24 patients postoperatively. (1) Improvement of regional left ventricular function at the VEGF treated myocardial sector (5/24 pts). (2) Newly visible vessels considered as collaterals (8/24 pts). (3) Earlier filling of parent vessels (6/24 pts). (4) An increase in diameter of preoperatively existing collateral vessels (7/24). An increased perfusion at rest in the region of gene application was detected in 3/24 patients by early postoperative ^{99m}Tc-sestamibi-SPECT investigation. In six additional cases, local perfusion increased markedly until the late examination. No perioperative myocardial infarctions and no signs of inflammation were observed. Newly developed abnormal vasculature was not detected in any patient. **Conclusions**: Direct intramyocardial administration of $VEGF_{165}$-DNA and $VEGF_{167}$-DNA may result occasionally in an enhancement of collateral vascularization in regions with diffuse peripheral coronary artery disease not surgically amenable. During midterm follow-up no adverse effects of VEGF-DNA application are observed so far. The very slight midterm improvements caused us to stop further VEGF-DNA application and, in our opinion, do not justify a prospective, and randomized study with a control group.

Keywords: Coronary disease; Bypass grafting; Gene therapy; Angiogenesis; Collateral revascularization

1. Introduction

The treatment of symptomatic coronary artery disease is based on three principles: medical treatment, percutaneous coronary interventions (PCI), and surgical revascularization. However, in an increasing number of patients presenting with severe angina pectoris refractory to medical therapy and unsuitable for surgical revascularization, PCI, or heart transplantation, additional therapeutic options are required.

Transmyocardial laser revascularization is used in such situations, however, the mechanisms leading to clinical improvement are yet poorly understood and long-term benefit is not proven.

A very recent approach is induction of new vessel formation and thus enhanced blood flow to ischemic areas. In recently published studies, it has been demonstrated that exogenously administered angiogenic growth factors can promote new vessel formation [1–3]. Vascular endothelial growth factor (VEGF) was identified as the most potent angiogenic cytokine and endothelial cell-specific mitogen [4,5]. Three distinct genes denominated VEGF (or VEGF-A), VEGF-B, and VEGF-C are known. The most ubiqui-

* Corresponding author. Tel.: +49-6898-12-2473; fax: +49-6898-12-2258.
E-mail address: huwer.vk@shg-kliniken.de (H. Huwer).

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tously expressed isoform is the 165-amino acid splice-variant of VEGF-A. This molecule was detected as both soluble and matrix-associated [6]. VEGF-B plays a role in angiogenesis and endothelial cell growth particularly in the heart and in the skeletal muscle [7,8]. VEGF differs from other angiogenic factors by the presence of a signal sequence at its amino terminus, enabling a natural secretion by intact cells [9]. This unique feature initiated studies using gene transfer as therapeutic strategy. Unlike genes encoding for proteins to remain intracellular, genes encoding for a secreted protein may have biological effects even at low-efficiency transfection [10]. In addition, local intramyocardial angiogenic gene transfer represents a technique providing the induction of collateral vessel formation particularly in the area of interest [3].

This report describes the use of intramyocardial gene transfer as an adjunct to surgical revascularization in patients with proximal stenosis of the major epicardial coronary arteries and concomitant diffuse distal disease. We applied both the plasmid DNA encoding the 165-amino acid variant of human VEGF-A as it represents the most ubiquitously expressed isoform and the 167-amino acid isoform of human VEGF-B as it is most abundant in heart muscle.

2. Methods

2.1. Patients

Twenty-four patients with multivessel disease scheduled for elective coronary artery bypass surgery were included in a prospective, non-randomized study. All patients had one myocardial area supplied by a major epicardial coronary artery that was not amenable to bypass revascularization due to diffuse, distal disease. In six patients, it was the circumflex artery, in 13 patients the right coronary artery, and in five patients, the left anterior descending artery (LAD). Preoperative ^{99m}Tc-sestamibi-SPECT at rest showed decreased marker activity in the regions of interest. Transmural scaring in these areas was ruled out by intraoperative inspection. The 21 male and three female patients had a mean age of 64.8 years (47–79).

Exclusion criteria from the study were: class IV NYHA, a left ventricular ejection fraction (EF) $<30\%$, any known or previous malignancy, and diabetic retinopathy.

This clinical trial was approved by the Ethics Commission of the General Medical Council of Saarland on May 5, 1999 (No. 122/98). All patients gave written informed consent preoperatively.

2.2. Plasmid DNA

The cDNA's coding for VEGF-B_{167} and $VEGF_{165}$ were amplified by polymerase chain reaction (PCR) using Pfu-Polymerase (Stratagene). Oligonucleotides used for PCR to amplify VEGF-B_{167} and VEGF-A_{165} were: 5′-GCGAATTCATGAGCCCTCTGCTCCGC-3′ and 5′-GCTCTAGATCACCTTCGCAGCTTCCG-3′ (VEGF-B_{167}), and 5′-GCGAATTCATGAACTTTCTGCTGTCTTGGG-3′ and 5′-GCTCTAGATCTGTCGATGGTGATGGTGT-3′ (VEGF-A_{165}) containing EcoRI and XbaI sites. The fragments amplified by PCR were digested with EcoRI and XbaI. The digested fragments were ligated in EcoRI/XbaI digested pCI mammalian expression vector (Promega), yielding $pVEB_{167}$ and pVE_{165}. The reading frame and the total DNA sequence of the ligated VEGF-B and VEGF-A fragments were checked by sequence analysis. The plasmids were prepared and tested for absence of bacterial endotoxins by Bayou Biolabs (LA, USA). The purified plasmids were reconstituted in sterile saline. The DNA concentration was determined by UV absorbance and by fluorometry. Both methods gave the same concentration, confirming an absence of nucleotide contamination (Abs280/Abs260, ratio = 1.80). Ethidium bromide staining after agarose-gel electrophoresis confirmed that most of the nucleic acid was in the closed, circular supercoiled form. The stained gel showed no nicked or chromosomal DNA contamination.

2.3. Intramyocardial gene transfer

Aliquots of 1000 μg of $VEGF_{165}$ plasmid DNA (400 μl) and 1000 μg of $VEGF_{167}$ plasmid DNA (360 μl) were filled into one syringe and diluted in 2 ml sterile saline. The fluid was divided into equal parts and injected at two time points into the area of interest. The first injection was given immediately after crossclamping and the second one after completion of the distal anastomoses. After reinstitution of coronary blood flow, two epicardial countershocks of 50 J each were given to every patient.

2.4. VEGF assay

Base levels of plasma VEGF were determined 24 h preoperatively and postoperatively every week for 2 months. We used a commercially available immunoassay (Quantikine human VEGF, R&D Systems, Minneapolis, MN, USA).

2.5. Imaging studies

Patients underwent preoperative coronary angiography within the last 2 months before the operation and 80–100 days after revascularization and gene transfer. The angiograms were interpreted by three reviewers. Posttreatment global and regional left ventricular function were evaluated in comparison to preoperative function and graded as poorer (−1), unchanged (0), improved (1), and considerably improved (2). Newly visible vessels in the area of interest fulfilling the criteria to be classified as collaterals [11] were graded as absent (0), few (1), and many (2). Differences of the filling velocities of parent vessels in the area of interest were compared to the preoperative angiogram as: poorer filling (−1), no change in filling velocity (0), earlier filling (1), and prompt filling of the parent vessel (2). Differences of the diameters of preoperatively visible vessels classified

Table 1
Laboratory testing after simultaneous CABG and VEGF-gene transfer

	op. day	p. op. day 1	p. op. day 2
CPK[a] (U/l)	201 ± 43	372 ± 164	270 ± 239
MB (U/l)	22 ± 8	21 ± 12	12 ± 3
SGOT (U/l)	25 ± 17	37 ± 22	25 ± 19
SGPT (U/l)	18 ± 14	22 ± 14	24 ± 30

[a] CPK, creatine kinase; MB, creatine kinase of MB subtype; SGOT, glutamic oxalacetic transaminase; SGPT, glutamic pyruvic transminase.

as collaterals were graded as follows: diameter decreased (−1), diameter unchanged (0), diameter distinctly increased (1), and diameter considerably increased (2).

Scintigraphic studies using ^{99m}Tc-sestamibi-SPECT were performed preoperatively, 1 week postoperatively, and 80–100 days postoperatively. Investigations were performed at rest and in the case of decreased marker activity in the region of interest the patient was provided for the study. The final decision to apply VEGF-DNA was made intraoperatively regarding the visual and tactile aspect of the region of interest. Lack of scars and the finding of diffusely diseased coronary arteries led to VEGF-DNA administration.

Postoperative scintigraphic changes in myocardial perfusion at rest in the center of the region of VEGF-DNA administration were graded as follows: worsening (−1), no change (0), slight improvement (1), and marked improvement (2). The reviewers of the scintigraphies were blinded so far, as they had no information concerning the operative procedure and the angiographic results as well.

2.6. Statistical analysis

Values are given as means ± standard deviation (SD). Differences between groups were tested by Student's *t*-test with statistical significance defined as $P < 0.05$.

3. Results

3.1. Postoperative course

No deaths occurred in the study group. Postoperative low cardiac output syndrome occurred in one patient. He needed catecholamines at higher dosages and an intraaortic balloon pump for 3 days. Late angiographic study showed a good left ventricular EF and three patent bypasses. Inadequate intraoperative myocardial protection has to be suspected in this case.

By serial electrocardiographics (ECG) no Q-wave myocardial infarction was detected in any patient. There were no enzymatic signs of infarction or inflammation during serial postoperative laboratory investigations (Tables 1,2).

3.2. Coronary angiography

Controls were performed 3.0 ± 0.3 months postoperatively. At that time of follow-up all patients presented without angina and were in NYHA class I or II. Patency rate of the 19 left internal artery bypasses and 51 vein grafts (2.9 bypasses/patient) was 64/70 (91%). Posttreatment global left ventricular EF improved in 8/24 patients (Fig.2A). No pathological vasculature was seen in the VEGF-DNA treated areas. Collaterals, not present before, could be demonstrated in 8/24 patients. Earlier filling of parent vessels in the area of gene therapy was noticed in 6/24 patients and increased diameters of vessels existing preoperatively and classified as collaterals were seen in 7/24 cases (Fig. 1A–C). An improved regional contractility of VEGF-DNA treated myocardium was found in 5/24 patients (Fig. 2B).

3.3. ^{99m}Tc-sestamibi scans

An increased perfusion rate at rest in the center of the region of VEGF-DNA administration was seen in 3/24 patients at p.o. day 7. In 6/24 cases perfusion at rest increased significantly until the 80–90th postoperative day(Fig. 3A,B). Overall, an improved perfusion in the area of interest was observed in 9/24 patients (38%).

Cumulative assessment of the four angiographic features (new vessels, earlier filling, increase of diameter of collaterals, regional function) and the nuclear scanning (perfusion improvement between postoperative day 7 and 90) showed an improved perfusion in 16/24 patients (67%) (Fig. 4).

3.4. Transgene expression

The baseline plasma VEGF protein level was 24.5 ± 11.9 pg/ml. Individual peak values were obtained after 14 days in four patients, after 21 days in 13 patients, and after 28 days in seven patients. The mean value of these peaks was 61.6 ± 28.8 pg/ml ($P < 0.001$).

4. Discussion

The endogenous mechanisms leading to collateral formation are the continuous presence of ischemic metabolites

Table 2
Laboratory testing for inflammatory reactions after simultaneous CABG and VEGF-gene transfer

	p. op. day 1	p. op. day 3	p. op. day 5	p. op. day 7
Leucocyte count (10^3 μl^{-1})	11 ± 4	11 ± 5	12 ± 5	9 ± 2
CRP[a] (mg dl^{-1})	7 ± 4	18 ± 6	11 ± 5	4 ± 2

[a] CRP, C-reactive protein.

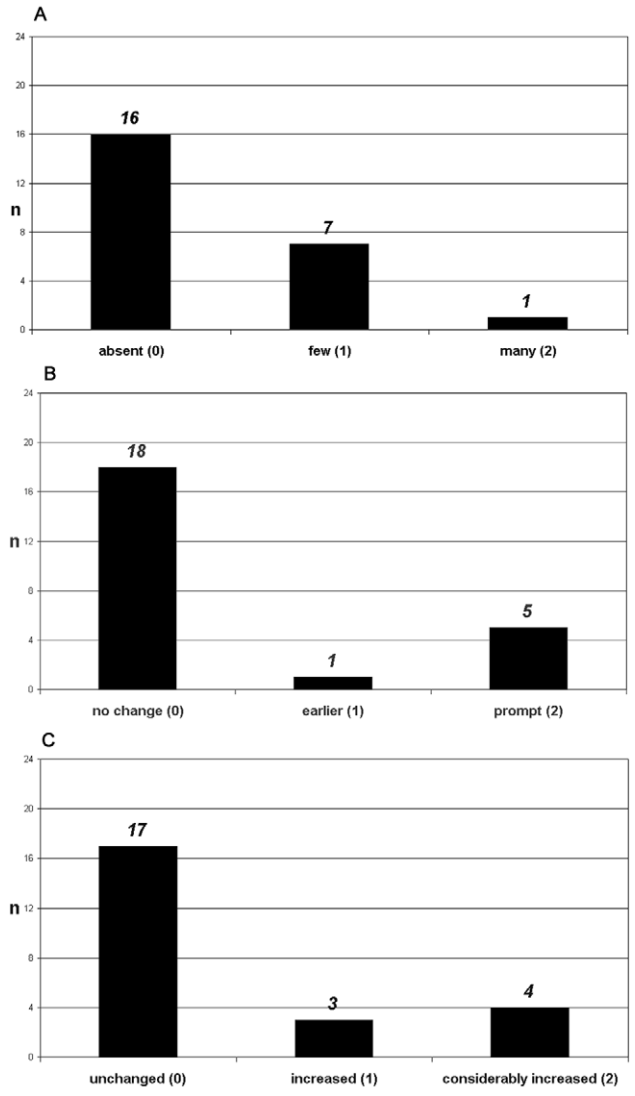

Fig. 1. Angiographic parameters of neorevascularization in the area of VEGF-DNA administration: newly visible vessels considered as collaterals after VEGF-DNA administration (A), filling of preexisting vessels after VEGF-DNA administration (B), and diameters of preoperatively existing collaterals (C).

and the activation and up-regulation of receptors that are responsive to endogenous growth factors. Exogenous growth factor delivery in ischemic tissue causes additional angiogenesis and vasculogenesis [12–14]. Based on these investigations, our pilot study directed towards the question, if short and midterm effects of the intramyocardial administration of plasmid DNA encoding the 165- and 167-amino acid isoform of human VEGF can principally occur in patients with localized distal coronary vascular disease not amenable to surgical revascularization.

4.1. Role of VEGF

In animal models of acute and chronic myocardial ischemia, local growth factor delivery significantly stimulates angiogenesis [1,15,16]. Of many angiogenetic cytokines, VEGF is the most potent and in addition, the most cell-specific endothelial mitogen [4,5]. The 165-amino acid isoform of VEGF-A was demonstrated to promote angiogenesis and neovascularization in humans with limb ischemia [17] and in ischemic myocardium [10], respectively.

VEGF-B is structurally closely related to VEGF-A and binds one of its receptors. It has a wide tissue distribution but is most abundant in heart muscle. Myocytes are the principle cells expressing and secreting VEGF-B transcripts [7]. VEGF-A and VEGF-B are coexpressed and are able to heterodimerize with each other [18].

Pioneered by Wolff et al. [19] and further developed by Isner and coworkers [10], the intramuscular injection of plasmid DNA was revealed to be associated with an expression of the transgene. No special delivery system was required to achieve these effects. As VEGF protein can be secreted by intact cells, an amplified angiogenic effect can be expected despite a low transfection rate. In our study, we administered VEGF-DNA in a standardized way and a standardized defibrillation protocol was additionally applied to avoid different transfection rates by electroporation effects.

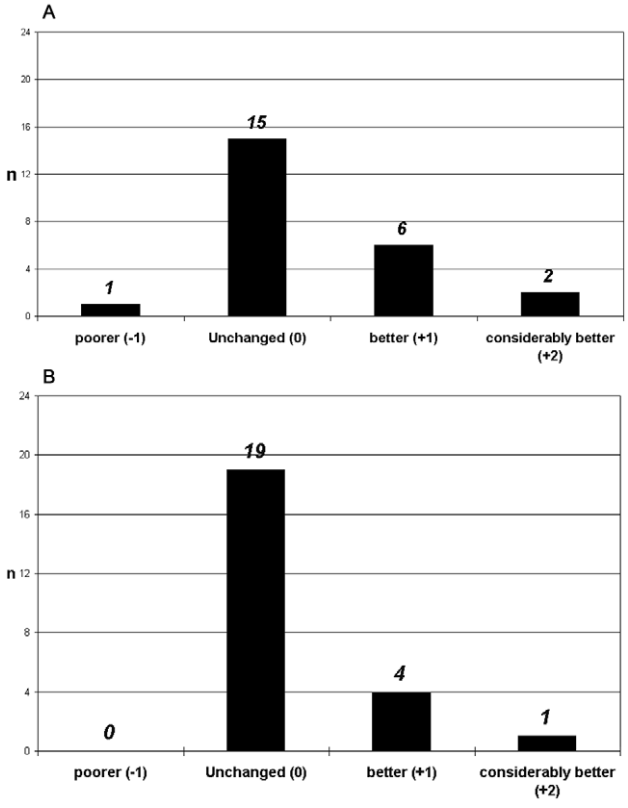

Fig. 2. LV function after VEGF-DNA administration: global LV function after VEGF-DNA (A), and regional contractility of VEGF-DNA treated myocardium (B).

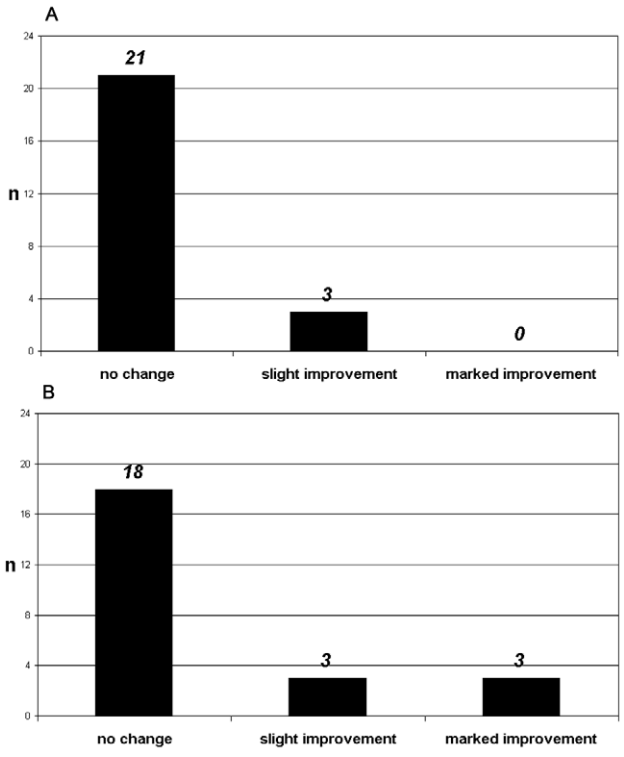

Fig. 3. Scintigraphic changes at rest in the center of the region of VEGF-DNA administration: early changes (preoperative vs. 7th day p.op.) (A), and late changes (7th day vs. 90th day p.op.) (B).

4.2. Study limitations

The effects of VEGF-DNA in ischemic myocardium are proven, but the most effective way of application is not yet defined. It is unknown whether many small depots or fewer but larger ones will be more effective. We decided to divide the volume into two equal parts and injected the aliquots at two time points centrally into the ischemic myocardium, not amenable to surgical revascularization, as we intended to observe the effects in a very circumscript region of the heart.

The postoperative slight differences in the local blood distribution and vascularization cannot unambiguously be assigned to the VEGF-DNA administration yet, as it is well known, that surgical revascularization may cause effects in remote myocardial areas as well, particularly as the most important coronary artery, the LAD, was surgically revascularized in 19/24 patients in this study.

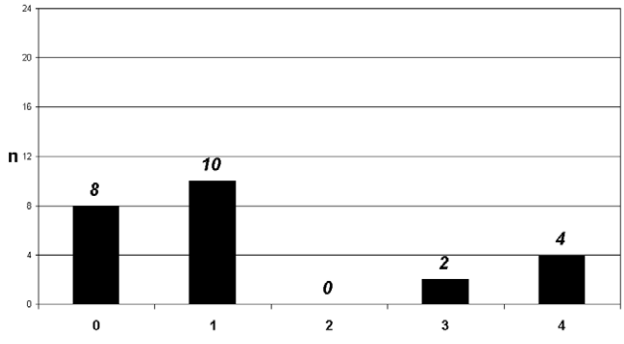

Fig. 4. Cumulative assessment of collateral revascularization by angiography and nuclear scanning. *n*, number of patients; 0–5, number of items per patient indicating improvement of perfusion in the VEGF-DNA treated area. See Figs. 1A–C, 2B, and 3B.

The dosage of plasmid DNA that will initiate collateral revascularization is still unknown. The dosage of both isoforms of VEGF-DNA (VEGF-A_{165} and VEGF-B_{167}) that we used was 1000 μg each. Compared to the dosages reported by others, this seems high [10,12,13], but the expected low transfection rates led us to the use of these dosages. From a clinical point of view, no adverse reactions took place, in particular, no perioperative myocardial infarctions and local or systemic inflammations were observed.

Although scores for quantification of angiographic and scintigraphic perfusion studies exist, this quantification remains subjective and depends on the investigator's experience.

4.3. Clinical relevance

Coronary angiography revealed an improvement of perfusion in VEGF-DNA treated areas, i.e. exhibition of newly visible vessels considered as collaterals (8/24, i.e. 33%), earlier filling of parent vessels (6/24, i.e. 25%), and increased diameter of preoperatively existing vessels considered as collaterals (7/24, i.e. 29%). An improved regional contractile function was seen in 5/24 patients (21%). At least one of these items was found in 16/24 patients (67%).

Using standard angiographic equipment, only collateral vessels with diameters of more than 80–100 μm can be visualized. Considering this aspect, the results of coronary angiography indicate vascular remodeling and collateral vasculogenesis in 29 and 33% of the VEGF-DNA treated areas, respectively. The changes were, on the other hand, not particularly impressive and it is known, that all these improvements in blood circulation can be observed in patients with mere surgical revascularisation as well.

Rest-Technetium sestamibi nuclear scanning was performed preoperatively, on postoperative day 7, and between the 80th and 100th day after combined surgical revascularization and gene transfer. As CABG can effect perfusion in remote myocardium as well true baseline to evaluate VEGF-induced enhancement of perfusion will be more likely the early postoperative nuclear scanning and not the preoperative investigation. Myocardial perfusion imaging by ^{99m}Tc-sestamibi now exhibited an improvement comparing 'true baseline' (7th postoperative day) with the results 80–100 days later in only six of the 24 patients (25%). In these six patients, no enhanced perfusion of the region of interest was seen comparing the early postoperative scanning with the preoperative investigation. This may indicate angiogenesis/collateral revascularization by VEGF-administration. In three patients, with an enhance-

ment of perfusion at early postoperative investigation, compared to the preoperative scanning, an effect of surgical revascularization leading to improved collateral circulation from remote areas to the ungrafted territory can be assumed.

The inconsistencies between real, low, and completely lacking effects may lead to the assumption that the dosages or transfection rate might be individual or inconstant, and mainly too low, respectively. In addition, the surgical revascularization procedures may influence the degree of activation of the local VEGF receptors.

Recent studies suggest that unregulated expression of VEGF is associated with formation of vascular tumors [20]. At postoperative control coronary angiography, no pathological vasculature was seen.

Biomechanical mechanisms are the usual explanation of collateral vessel growth [21], whereas VEGF is known to be endothelium-specific and to stimulate angiogenesis, i.e. capillary sprouting. In addition, molecular models of collateral vessel growth mediation must be taken into account. They are based on the interdependence of VEGF and bFGF [22], VEGF caused up-regulation of nitric oxide production [23], and VEGF induced endothelial progenitor cell mobilization [12]. Recent studies using myoblast-mediated VEGF delivery suggest that the induction of angiogenesis or vasculogenesis by VEGF may be dose-dependent. At low concentrations, angiogenesis will prevail, whereas at high concentrations, vasculogenesis dominates [20,24].

There is no doubt that VEGF is able to initiate capillary sprouting in ischemic tissue [3,25], leading to an improvement of nutritive perfusion. Angiographic techniques fail to depict the extent of this 'true angiogenesis', while radiopharmaceuticals accumulate proportionally to the regional macro- and micro-vasculature.

Cumulative assessment of the angiographic and the nuclear scanning data reveal an improved perfusion in 16/24 patients (i.e. 67%), but only in six patients this improvement can possibly be assigned to VEGF-application. In 8/24 patients, no perfusion improvement in the VEGF-DNA treated regions was detected at all. In ten patients, the effects can be mainly explained by collateral perfusion from the grafting of remote coronary arteries.

5. Conclusions

Administration of VEGF-DNA may improve perfusion in ischemic areas, not amenable to surgical revascularization, but the success rate is low.

The dosages as well as the application mode need to be further defined, and are perhaps individual.

No adverse side effects attributable to VEGF-DNA application were observed in this study. The inconsistent results of the pilot study caused us to stop VEGF-DNA application and prevented us from performing a randomized prospective study with a control group. Due to the slight effects observed, study populations of more than 60 patients per group would be necessary to have a questionable chance to obtain statistically significant results.

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