

AdVEGF₁₆₅ gene transfer increases survival in overdimensioned skin flaps

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Received: 4 May 2004
Revised: 29 July 2004
Accepted: 2 August 2004

Abstract

Background Vascular endothelial growth factor (VEGF) is a key regulator of angiogenesis. VEGF A also plays an important role in wound healing of the skin by promoting angiogenesis and by stimulating blood vessel growth. Therefore we tested the hypothesis that flap survival could be increased by the preoperative injection of AdVEGF₁₆₅.

Methods We studied the effect of AdVEGF₁₆₅ in an overdimensioned ischemic random-pattern-flap model in the rat (n = 50) with a length-to-width ratio of 4:1. VEGF cDNA was administered in two concentrations of 5×10^8 plaque-forming units (pfU) and 1×10^9 pfU using a recombinant adenoviral vector. Recombinant virus was injected subdermally 7, 3 or 0 days prior to flap harvest for the lower concentration and 7 days prior for the higher concentration. Flap survival and necrosis were observed at day 7, the day the animals were sacrificed.

Results Adenoviral gene transfer with VEGF₁₆₅ 3 and 7 days before flap harvest showed a significantly increased flap survival of 50% together with a significantly reduced necrosis ($p < 0.01$). Injection using a titer of 1×10^9 pfU 7 days prior to surgery increased flap survival even more, though failing to reach statistical significance compared to the lower concentration. VEGF protein concentration in the injected skin was significantly higher than in controls ($p < 0.01$). Flap perfusion was increased as well, demonstrated by indocyanine green (ICG) fluoroscopy ($p < 0.001$).

Conclusions Our results confirm the important role of VEGF₁₆₅ on angiogenesis in ischemic flaps. Indeed by injecting VEGF₁₆₅ at 3 to 7 days preoperatively in a concentration of 1×10^9 pfU our data show that length-to-width ratio for random-pattern-flaps could be increased from 2:1 to 3:1 and therefore may allow a wider range of applications of this simple flap technique. Copyright © 2004 John Wiley & Sons, Ltd.

Keywords VEGF; gene therapy; skin flap; angiogenesis; adenovirus; gene transfer

Introduction

Adenoviral gene transfer with vascular endothelial growth factor (VEGF) is a promising tool to stimulate angiogenesis [1,2]. The following members of the VEGF family have been cloned and characterized so far: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factors, but VEGF-A seems to be the key regulator of blood vessel growth. There are four different isoforms (VEGF₁₂₁, VEGF₁₆₅, VEGF₁₈₉ and VEGF₂₀₆) having 121, 165,

189 and 206 amino acids, of which VEGF₁₆₅ is supposed to have optimal characteristics of bioavailability and biological potency [1]. VEGF₁₆₅ binds to tyrosine kinase receptors Flt-1, KDR, and Flt-4. VEGF has the important ability to induce growth of new blood vessels in ischemic tissues in addition to having cytoprotective effects on the endothelium. Growth of new blood vessels is due to stimulation of receptor tyrosine kinase that transmits signals for endothelial cell migration and proliferation, followed by endothelial tube formation and maturation of new blood vessels. VEGF₁₆₅ plays important roles in embryogenesis, angiogenesis and blood vessel formation in normal physiological (wound healing, menstrual cycle, placenta) and pathological (tumor neovascularization, retinopathy) conditions.

AdVEGF₁₆₅ has already been used up to phase II clinical trials for the treatment of ischemic heart muscle and peripheral arterial disease [2–8]. VEGF-induced angiogenesis could play a role in various clinical applications in plastic and reconstructive surgery [9], of which we consider the pre-treatment of local flaps to increase flap size to be the most appropriate at this time.

Random-pattern-flaps are commonly used in plastic surgery to cover skin defects. The disadvantage of this easy performed technique still remains the limited and long-term established [10,11] length-to-width ratio of 2:1 that this local tissue rearrangement can tolerate without jeopardizing blood supply and risking ischemia and necrosis. Thus, in many cases, the reconstructing surgeon would have to switch to alternative procedures such as free tissue transfers that will create additional donor-site defects in other parts of the body and are more time-consuming, costly and put a greater strain on the patient. A larger length-to-width ratio of random-pattern-flaps would implicate a far wider range of clinical indications for this simple technique.

Hence methods to improve local perfusion and promote angiogenesis have been studied for years. From the different proteins involved in the process of angiogenesis, VEGF has crystallized to be amongst the most promising.

First studies in plastic surgery examined local administration of VEGF in ischemic axial pattern muscle or skin flaps under experimental conditions [12–18]. These studies showed a higher survival rate with intramuscular injection in a transverse rectus abdominis flap (TRAM-Flap) model [12] and in other musculocutaneous flaps [16].

Encouraged by these positive results various techniques for gene transfer were used to enhance the biological effect of increased flap survival through a further increased VEGF production. Techniques included the local administration of cDNA encoding VEGF [9,19,20], using liposomes as a vector [21,22], and finally the adenovirus-mediated gene transfer of VEGF [9,23–26]. The latter appears to be currently the most promising approach since high transgenic expression can be achieved. *In vivo* adenoviral gene transfer of VEGF in retroperitoneal adipose tissue of the rat showed increased VEGF expression after 48 h with peak expression being more

than five times higher 5 days after transfection [25]. Consequently, the process of angiogenesis initiated by an increased production of VEGF requires even more time to result in an increased tissue perfusion. Despite this, Gurunluoglu and co-workers [24] could not find a statistical difference in the amount of reduced flap necrosis when adenoviral gene transfer with VEGF was performed at 12 h, 3, 7 or 14 days prior to flap harvest. However, their experimental model of an axial-pattern-flap on the anterior abdominal wall of the rat showed an average flap necrosis of 30 (21–36) % in controls. Overdimensioned random-pattern-flap models, where the clinically long-term established rule of a biologically possible length-to-width ratio of 2:1 is exceeded, seem to be more appropriate because they reliably cause ischemia in the clearly defined overdimensioned flap areas [20,23].

In this study we therefore investigated the effect of locally administered adenoviral-mediated gene transfer of VEGF₁₆₅ in an overdimensioned random-pattern-flap model with a length-to-width ratio of 4:1 where the distal half should necrotize due to insufficient perfusion. From a surgical point of view an intraoperative application would be most desirable but angiogenesis and improved perfusion as a consequence will need time to develop. Most previous studies demonstrated increased flap survival, but failed to show evidence of increased VEGF expression, increased flap perfusion or improved angiogenesis [9,24,25]. We show that the delivery of AdVEGF₁₆₅ to the preoperative flap site 3 or 7 days before surgery indeed leads to increased VEGF concentration in the skin, increased perfusion of the injected area, and an increased flap survival size together with a reduced necrosis.

Material and methods

Study design

Fifty male Sprague-Dawley rats with an average weight of 350 g were used for this study. Animals were caged individually and standard laboratory food for rats and water was provided *ad libitum*. The procedures involving animal treatment and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies (EEC council directive 86/609, OJ L 358, 1, Dec. 12, 1987; NIH guide for the care and use of laboratory animals, NIH publication no. 85-23, 1985).

All surgical procedures were performed under standard aseptic conditions. In all experiments anesthesia was induced and maintained by spontaneous inhalation of isofluran and oxygen.

Prior to injection of vectors the abdominal skin was shaved under anesthesia from sternum to the pubic region. 200 µl 0.9% NaCl alone (group 1; controls) or with 5×10^8 pfU Ad3/12 control vector (group 2), or 5×10^8 pfU AdVEGF₁₆₅ (groups 2–5) were injected subcutaneously in four aliquots into the distal half of



Figure 1. Cranial pedicled random-pattern-flap on the anterior abdominal wall with a length-to-width ratio of 4:1. Four injection sites are marked in the distal half of the flap

Table 1. Control and experimental groups each with 200 μ l (NaCl, Ad3/12 or AdVEGF₁₆₅) fluid injected in the distal half of the flap in four equal portions

Group	n	Treatment	Transfection time
1	6	0.9% NaCl, 200 μ l	7 days
2	6	Ad3/12 (5×10^8 pfU), 200 μ l	7 days
3	6	AdVEGF ₁₆₅ (5×10^8 pfU), 200 μ l	0
4	6	AdVEGF ₁₆₅ (5×10^8 pfU), 200 μ l	3 days
5	6	AdVEGF ₁₆₅ (5×10^8 pfU), 200 μ l	7 days
6	6	AdVEGF ₁₆₅ (1×10^9 pfU), 200 μ l	7 days
E1	7	0.9% NaCl, 200 μ l	7 days, no surgery
E2	7	AdVEGF ₁₆₅ (1×10^9 pfU), 200 μ l	7 days, no surgery

the future flap (Figure 1). Group 6 received a doubled virus concentration (1×10^9 pfU, Table 1). Surgery was performed just before subcutaneous injection (group 3), 3 days (group 4) or 7 days later (groups 1, 2, 5, 6), under the same conditions, with transfection time being 0, 3 and 7 days, respectively.

Additional animals (group E1, vehicle only; and E2, 5×10^8 pfU AdVEGF₁₆₅) were used to assess VEGF expression and histology 7 days after injection.

Surgical technique

A quadrangular flap with a cranial pedicle was outlined on the left abdominal wall. The median line ranging from the xiphoid process to the pubic determined the medial margin. The cranial margin was cranial to the lower chest border; the skin was not divided here. The flap was outlined with a width of 2 cm and a length of 8 cm with its caudal margin being in the inguinal region. After

depilation of the anterior abdominal wall and marking of the flap design, the flap was harvested in the subcutaneous layer between skin and muscle fascia until its cranial border overlying the lower ribs. All perforating vessels reaching the skin from the inferior and superior epigastric artery were carefully dissected. After haemostasis was obtained the flap was repositioned and sutured back into place with interrupted sutures.

Gene transfer

Recombinant E1-E3-deleted adenoviral vectors encoding VEGF₁₆₅ (AdVEGF) under the CMV promoter were propagated in 293 cells. Adenoviruses were concentrated and purified by caesium chloride gradient. Standard plaque assays on 293 cells were used to determine the vector titers. A polymerase chain reaction (PCR) was conducted to ensure the deletion of E1A in the AdVEGF genome. A positive control for E2A was carried out as well [27,28].

VEGF₁₆₅ ELISA

Normal rat epithelial cells from the small intestine (cell line IEC-6) were transfected with 50 pfU AdVEGF per 3×10^5 cells. Cell culture supernates were collected every 24 h post-transfection for the ensuing 10 days and stored at -80°C . Then a quantitative sandwich enzyme immunoassay (Quantikine[®] Human VEGF Immunoassay, R&D Systems Minneapolis, USA) was performed for the quantitative determination of VEGF₁₆₅.

Cell culture supernates were thawed, particulars were removed by centrifugation, and the samples were diluted 1:500 in a three-step dilution series and added to the kit. Upon completion of the assay procedure standard and samples were read at 450 nm and 550 nm λ correction.

Indocyanine green fluoroscopy

Indocyanine green (ICG, Pulsion Medical Systems AG, Munich, Germany) is a tricarboyanine dye that binds almost completely to globulins and is exclusively distributed in the intravascular space. Indocyanine green has a normal plasma half-life of 3–4 min, is not metabolized in the body and is eliminated exclusively through the liver and excreted into the bile. ICG fluorescence was induced and recorded using a laser-fluorescence imaging device (IC-View[®], Pulsion Medical Systems AG, Munich, Germany), comprising a NIR laser light source (0.16 W, wavelength λ 780 nm) and a NIR-filtered digital camcorder. With the resutured flap in position a bolus of 0.3 mg/kg bodyweight was injected into a tail vein [25]. The fluorescence was recorded with a digital camera and the grey scale images transferred to an image analyzing system (IC-Calc[®], Pulsion Medical Systems AG, Munich, Germany). The distal half of the flap was evaluated as a region of interest (ROI).

Perfusion maximum and perfusion index (increase of grey value/time) were recorded in relation to the surrounding skin with unchanged blood flow. A standard sponge with ICG-bound globulins provided exclusion of artefacts due to automatical adaptation of the camera's shutter. ICG fluoroscopy was performed immediately postoperative.

Measurement of flap size

Digital images were taken perpendicular to the flap together with a scale during flap elevation, postoperatively and 7 days post-surgery. Pictures were analyzed with NIH Image software (National Institutes of Health, USA).

Tissue extraction and radioimmunoassay (RIA) for VEGF (groups E1, E2)

With 7 days of transfection time only the distal half of the future flap was raised, resected and immediately shock-frozen in liquid N₂. VEGF expression was measured separately in the skin and in the rectus abdominis muscle.

The tissue (100 mg wet weight) was transferred into 10 volumes of ice-cold phosphate-buffered saline (PBS) containing one complete mini-tablet of bovine serum albumin (BSA, Boehringer, Mannheim, Germany). This tablet contained both reversible and irreversible protease inhibitors, and inhibited a broad spectrum of serine, cysteine and metalloproteases.

The tissue was then homogenized in a Fast Prep[®] FP 120 cell disrupter (Qbiogene, Carlsbad, CA, USA) for 40 s (6 m/s) and allowed to settle down for 30 min at 4 °C. The homogenate was subsequently centrifuged for 15 min at 10 000 rpm at 4 °C. The supernatant was separated in aliquots and kept frozen until analysis.

Concentrations of VEGF in homogenate supernatant were measured in 200 µl by RIA using a rabbit antiserum raised against recombinant bovine VEGF₁₆₄ (prepared in our laboratory [29]). The antibody cross-reacts with all four human isoforms of VEGF (VEGF_{121,165,189,206}). The cross-reactivities to the other platelet-derived growth factors (PDGF)-AA, PDGF-BB, PDGF-AB, FGF1, FGF2 and transforming growth factor were below 0.1%. Labelled VEGF was separated from free iodine with a prepacked disposable NAP-1 column containing Sephadex G-25 medium (Amersham-Pharmacia, Freiburg, Germany). Glycerine was added to fractions with labelled VEGF up to 50% and they were stored at -20 °C until use in RIA. The tracer is stable for 3–4 months. The incubation buffer for RIA was 3 M NaCl containing 1% BSA, 0.1% Triton X-100, pH 7.5. The antiserum was used at a final dilution of 1/400 000. Separation of bound and free VEGF was completed using the double antibody technique and 6% polyethylene glycol 6000 (Serva, Heidelberg, Germany). The intraassay and interassay variations are below 6 and 14%, respectively. The ED₅₀ of the assay was 0.6 ng/ml.

Histology (groups E1, E2)

For histological analysis, in these animals the complete anterior abdominal wall (skin, subcutaneous and muscular layer) was resected just proximal, fixed in formalin and embedded in paraffin. The tissue was cut into 5 µm sections and stained with H&E and Elastica van Gieson. Furthermore, both group 1 and group 5 were evaluated 7 days after flap harvest when animals were sacrificed.

Flap microvessel density was assessed in additional animals treated like those in groups 1 and 5 seven days after injection using a monoclonal CD31 antibody (Serotec, mouse anti rat, MCA 1334G). For this, the presumptive flap of the anterior body wall was harvested after sacrificing the animals. The skin was separated along the fascia from the underlying musculature. From each flap at least three skin and three muscle samples were isolated and frozen in liquid nitrogen. After cryosectioning and incubation with the antibody, hematoxylin was used for nuclear counterstaining. From each specimen three representative images with a primary magnification of ×100 were taken with an Axiophot photomicroscope (Carl Zeiss, Oberkochen, Germany) and downloaded into a morphometry and image analysis program (Diskus 4.50.20, Hilgers, Königswinter, Germany).

Microvessel density was determined with a modified 25-point Chalkley graticule [30,31], a validated, rapid and reproducible method for clinical use. The Chalkley grid was superimposed on the TFT display of the image analysis program. With the final magnification of ×345 the Chalkley grid has a diameter of 350 µm. The grid was orientated so that the maximum number of points was on or within areas of highlighted vessels.

Statistical evaluation

Statistical evaluation between groups was performed using the non-parametric Kruskal-Wallis and Mann-Whitney tests. Multiple testing was taken in account according to the method of Marcus *et al.* [32].

Results

VEGF₁₆₅ ELISA *in vitro*

VEGF *in vitro* expression measurements in rat epithelial cells (Figure 2) showed peak VEGF expression 2 days after transfection followed by a successive reduction.

Flap survival and necrosis (Figure 3)

Except for groups E1 and E2 all rats were sacrificed on the seventh postoperative day. Flap measurement immediately postoperative after harvest and repositioning of the flap revealed a constant flap size (average of

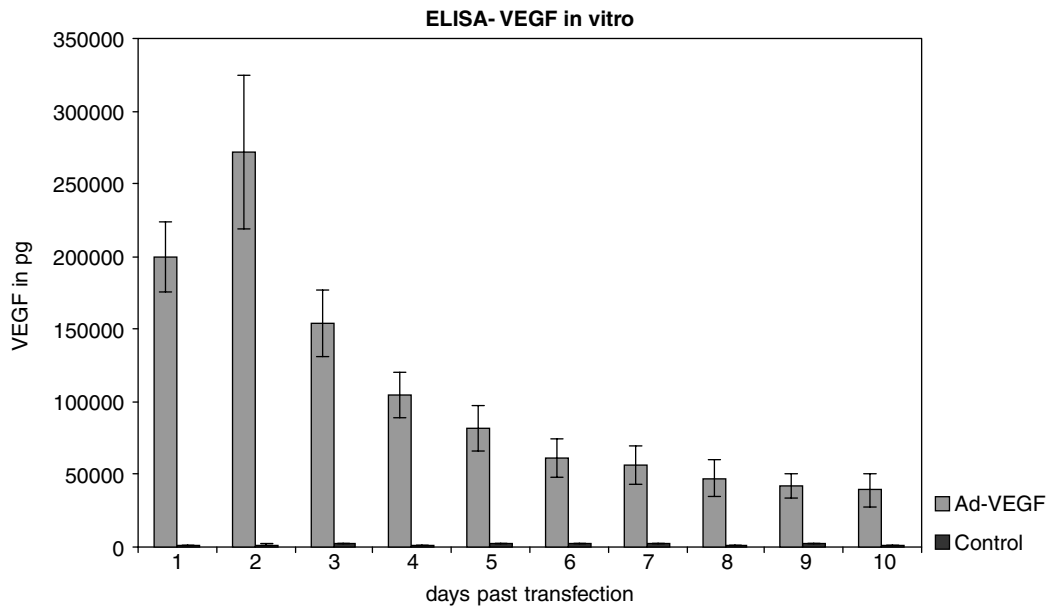


Figure 2. Elisa VEGF *in vitro* measurement. 3×10^5 cells per well ($n = 6$), supernates harvested days 1–10 post-transfection with 50 pfU Ad-VEGF per well

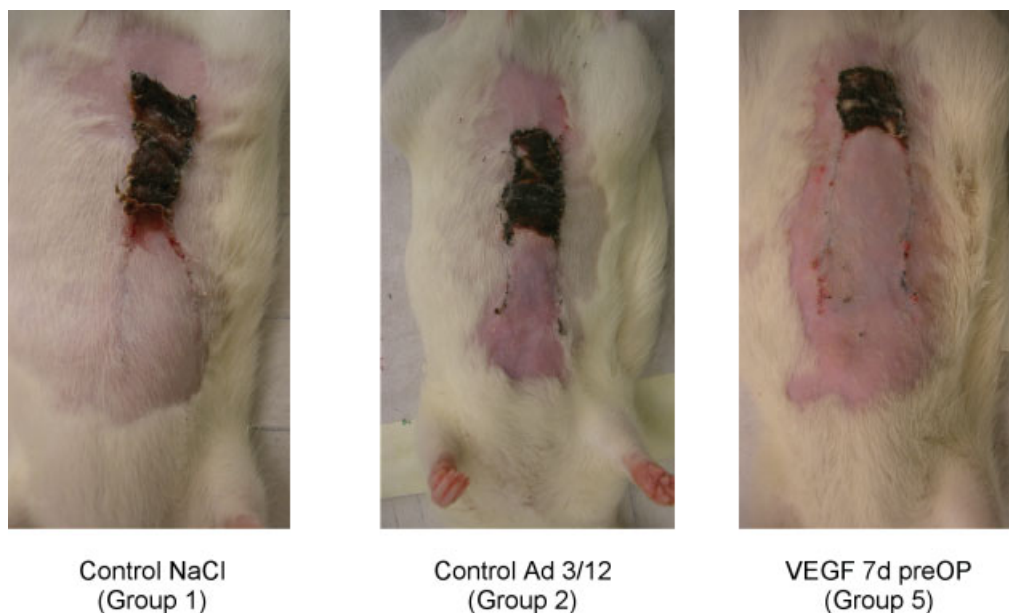


Figure 3. In both control groups (NaCl left, Ad3/12 middle) a flap with a length-to-width ratio of 2 : 1 survived. The distal half of the flaps became necrotic. In experimental groups (right: group 5: AdVEGF₁₆₅ 7 days prior to flap harvest) an average of 74% of the flap survived (length-to-width ratio 3 : 1)

14.3 cm²) with no statistical differences (Kruskal-Wallis test, $p = 0.581$).

In vehicle-only controls (group 1: 50.1%) and control-vector-only (group 2: 48.1%) approximately half of the flap area became necrotic. AdVEGF₁₆₅ administration increased the surviving flap area in a time-dependent manner to 80.1% (group 6). AdVEGF₁₆₅ application 3 (group 4: 70.1%) and 7 days (groups 5: 74.1%) before surgery resulted in statistically significant increases of viable, surviving flap area. Doubling of the dosage 7 days before surgery (group 6: 80.1%) showed a tendency towards higher values without being statistically

significant. As expected, intraoperative application of AdVEGF (group 3: 57.3%) did not result in significant differences when compared to the controls.

Sizes of the surviving and necrotic flap areas in cm² of the individual groups are shown in Figures 4 and 5. Statistically significant differences were found between the groups by Kruskal-Wallis test ($p < 0.001$). No statistical difference was found between controls ($p = 0.310$). Compared to the control groups differences were statistically significant in the Mann-Whitney U-test for groups 3, 4, 5 and 6 ($p < 0.005$). Between groups 5 and 6 no statistical significance ($p = 0.394$) was demonstrated.

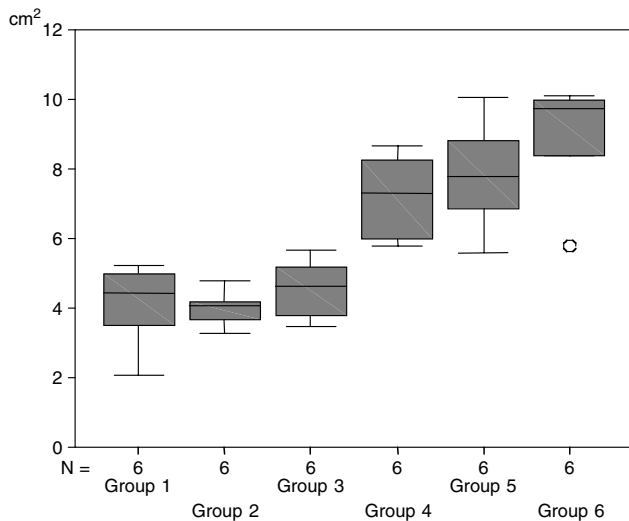


Figure 4. Box plot of surviving flap areas measured in cm^2 in the two control groups and four experimental groups (each $n = 6$). Statistically significant differences ($p < 0.005$) to controls are present for groups 4, 5 and 6 (o Outliers: cases with values between 1.5 and 3 box lengths from the upper or lower edge of the box. The box length is the interquartile range)

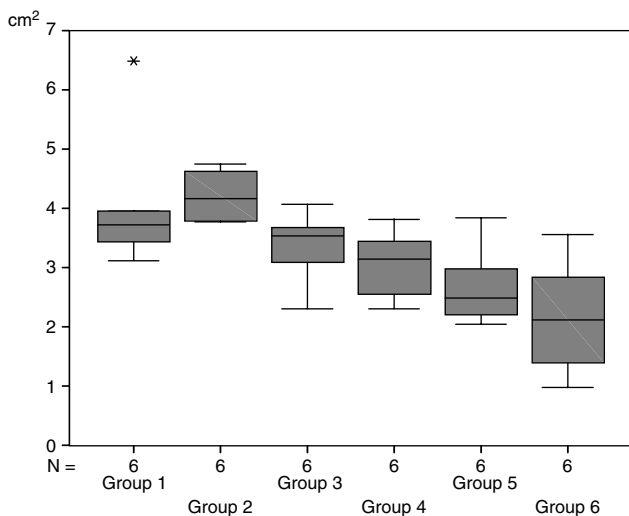


Figure 5. Box plot of necrotic flap areas measured in cm^2 in the two control groups and four experimental groups (each $n = 6$). Statistically significant differences ($p < 0.005$) to controls are present for groups 4, 5 and 6 (* Extremes: cases with values more than 3 box lengths from the upper or lower edge of the box. The box length is the interquartile range)

Flap perfusion

Flap perfusion was measured immediately postoperative by means of perfusion index (ratio of arterial inflow: time) in the distal half of the flap. In controls the mean perfusion index was 21% (group 1) and 22% (group 2). Mean flap perfusion in the experimental groups was 23, 30, 32 and 35% (groups 3–6). Differences were statistically significant in all groups (Kruskal-Wallis test, $p < 0.001$), with differences between the two control groups not being significant (Mann-Whitney U-test). Experimental

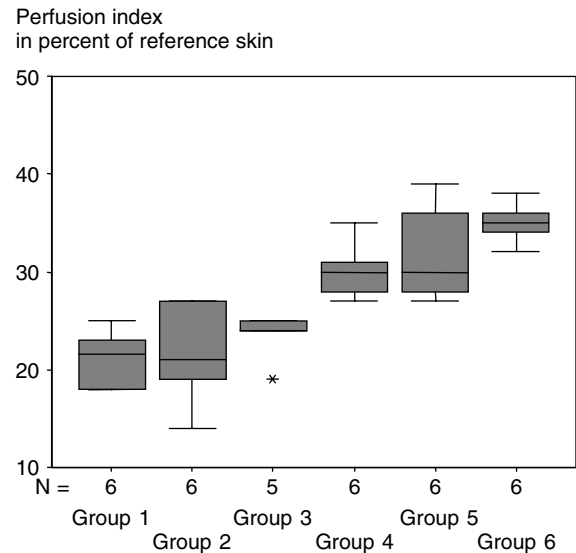


Figure 6. Box plot of indocyanine perfusion index measured in the distal part of the flap immediately postoperatively. Statistically significant differences ($p < 0.005$) to controls are again present for groups 4, 5 and 6 (* Extremes: cases with values more than 3 box lengths from the upper or lower edge of the box. The box length is the interquartile range)

groups 4–6 showed statistically significant differences ($p < 0.005$), whereas between groups 5 and 6 differences were not significant (Figure 6).

VEGF expression *in vivo*

The control group E1 showed a VEGF_{165} concentration of 0.83 (0.3–1.0) ng/g tissue in the skin. The experimental group E2 averaged with 1.38 (1.0–1.6) ng VEGF/g skin tissue (Figure 7). A Mann-Whitney U-test (2-tailed) showed statistical significance ($p = 0.002$).

In the muscle tissue, control group E1 had a VEGF_{165} expression of 1.63 (0.7–2.8) ng/g tissue. The experimental group E2 averaged with 2.24 (1.1–3.5) ng VEGF/g muscle tissue. A Mann-Whitney U-test (2-tailed) failed to show statistical significance ($p = 0.209$).

Flap histology

Histopathological analysis of flaps from group 1 (vehicle-only) and group 6 (7-day AdVEGF_{165}) 7 days after surgery revealed typical wound healing signs such as lymphocyte reaction sites and focal haematomas to comparable extents. In both groups conventional histology did not reveal qualitative changes and a vessel morphology shift towards the immature phenotype. The flaps of group E1 and E2 animals, which received the same regimen as group 1 (vehicle-only) and group 6 (7-day AdVEGF_{165}), however, without undergoing subsequent surgery, also did not show qualitative differences.

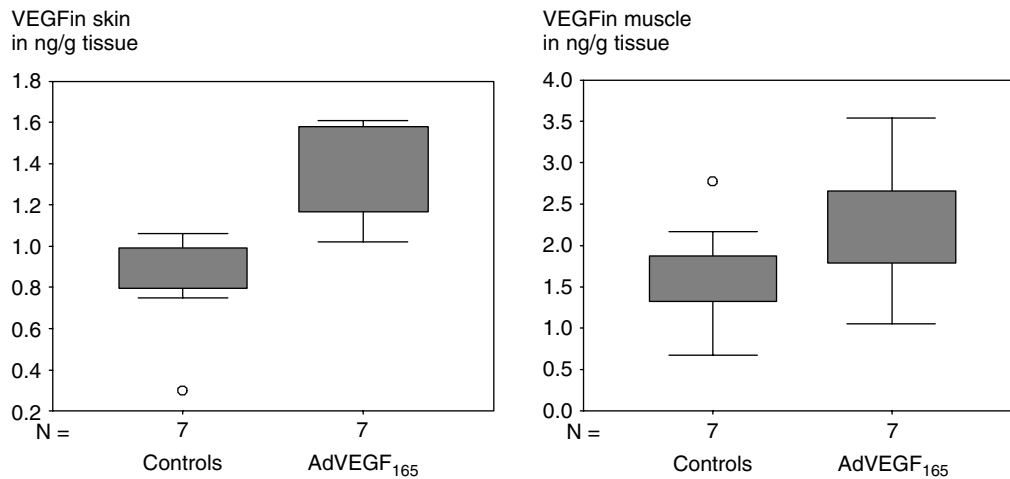


Figure 7. Box plot of VEGF concentration measured with RIA in the distal half of the flap after 7 days of transfection time in ng/g tissue. Controls (n = 7) and group 5 (n = 7) have statistical differences (Mann-Whitney U-test, $p < 0.005$) in skin (left) but not in the rectus muscle tissue (right) (o Outliers: cases with values between 1.5 and 3 box lengths from the upper or lower edge of the box. The box length is the interquartile range)

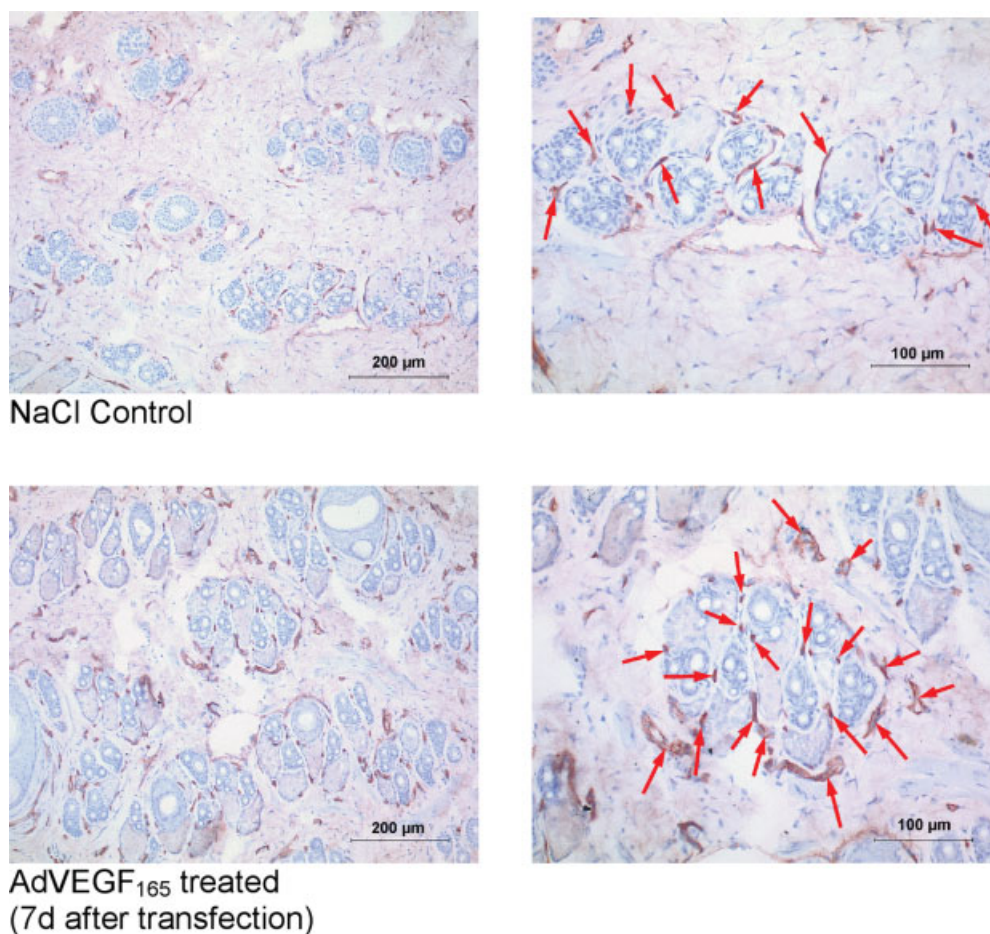


Figure 8. Histological sections (5 μm) with CD31 immunostaining through the anterior abdominal wall 7 days post-transfection (groups E1 and E2). Experimental group E2 (overview lower left) shows increased deposition of CD31 in angiogenic cells around hair follicles (marked with arrows in higher magnification, lower right) compared to controls (upper two)

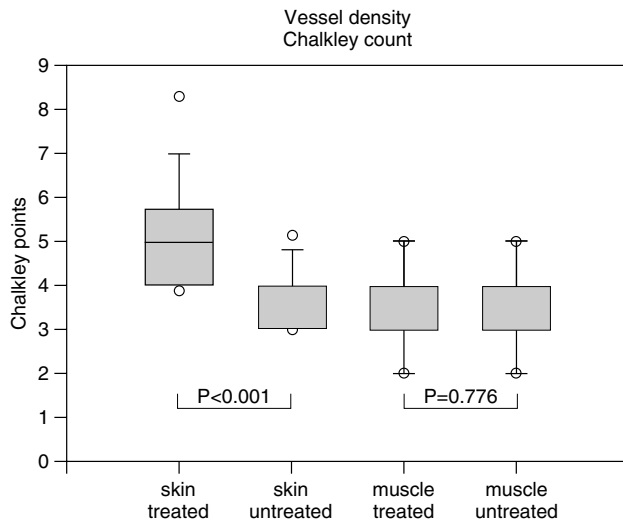


Figure 9. Box plot of vascular density counts (Chalkley count) in skin (two left bars) and rectus abdominis muscle (two right bars). Differences in skin are significant ($p < 0.001$) but not in muscle ($p = 0.776$) (o Outliers: cases with values between 1.5 and 3 box lengths from the upper or lower edge of the box. The box length is the interquartile range)

Microvessel density

Immunostaining with the pan-endothelial cell marker CD31 showed an increase of skin vessel density, especially in the perifollicular dermis areas (Figure 8). Quantitative morphometry (Figure 9) proved an increase of microvessel densities from 3.6 Chalkley points in the controls to 5.2 points 7 days after administration of AdVEGF₁₆₅. The vessel densities in the underlying musculature were not affected by the treatment, indicating the strictly local effect of the AdVEGF₁₆₅ treatment.

Discussion

Most research of angiogenesis has been done for ischemia of the human heart and the lower limbs. Research in this field has already reached stage II clinical trials [4–6]. However, in these patients, a chronic process of coronary heart disease or arteriosclerosis has to be treated. Compared to this long-term treatment, flap ischemia is an acute issue and needs short-term stimulation of angiogenesis since flap necrosis may occur before a gene transfer may be effective. Our study confirms the potential of stimulating angiogenesis by adenoviral-mediated VEGF gene transfer in acute flap ischemia when used for preconditioning of the flap.

The adenoviral vector produced VEGF concentrations with a biological effect on flap survival and was sufficient to modify the perfusion features of the flap. These findings provide evidence that recombinant AdVEGF gene transfer alone can be used to increase flap survival of random-pattern-flaps.

Our *in vitro* measurement of VEGF₁₆₅ expression confirms that adenoviral gene transfer needs approximately 48 h before reaching its peak expression. Hence intra-operative application is unlikely to be efficient due to VEGF-induced angiogenesis, since it may need further time. Although application 3 days prior to surgery showed statistical significance for increased flap survival and decreased flap necrosis, best results in terms of survival rates and flap perfusion are obtained with an application 7 days prior to surgery. However, these results contradict the findings of Gurunluoglu and co-workers [24], who also found significant decrease of flap necrosis when adenoviral VEGF gene transfer was performed within 12 h before surgery and did not find any significant differences to an application 3, 7 and 14 days prior to surgery. One possible reason for the different findings is that these authors did not report absolute values of flap survival in cm² and evaluated only the relative percentage of skin necrosis to total flap area. Our results demonstrate that skin elasticity leads to reduced flap size immediately post-operatively (average of 14.3 cm² with $8 \times 2 = 16$ cm² prior to flap harvest) and scar contracture further reduces areas subsequently. This leads us to believe that absolute values are better and more predictive parameters.

Histological evaluation in previous immunohistochemical studies had shown increased deposition of VEGF protein in flaps [19], but many investigations could not demonstrate quantitative differences as a sign of enhanced angiogenesis in light microscopy [25] and microvessel counts failed to produce evidence of angiogenesis [9]. Some researchers found a qualitatively greater amount of granulation tissue and neovascularization in their experimental group with subdermal VEGF protein application [17] and adenovirus-mediated VEGF gene transfer in random-pattern-flaps [23]. Our results provide evidence of enhanced angiogenesis 7 days after vector injection in skin tissue. Immunostaining with CD31 confirms a higher degree of stained angiogenic cells in skin, but not in the underlying muscle tissue.

However, VEGF may not only induce angiogenesis, but also have a vasodilatory effect on skin vasculature. Ashrafpour and co-workers [33] reported that VEGF₁₆₅ induced vasorelaxation and that this effect could be blocked with a specific antibody to VEGF receptor-2 or a specific VEGF receptor-2 inhibitor. Vasorelaxation might be a short-term effect of VEGF, enhancing perfusion and hence allowing better survival rates.

Despite this, our skin perfusion measurements in the distal half of the flap with indocyanine green fluorescence showed an increased perfusion in the experimental groups 4–6 to over 30%. Our previous studies in the same model had shown that a perfusion index of less than 25% in relation to the reference skin led to flap necrosis with a sensitivity and specificity of both 100%. Immediate postoperative application of AdVEGF₁₆₅ did not show increased perfusion over the critical value of 25%. An immediate short-term effect of vasorelaxation is thus improbable in our model.

Previous studies had shown an increased flap survival, but failed to prove increased VEGF expression or angiogenesis in a random-pattern-flap model. Our results show a statistically increased VEGF expression in flap skin of approximately 65% over control. Increased VEGF expression and increased vessel counts are both limited to skin; in the underlying muscle there was no statistically significant difference. Thus, the angiogenic effect induced with subdermal injection seems to remain localized and does not reach the underlying muscle. Increasing the vector concentration increased flap survival but did not reach statistical significance.

In conclusion, this study demonstrates for the first time that VEGF increased flap survival size from 50 to 75% of initial flap size in a random-pattern-flap model with a length-to-width ratio of 4:1. These results show that an increased length-to-width ratio of random-pattern-flaps of 3:1 is a realistic goal, with VEGF gene transfer being performed 3, better 7, days prior to surgery. This could have a clinical impact since indications for random-pattern-flaps may now overcome limitations due to flap size and arc of rotation. There certainly is a practical limitation in acute postoperative flap ischemia or in flaps used as emergency procedures. However, many of the flaps used in plastic surgery are secondary procedures (i.e. in diabetic ulcers or pressure sores) giving enough time to discuss and plan with the patient. A 'preconditioning' of the flap by injecting the recombinant virus 3 or 7 days prior to the operation is hence feasible. However, the exact molecular mechanism in skin and subcutaneous tissue must be further elucidated prior to consideration of a clinical trial to enhance flap survival in humans.

Acknowledgements

The statistical evaluation was performed in cooperation with Dipl. Math. Mrs. Raymonde Busch, Institute of Biomedical Statistics and Epidemiology (Head: Univ.-Prof. Dr. A. Neiß), University of Technology Munich.

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