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ORIGINAL ARTICLE

Plasmid-mediated VEGF gene transfer induces cardiomyogenesis and reduces myocardial infarct size in sheep

G Vera Janavel[1], A Crottogini[1], P Cabeza Meckert[2,3], L Cuniberti[2], A Mele[4], M Papouchado[5], N Fernández[5], A Bercovich[5], M Criscuolo[5], C Melo[5] and R Laguens[2]

[1]Department of Physiology, Favaloro University, Buenos Aires, Argentina; [2]Department of Pathology, Favaloro University, Buenos Aires, Argentina; [3]Scientific Investigation Commission (CIC), Buenos Aires Province, Argentina; [4]Institute of Cardiology and Cardiovascular Surgery of the Favaloro Foundation, Buenos Aires, Argentina and [5]Bio Sidus, Buenos Aires, Argentina

*We have recently reported that in pigs with chronic myocardial ischemia heart transfection with a plasmid encoding the 165 isoform of human vascular endothelial growth factor (p*VEGF$_{165}$*) induces an increase in the mitotic index of adult cardiomyocytes and cardiomyocyte hyperplasia. On these bases we hypothesized that* VEGF *gene transfer could also modify the evolution of experimental myocardial infarct. In adult sheep p*VEGF$_{165}$ *(3.8 mg,* n = *7) or empty plasmid (*n = *7) was injected intramyocardially 1 h after coronary artery ligation. After 15 days infarct area was 11.3*± *1.3% of the left ventricle in the VEGF group and 18.2*± *2.1% in the empty plasmid group (*P < *0.02). The mechanisms involved in infarct size reduction (assessed in additional sheep at 7 and 10 days after infarction) included an increase in early angiogenesis and arteriogenesis, a decrease in peri-infarct fibrosis, a decrease in myofibroblast proliferation, enhanced cardiomyoblast proliferation and mitosis of adult cardiomyocytes with occasional cytokinesis. Resting myocardial perfusion (^{99m}Tc-sestamibi SPECT) was higher in VEGF-treated group than in empty plasmid group 15 days after myocardial infarction. We conclude that plasmid-mediated* VEGF *gene transfer reduces myocardial infarct size by a combination of effects including neovascular proliferation, modification of fibrosis and cardiomyocyte regeneration.*
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Keywords: *myocardial infarction; VEGF; heart regeneration; cardiomyocytes*

Introduction

Despite that the prognosis of patients with coronary artery disease has improved with the incorporation of new drugs and reperfusion strategies, heart failure resulting from myocardial infarction with extensive cardiomyocyte loss has an ominous outcome. For this reason, therapeutic interventions tending to reduce infarct size are needed.

Recently, in an animal model of chronic myocardial ischemia we reported that administration of plasmid encoding recombinant human vascular endothelial growth factor (VEGF)$_{165}$ (p$VEGF_{165}$) induces a significant increase in the mitotic index[1] and hyperplasia[2] of adult cardiomyocytes. Therefore, we hypothesized that VEGF could display a beneficial effect on acute myocardial infarction (AMI) by promoting cardiomyogenesis. We thus studied in adult sheep the effect of human $VEGF_{165}$ gene transfer on the evolution and size of AMI during the first 2 weeks after acute, permanent coronary artery occlusion.

Correspondence: Dr R Laguens, Department of Pathology, Favaloro University, Solís 453 (1078) Buenos Aires, Argentina.
E-mail: rlaguens@ffavaloro.org
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Results

Vascular endothelial growth factor reduces infarct size

Determination of infarct size was carried out on 26 adult sheep with AMI achieved by ligating the left anterior descending (LAD) artery at its distal third. At 1 h after LAD occlusion, the animals were randomized to receive 3.8 mg of a plasmid encoding the human $VEGF_{165}$ gene ($pVEGF_{165}$, $n = 13$) or empty plasmid (placebo, $n = 13$), in 10 intramyocardial injections distributed along the peri-infarct area. The nature of the injectates was kept blind until the end of data processing. Twelve animals ($n = 6$ per group) were killed at 10 days and 14 ($n = 7$ per group) at 15 days.

In sheep killed at 10 days, AMI size was similar for placebo (17 ± 1.8% of the left ventricle) and VEGF groups (18.7 ± 3.7%, P = NS), but in sheep killed at 15 days it was significantly smaller for VEGF group (placebo: 18.2 ± 2.1%, VEGF: 11.3 ± 1.3%, $P < 0.02$, Figure 1). A similar result was observed for absolute values of infarct area (10 days: placebo 9.7 ± 0.9 cm^2, VEGF 11.6 ± 1.6, P = NS; 15 days: placebo 9 ± 1, VEGF 6.1 ± 0.8, $P < 0.05$).

Vascular endothelial growth factor enhances adult cardiomyocyte division

On account that cardiomyocyte mitosis is known to occur after AMI[3] and that *VEGF* gene transfer induces entrance

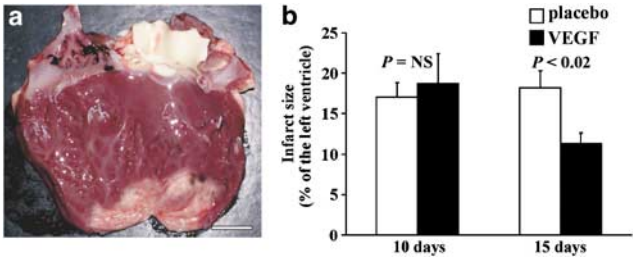

Figure 1 Infarct aspect and size. (**a**) At 10 and 15 days after coronary artery occlusion the infarct appears as a white area sharply demarcated from the surrounding myocardium. Bar: 20 mm. (**b**) Infarct size, as a percentage of left ventricular (LV) area, was smaller in *vascular endothelial growth factor (VEGF)*-treated sheep at 15 but not at 10 days after coronary artery occlusion.

in mitosis and hyperplasia of adult cardiomyocytes in chronic myocardial ischemia,[1,2] we studied the mitotic index (number of mitosis per 10^6 cardiomyocyte nuclei (CMN)) in the 10 rows of viable myocardium bordering the infarct. Although in some reports it is held that confocal microscopy with fluorescent dyes is the gold standard for identifying cardiomyocyte mitosis,[4] we employed Nomarski optics or conventional light microscopy on account that confocal microscopy does not allow to clearly visualize the nuclear envelope. This limitation can lead to confuse true mitosis with endomitosis and/or chromatin fragmentation, as has been reported and illustrated (see Figure 3 of Laguens *et al*.[1] and Figure 1 of Cabeza Meckert *et al*.[5]) in studies using both techniques in chronically ischemic pig hearts and in infarcted human hearts.[1,5] Given the thinness of the tissue sections (half the diameter of a small lymphocyte) and the fact that under Nomarski optics the cell boundaries are readily noticeable, the chance of confusing cycling superimposed cells was unlikely. Adult cardiomyocytes were identified by their size, shape, presence of cross-striations and sarcomeric α-actin.

At 10 days after LAD ligation cardiomyocyte mitotic index was significantly higher in *VEGF*-treated animals (190 ± 27 mitosis/10^6 CMN) than in placebo group (62 ± 10, $P < 0.004$, Figure 2a). Examples of cardiomyocytes in different phases of mitosis are shown in Figure 2. Furthermore, we observed occasional images of ongoing cytokinesis (Figure 2h). At 15 days, mitotic activity decreased in the *VEGF*-treated group (VEGF: 47 ± 28 mitosis/10^6 CMN, placebo: 60 ± 30, $P =$ NS).

To see whether the presence of AMI is necessary for VEGF to induce cardiomyocyte proliferation, we searched for Ki67+ CMN and mitotic figures in 12 additional sheep with no LAD ligation but injected with p$VEGF_{165}$ ($n = 6$) or empty plasmid ($n = 6$) in the same zone and with the same doses as in the protocol sheep. Also in this case the nature of the injectates was kept blind until the end of data analysis. After 10 days, neither in placebo nor in *VEGF*-treated sheep cardiomyocyte mitosis or Ki67+ nuclei were found, suggesting that myocardial ischemia is a precondition to allow for the VEGF mitogenic effect.

Vascular endothelial growth factor increases peri-infarct density of cardiomyoblasts

At 10 days after LAD occlusion, small cells (6–10 μm in diameter) with nuclei stained for the Ki67 antigen, positive for sarcomeric α-actin and connexin 43 and negative for smooth muscle actin and Ulex lectin, were present within the area between dead and surviving myocardium. On account of these features they were considered to be cardiomyoblasts.

The *MDR-1*-gene-encoded P170 glycoprotein was found to be positive in cardiomyoblasts, but also in the sarcolemma of peri-infarct adult cardiomyocytes, confirming previous observations.[6] Sca-1 and c-kit immunostainings of cardiomyoblasts were negative because the commercial kits we used did not react with ovine antigens. The same antibodies did react with murine- and human-positive controls. The density of cardiomyoblasts was twofold higher in *VEGF*-treated (1175 ± 141 cells/mm^2) than in placebo sheep (529 ± 86, $P < 0.003$). The percent cardiomyoblasts displaying mitotic figures was $12 \pm 0.8\%$ in *VEGF*-treated sheep and 9.5 ± 2.6 in placebo sheep ($P =$ NS). Occasional cytokinesis of these cells (Figure 3) was observed. At 15 days post-AMI cardiomyoblasts were no longer present in either group.

Vascular endothelial growth factor gene transfer reduces myofibroblast proliferation and fibrosis

At 10 days after AMI, in the area between the dead and surviving myocardium, myofibroblasts, identified as smooth muscle actin-positive cells, were less numerous in the VEGF group (5038 ± 513 myofibroblasts/mm^2) than in the placebo group (8513 ± 573, $P < 0.002$, Figure 4a). This decrease persisted at 15 days post-AMI (placebo 7280 ± 1431, VEGF 3312 ± 269, $P < 0.05$, Figure 4b).

Concordantly, collagen content in the area between dead and surviving myocardium, as calculated from paraffin-embedded sections stained with picrosirius red, was lower in *VEGF*-treated sheep at 10 and 15 days post-AMI (10 days: placebo $70.1 \pm 1.7\%$ of total area, VEGF $43.5 \pm 4.5\%$, $P < 0.006$; 15 days: placebo $74.3 \pm 9.8\%$, VEGF: $22.3 \pm 3.4\%$, $P < 0.02$, Figure 4c and d).

Vascular endothelial growth factor increases arteriogenesis in peri-infarct surviving myocardium

At 10 and 15 days post-AMI, small-sized arterioles were clearly visible in the tissue sections stained with an antibody against smooth muscle actin (Figure 5a and b). At 10 days post-AMI *VEGF*-treated sheep showed increased number of arterioles located within 10 rows of peri-infarct viable myocardium (placebo 18.8 ± 5 arterioles/mm^2, VEGF 119.8 ± 32.3, $P < 0.03$). After 5 days, the arteriogenic effect of VEGF was preserved (placebo 26.5 ± 7.3, VEGF 83.3 ± 2.9, $P < 0.001$).

Vascular endothelial growth factor increases peri-infarct angiogenesis

At 10 and 15 days post-AMI, the fibrosis present between dead and surviving myocardium prevented accurate determination of reparative angiogenesis. This led us to study eight additional sheep with AMI randomized to blindly receive 3.8 mg p$VEGF_{165}$ ($n = 4$) or empty plasmid ($n = 4$), in order to investigate angiogenesis at 7 days after LAD ligation. We chose this time because it is known that it is about 1 week after AMI when the inflammatory infiltrate subsides and a reparative process starts, with angiogenesis preceding myofibroblast proliferation and fibrosis.[7] Moreover, it was at this time when the product of the transfected *VEGF* gene was

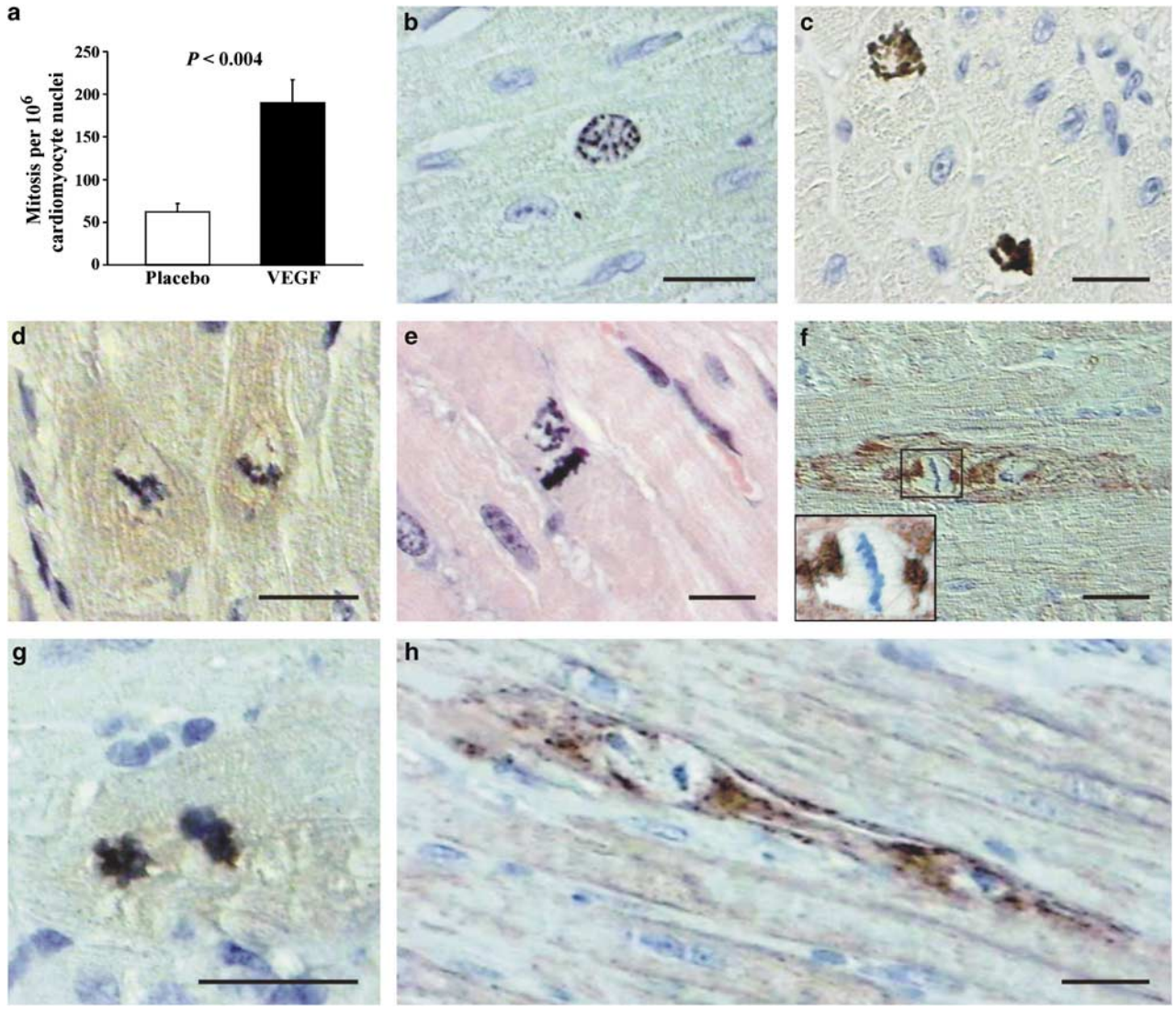

Figure 2 Adult cardiomyocyte mitosis and cytokinesis. (**a**) At 10 days after coronary artery occlusion the mitotic index was higher in *vascular endothelial growth factor (VEGF)*-treated sheep. (**b**) Late prophase. (**c–f**) Metaphase plates in different projections. Occasionally, the metaphase plates were oriented perpendicularly to each other (**e**) and the mitotic spindle, extended between sarcomeric α-actin condensations, was patent (**f**, inset). (**g**) Late anaphase. (**h**) Late telophase with ongoing cytokinesis. (**b–e** and **g**) Anti-Ki67 immunohistochemistry and hematoxylin counterstain. (**f** and **h**) Anti-sarcomeric α-actin immunohistochemistry and hematoxylin counterstain. Bars: 20 μm.

present in the myocardium, as shown by immunohistochemistry and Western blot (see below).

In the area between the remnant of coagulation necrosis tissue and viable myocardium a marked angiogenic response with formation of a network of closely packed small capillaries was observed. Since in our material search of CD34 and von Willebrand factor for identification of endothelial cells with commercial antibodies yielded erratic results, probably because these antibodies are developed to react with other mammalian species, we employed biotinilated Ulex lectin and avidin–peroxidase stain for endothelial cell identification. Ulex lectin specifically binds α-L-fucosyl residues on the endothelial cell membrane and is usually employed for specific identification of these cells.[8] Quantification after Ulex lectin staining (Figure 5c and d) showed that capillary density was significantly higher in VEGF (1805 ± 221 capillaries/mm^2) than in placebo group (703 ± 35, $P < 0.04$).

Vascular endothelial growth factor improves myocardial perfusion

In the animals killed at 15 days post-AMI ($n = 14$), resting myocardial perfusion and ventricular function were studied with gated single-photon emission computed tomography (G-SPECT) 2 days before surgery, 2 days after AMI and just before killing (Figure 6). In one *VEGF*-treated sheep the myocardial perfusion images had unacceptable quality. Therefore, VEGF group results are based on six animals. Basal, preocclusion perfusion was normal in all animals. Although after LAD ligation perfusion was equally altered in both groups (placebo: $73.5 \pm 1.8\%$, VEGF: $69.2 \pm 4.1\%$, $P =$ NS), 15 days later *VEGF*-treated sheep showed higher perfusion ($78.3 \pm 3.6\%$) than placebo animals ($67.8 \pm 3.7\%$, $P < 0.05$). The percent change in myocardial perfusion between days 2 and 15 after transfection was $16 \pm 10.8\%$ in *VEGF*-treated sheep and $-8 \pm 3.5\%$ in placebo sheep ($P < 0.05$).

In two animals from each group, the gated study failed as a consequence of frequent premature ventricular contractions. Function data from five animals per group are thus reported. In both groups wall motion scores were similar 2 days post-AMI and showed a tendency to improve 15 days later (placebo: 10.4 ± 1.6 and 7.6 ± 1.2; VEGF: 9.6 ± 2.2 and 6 ± 1.2; $P = \text{NS}$, paired comparisons). Although this tendency was more pronounced in *VEGF*-treated sheep, the differences with placebo did not reach statistical significance ($P < 0.15$, unpaired comparison).

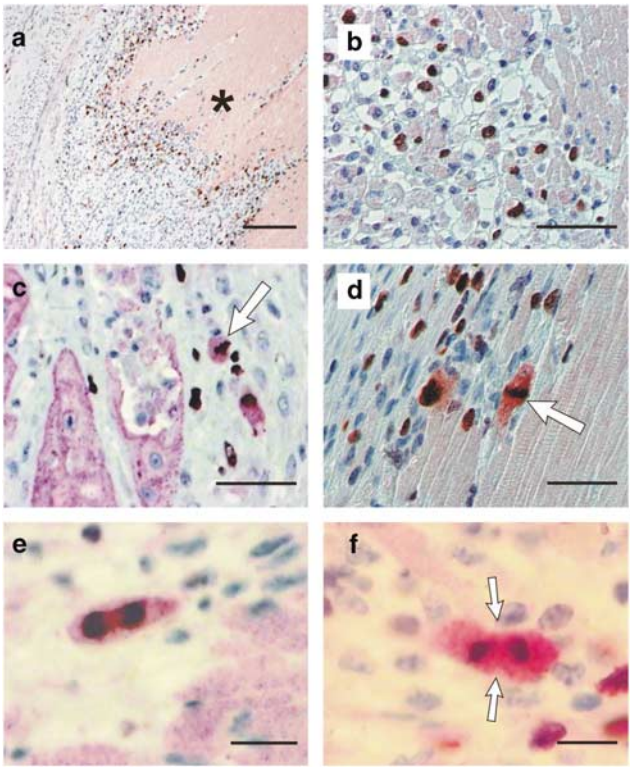

Figure 3 Cardiomyoblasts. (**a**) In the 10-day-old infarcts numerous small cells with Ki67+ nuclei were found adjacent to the dead myocardium (*). (**b**) In many of those Ki67+ cells the cytoplasm was stained with an antibody against sarcomeric α-actin. (**c** and **d**) Cardiomyoblasts in metaphase (arrows). (**e**) Anaphase. (**f**) Late telophase with symmetrical opposite invaginations of the plasma membrane (arrows), suggesting ongoing cytokinesis. (**b** and **d**) Nomarski optics. Bars: (**a**) 200 μm, (**b**) 100 μm, (**c** and **d**) 40 μm, (**e**) and (**f**) 20 μm.

Gene expression

In a group of 10 additional sheep with LAD ligation we assessed *VEGF* gene transfer and expression. In all hearts receiving p$VEGF_{165}$ a positive PCR for a specific portion of the plasmid DNA was found 3, 7, 10 and 15 days later ($n = 2$ for each time point). Reverse transcription-polymerase chain reaction (RT-PCR) with specific primers for human VEGF mRNA was positive at 3 days ($n = 2/2$) after p$VEGF_{165}$ administration, but negative at 7, 10 and 15 days postinjection as well as in two placebo animals studied 3 days after injection of empty plasmid (Figure 7a). These negative results confirm specificity of RT-PCR for human (versus endogenous) VEGF mRNA.

In the smooth muscle media layer of some intramyocardial vessels and in the cytoplasm of scattered cardiomyocytes VEGF protein was found by immunohistochemistry at 7, 10 and 15 days after p$VEGF_{165}$ injection (Figure 7b). Consistently negative reactions in control tissue samples established the specificity of the analysis. These results were confirmed by Western blot (Figure 7c). Peak VEGF mass was observed at 10 days post-transfection ($n = 2/2$), but positive reactions were also present at 7 ($n = 2/2$) and 15 ($n = 2/2$) days. Negative results were found at 3 days after p$VEGF_{165}$ injection ($n = 2$) and in placebo-treated hearts ($n = 2$). In infarcted sheep from previous safety studies using equal doses and transfection strategy, we found that Western blot analysis was positive at 35 days ($n = 2/2$) but negative at 450 days ($n = 2$) after transfection (results included in Figure 7c). Western blot for sarcomeric α-actin was employed as internal loading control.

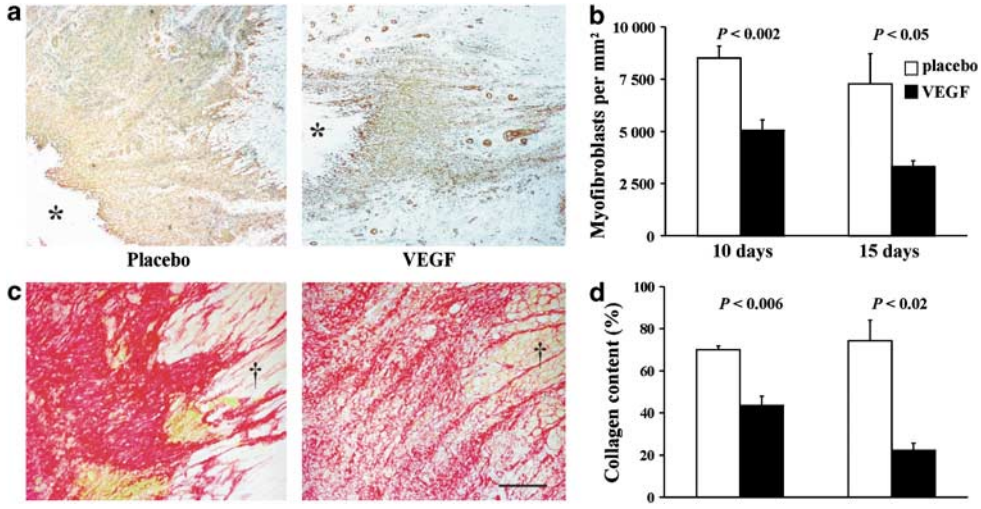

Figure 4 Myofibroblast proliferation and fibrosis. (**a**) Myocardial tissue sections stained with immunohistochemistry against smooth muscle actin in the zone between dead (*) and surviving myocardium showing decreased myofibroblast proliferation in the *vascular endothelial growth factor (VEGF)*-treated animal. (**b**) Group results for myofibroblast density at 10 and 15 days after coronary artery ligation. (**c**) Myocardial tissue sections stained with picrosirius red in the peri-infarct zone adjacent to the viable myocardium (†) showing decreased collagen content in the *VEGF*-treated animal. (**d**) Group results for collagen content as a percent of total scanned area. Bar: 100 μm.

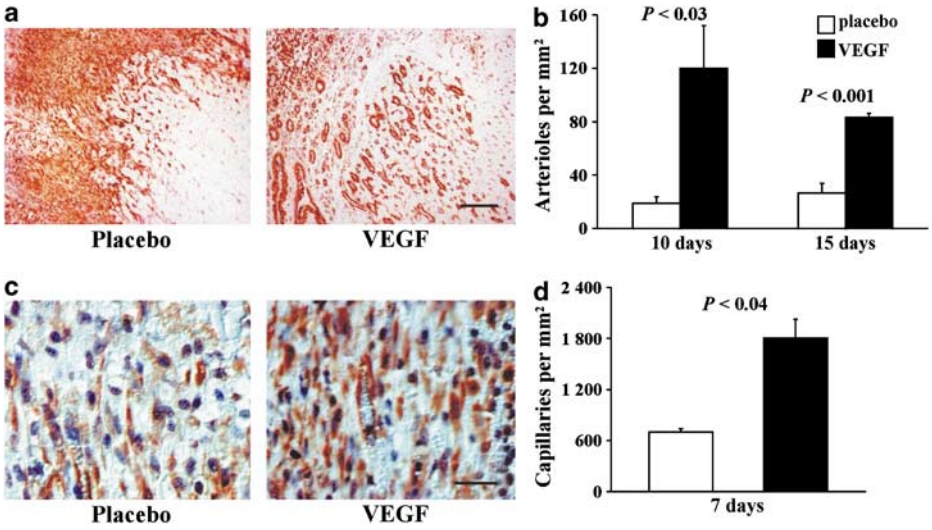

Figure 5 Neovascular proliferation. (**a**) Immunostaining against smooth muscle actin in the peri-infarct viable myocardium of a placebo- and a *vascular endothelial growth factor (VEGF)*-treated sheep. Arteriolar proliferation is enhanced after *VEGF* gene transfer. Bar: 100 μm. (**b**) Group results for arteriolar density. (**c**) Ulex lectin-stained myocardial tissue sections of the zone between dead and viable myocardium at 7 days after coronary artery ligation in a placebo- and a *VEGF*-treated animal. Closely packed capillaries are more abundant after *VEGF* gene transfer. Bar = 50 μm. (**d**) Group results for capillary density.

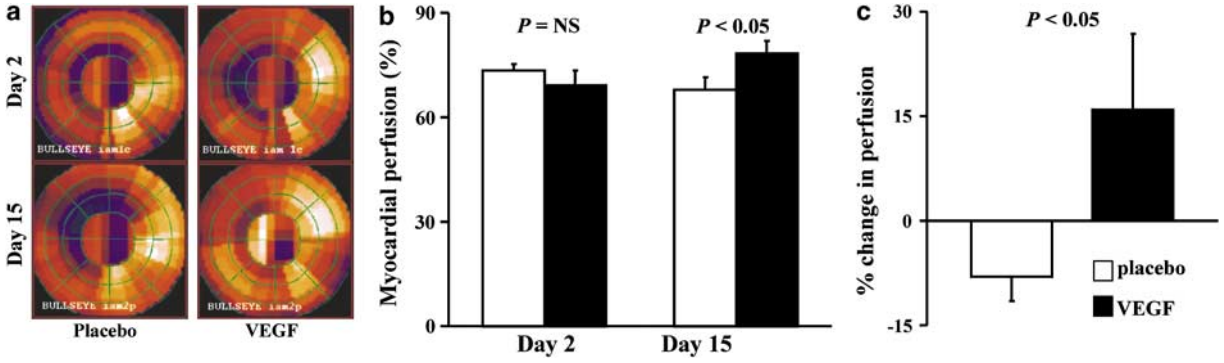

Figure 6 Left ventricular (LV) perfusion (^{99m}Tc-sestamibi SPECT). (**a**) Polar plots at 2 and 15 days after coronary artery ligation show that while in the placebo-treated animal the left anterior descending (LAD)-dependent perfusion defect increased overtime, it decreased markedly in the *vascular endothelial growth factor (VEGF)*-treated animal. (**b**) Group analysis shows that the deterioration of perfusion immediately after myocardial infarction was similar in both groups, but 15 days later *VEGF*-treated sheep had significantly higher resting perfusion than placebo animals. (**c**) The percent change between days 2 and 15 shows an improvement in *VEGF*-treated sheep and a worsening in placebo sheep, the difference being significant.

Immunoassay specificity was confirmed with a standard of human-purified VEGF.

Discussion

Infarct size

The first study on the effect of angiogenic growth factors on AMI was reported more than 10 years ago.[9] Later, VEGF protein was shown to increase myocardial blood flow in porcine hearts,[10] and growth factors were proposed as potential tools for reducing infarct size.[11] On account of the short half-life and potential harmful effect of recombinant growth factors, efforts were oriented towards gene therapy.

To our knowledge, the present study is the first to show in a large mammalian model of AMI that transfecting the heart with the human *VEGF* gene significantly reduces infarct size 15 days after LAD occlusion. Most studies on the effect of transfection of genes encoding for angiogenic growth factors on experimental AMI have been made in rodents[12–17] and only a few in large mammals. Moreover, the latter studies have used a model of chronic ischemia rather than AMI,[18] or assessed variables other than infarct size.[19]

When selecting a large mammalian model of AMI we chose the sheep because, unlike pigs, whose cardiomyocytes have up to 32 nuclei,[1,2] ovine cardiomyocytes have only 1–4 nuclei, thus being more similar to human.[20] Dogs, on the other hand, have extensive innate collaterals, which may influence the size of AMI independently from the tested intervention. This is not the case for ovine coronary circulation, where no collateral vessels would confound the determination of infarct size.[21]

Given that in our study infarct size reduction was evident at 15 days but not at 10 days after LAD ligation, the infarct-limiting effect must have occurred in only

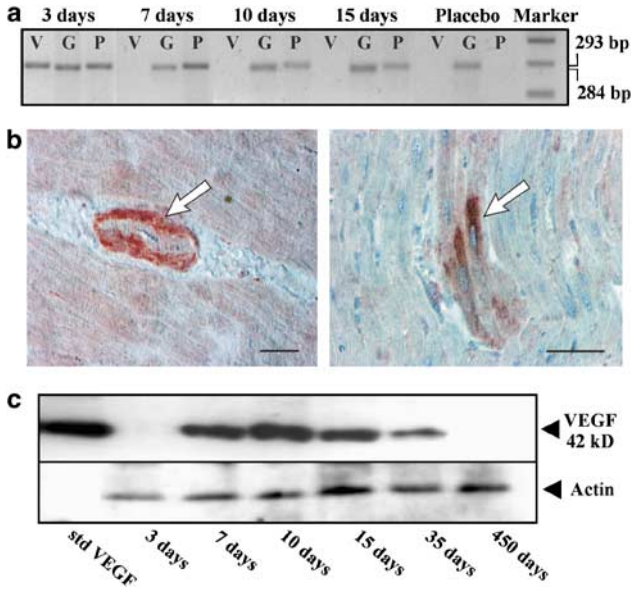

Figure 7 Time course of human vascular endothelial growth factor (VEGF) expression in ovine peri-infarct myocardium. (**a**) PCR for DNA of plasmid encoding *VEGF* (lane P) showed plasmid presence at all time points and reverse transcriptase-polymerase chain reaction (RT-PCR) for VEGF mRNA (293 bp, lane V) showed gene expression at 3 days after plasmid-mediated *VEGF* gene transfer. Polymerase chain reaction and RT-PCR were negative after 3 days of empty plasmid injection (placebo), whereas mRNA integrity and loading control was verified in all samples (RT-PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 284 bp, lane G). (**b**) Immunohistochemistry revealed location of VEGF protein (arrows) in the smooth muscle of arterioles (left) and in the cytoplasm of adult cardiomyocytes (right). Bars: 40 μm. (**c**) Western blot confirmed protein expression results at 7, 10 and 15 days after *VEGF* gene transfer in protocol sheep and at 35 and 450 days in additional sheep (see text). Loading control was assessed with sarcomeric α-actin analysis and specificity was validated with a standard of human-purified VEGF (std VEGF, 42 kDa).

5 days, and VEGF must have acted upon the mechanisms engaged in early infarct expansion, a phenomenon resulting from the interaction of cellular and extracellular post-AMI events that conduct to changes in left ventricular (LV) geometry.[22–24]

Neovascularization

At 7 days after infarction, coincident with the detection of human $VEGF_{165}$ in heart tissue, transfected sheep showed an increase in the neoangiogenesis that normally occurs in the infarct.[12] In addition, p$VEGF_{165}$ induced arteriogenesis, an effect reported in mice[14] and pigs.[25] At 10 and 15 days post-AMI, the number of arterioles in the surviving myocardium immediately adjacent to the infarct was higher in the *VEGF*-transfected group. Improved perfusion resulting from enhanced angio-arteriogenesis may have rescued cardiomyocytes that otherwise would have been lost, and this might be a likely reason for the reduction in infarct size observed later. This assumption is supported by two previous observations: VEGF is essential for cardiomyocyte survival through its effect on maintenance of the capillary bed;[26] and in humans late reperfusion after infarction may benefit remodeling and function.[27]

Fibrosis

Vascular endothelial growth factor transfection modified the pattern of the reparative process. At 10 and 15 days post-AMI, *VEGF*-treated animals exhibited decreased myofibroblast proliferation and collagen in the area between dead and viable myocardium. Although the mechanisms involved were not explored, it can be speculated that VEGF inhibited differentiation of fibroblasts into myofibroblasts and subsequent proliferation, as has been shown in rats with glomerulosclerosis and progressive renal failure, where VEGF reduced renal fibrosis.[28,29] Alternatively, increased angiogenesis occurring at 7 days postinfarction may have replaced or inhibited fibroblast proliferation, as supported by the fact that VEGF enhances experimental wound healing by promoting angiogenesis.[30,31]

Cardiomyogenesis

In addition to neovascularization and antifibrogenesis, VEGF increased the density of a population of small cells located in the area between dead and viable myocardium. Many of them were in the cell cycle, as demonstrated by Ki67 antigen expression, and showed mitotic bodies and occasional cytokinesis. Given their small size, presence of sarcomeric α-actin and connexin-43 and absence of smooth muscle and endothelial cell markers, it can be assumed that they were proliferating cardiomyoblasts. Unfortunately, the lack of commercial antibodies for stem cells markers in sheep prevented to determine if they were originated from resident progenitors[32,33] or cardiac stem cells.[34,35] In addition, the MDR-1-positive reaction cannot be considered as a proof for undifferentiated phenotype, since MDR-1 is also found in ischemic adult cardiomyocytes.[6] The presence of these cells was transient and they were not observed at 15 days post-AMI.

As has been reported for human AMI,[3] at 10 days after LAD occlusion all sheep showed mitotic activity of adult cardiomyocytes in the peri-infarct area. This phenomenon was markedly enhanced by *VEGF* gene transfer, confirming our previous results.[1] With the present data we cannot establish the mechanisms of this effect. The increased mitotic activity in adult myocytes and cardiomyoblasts can be due to VEGF-induced improved perfusion, as has been hypothesized for implantation of bone marrow stem cells[36,37] or skeletal myoblasts transfected with the *VEGF* gene,[38] or to a direct effect of VEGF.[1]

On account that growth factors may display pleiotropic actions,[39] the possibility that VEGF acted on diverse targets should be considered. In fact, in pigs with chronic myocardial ischemia, *VEGF* gene transfer induces not only angio-arteriogenesis in ischemic territories[25] but also increases the mitotic index of adult cardiomyocytes in ischemic and non-ischemic myocardium.[1] Moreover, the evidence that in this pig model VEGF induces hyperplasia of adult cardiomyocytes[2] was the reason that encouraged us to conduct the present study. Furthermore, a recent report in a similar model shows that transfer of human *FGF-5* gene increases cardiomyocyte mitotic index.[40] On these bases, cardiomyoblast proliferation and entrance in mitosis of adult cardiomyocytes, with evidence of occasional cytokinesis, may have also contributed to decreased infarct size.

Left ventricular function and perfusion

Vascular endothelial growth factor-treated animals showed a tendency for improved ventricular motility. The lack of significance could be due to the short time elapsed between the infarct-limiting effect and the moment of evaluation. If peri-infarct ischemia existed, it is reasonable to speculate that angio-arteriogenesis induced by *VEGF* gene transfer had not yet resulted in a degree of functional recovery high enough to yield significant differences with respect to placebo group. Contrarily, the effect of VEGF on myocardial perfusion was consistent with infarct size results. In effect, given that SPECT studies were carried out at rest, the higher perfusion values in *VEGF*-treated sheep represent larger amount of viable myocardium rather than augmented blood flow.

Gene expression

Presence of VEGF protein (confirmed by immunohistochemistry and Western blot) far beyond the disappearance of mRNA expression is interesting. Similar time lags between both phenomena were reported by Couffinhal *et al*.[41] in mice with hindlimb ischemia and by us in pigs with chronic myocardial ischemia.[2,25] The most likely explanation is the known ability of the secreted protein to remain bound to VEGF-binding sites for long periods.[42]

Conclusion

In adult sheep human $VEGF_{165}$ gene transfer reduces infarct size at 15 days after acute coronary artery occlusion. The mechanisms involved include neovascular formation, reduced fibrosis and increased cardiomyocyte regeneration. Vascular endothelial growth factor gene transfer may thus represent an approach for treatment of AMI and prevention of LV remodeling.

Materials and methods

Plasmid construct

The eukaryotic expression vector (p$VEGF_{165}$, deposited as pBSVEK3 at *Deutsche Sammlung von Mikroorganismen und Zellkulturen*, accession number DSM 14 346) is a 3930 bp plasmid that includes the human $VEGF_{165}$ coding gene, transcriptionally regulated by the cytomegalovirus promoter/enhancer, and a SV40 poly-A terminator. The placebo plasmid (pLUSK3, 3317 bp, accession number DSM 14 384) is obtained from p$VEGF_{165}$, by excision of the human $VEGF_{165}$ coding gene. Preparation procedures, purification and quality control analyses were performed under GMP conditions (Bio Sidus, Buenos Aires, Argentina).

Surgical preparation and experimental protocols

Twenty six Corriedale male sheep aged 12–16 months and weighing 23 ± 0.4 kg were operated. All procedures were carried out in accordance with the Guide for Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996). Following premedication with acepromazine maleate (5 mg, i.m.), anesthesia was induced with intravenous sodium thiopental (20 mg/kg) and maintained with 1.5% halothane in pure oxygen under mechanical ventilation (Neumovent, Córdoba, Argentina). After a sterile thoracotomy at the 4th intercostal space, the LAD artery was ligated at its distal third. The second diagonal branch was also ligated at a point in line with the LAD ligature.[21] This resulted in an apical infarct comprising approximately 20% of the LV mass. To reduce the incidence of ventricular arrhythmias, lidocaine (three bolus injections of 2 mg each and a 2 mg/kg infusion), amiodarone (150 mg infusion in 2 h) and atenolol (2 mg) were administered. After 1 h, 3.8 mg of p$VEGF_{165}$ or placebo plasmid was injected intramyocardially in ten 0.2 ml aliquots. The nature of the injectates was kept blind until the end of data analysis. Injections were distributed along the normoperfused tissue 10–15 mm distant from the border of the ischemic zone. This zone was readily recognized by the presence of cyanosis and dyskinesia.

Sheep were killed at two different time points: 12 (*VEGF* treated: $n=6$, placebo: $n=6$) at 10 days and 14 (seven per group) at 15 days post-AMI. The sheep were killed with an overdose of sodium thiopental followed by a bolus of potassium chloride. For angiogenesis assessment, eight additional sheep (*VEGF* treated: $n=4$; placebo: $n=4$) prepared and treated as previously described were killed at 7 days post-AMI. For transgene expression studies we employed 10 additional sheep with AMI: eight receiving p$VEGF_{165}$, killed at 3, 7, 10 and 15 days ($n=2$ for each time point), and two receiving placebo, killed at 3 days post-AMI. Five myocardial tissue specimens from each heart were removed from the peri-infarct area, snap frozen in liquid nitrogen and stored at -70°C for PCR, RT-PCR and Western blot, or fixed in 10% buffered formaldehyde for immunohistochemistry.

For assessing if VEGF-induced cardiomyogenesis could also take place in non-infarcted hearts, 12 additional sheep without coronary ligation were randomized to blindly receive p$VEGF_{165}$ ($n=6$) or empty plasmid ($n=6$) in the same doses. Injections were distributed in the anterolateral LV wall, and sheep were killed 10 days later for histological analysis of the injected myocardium.

Left ventricular function and perfusion

In the group of 14 sheep killed at 15 days post-infarction, myocardial function and perfusion were studied at normal, pre-LAD occlusion state, 2 days post-AMI and just before killing. Studies were carried out in an ADAC Vertex Dual Detector Camera System (Milpitas, CA, USA) using ^{99m}Tc-sestamibi (G-SPECT). The sestamibi injection was carried out at conscious, resting condition 2 h before acquisition.

Regional wall motion was visually evaluated by two independent observers using the 20 segments model[43] and a motion score in which 0 corresponds to normokinesis, 1 to mild hypokinesis, 2 to moderate hypokinesis, and 3 to akinesia and dyskinesia. The final score at each experimental condition results from the sum of the individual segmental scores. Given the reported inaccuracy of the QGS software to detect the endocardial surface in small hearts,[44] the volume and ejection fraction data were disregarded.

For LV perfusion, the circumferential count profiles (polar plots) determining the number of counts per segment were analyzed in each study, as previously described.[25] The analysis of the results was focused on the zones corresponding to the occluded LAD territory: apical and medial segments of the anterior and septal

sectors, and apical segment of the infero-septal sector. Percent change in myocardial perfusion was calculated as ((perfusion at day 15−perfusion at day 2)/perfusion at day 2) × 100.

Infarct size measurement

In animals killed at 10 ($n=12$) and 15 ($n=14$) days, the LV was opened through an incision parallel to the posterior interventricular sulcus and extended flat before fixation. Digital photographs were obtained for image processing (Image-Pro Plus 4.1, Media Cybernetics, Silver Spring, MD, USA) to determine LV and infarct areas. Infarct size was expressed as percent total LV area.

Histological and immunohistochemical studies

The whole heart was immersed in 10% buffered formaldehyde. After at least 48 h fixation, 5 mm thickness transversal slices of the whole LV wall, including the interventricular septum, were obtained at the level of the infarct center and at 10 and 20 mm above and below. The tissue slices were divided in four blocks that were embedded in paraffin, sectioned at 4 μm and stained with hematoxylin–eosin, Masson's trichrome and picrosirius red. For immunohistochemical studies tissue sections were deparaffinized and brought to phosphate-buffered saline, pH 7.2. After blocking endogenous peroxidase with 3% H_2O_2 in methanol and antigen retrieval pretreatment with citrate buffer in a microwave oven, the slides were incubated 1 h with rabbit anti-human $VEGF_{165}$ polyclonal antibody (BioGenex, San Ramon, CA, USA) or specific monoclonal antibodies against the Ki67 antigen (Novocastra, Newcastle upon Tyne, UK) and smooth muscle actin (BioGenex), and post-treated with biotinilated anti-mouse and anti-rabbit immunoglobulin antiserums (Multilink, Biogenex), followed by peroxidase-labeled avidin, and revealed with AEC as chromogen. In addition, tissue sections were double stained with monoclonal antibodies against the Ki67 antigen and sarcomeric α-actin (Dako, Carpinteria, CA, USA), connexin-43 (Zymed, San Francisco, CA, USA), sca-1 (Sigma, St Louis, MO, USA), c-kit (Biogenex) and the *MDR-1*-gene-encoded P170 glycoprotein (clone C494, Signet Laboratories, Dedham, MA, USA; clone MDR-88, Biogenex, San Ramon, CA, USA) as previously described.[1,6] Endothelial cells were identified by means of biotinilated *Ulex europaeus* lectin (Vector, Burlingame, CA, USA).[7]

Collection of quantitative data was carried out in two different zones. Adult cardiomyocyte mitosis and arteriolar density (vessels with smooth muscle actin-positive wall and measuring 50 μm or less in diameter) were determined in an area comprising 10 rows of myocytes of the surviving myocardium adjacent to the infarct border (2.3 ± 0.2 mm^2). Adult cardiomyocytes were identified by their size, shape, nuclear morphology and presence of cross-striations and cytoplasmic sarcomeric α-actin. The second zone corresponded to the area present between the remnant of coagulation necrosis tissue and the viable myocardium (2.1 ± 0.1 mm^2). At that level, cardiomyoblast proliferation, angiogenesis, myofibroblast proliferation (smooth muscle actin-positive cells) and fibrosis (red areas after picrosirius red staining) were determined.

Adult cardiomyocyte mitotic index was calculated according to previously described procedures for pig[1] and human[3] hearts. Number of mitosis was expressed respective to the number of CMN in the examined area. Other data (capillaries, arterioles, cardiomyoblasts and myofibroblasts densities) were expressed as number of structures per mm^2.

Gene expression

Presence of p$VEGF_{165}$ and expression of human $VEGF_{165}$ were assessed by PCR, RT-PCR and immunohistochemistry as above mentioned. In addition, human $VEGF_{165}$ protein mass was studied with Western blot at 3, 7, 10 and 15 days after *VEGF* gene transfer. Presence of human VEGF protein in ovine myocardium at longer times after transfection (35 and 450 days) was studied on specimens of infarcted sheep belonging to previous safety protocols where we used the same doses and transfection strategy as in the present protocol.

PCR: Total DNA was isolated (Qiagen, Hilden, Germany) and quantitated ($A_{260/280}$ nm spectrophotometry). Amplification was done using T*aq* polimerase (Perkin-Elmer, Boston, MA, USA) and previously reported *ad hoc* primers.[1]

RT-PCR: Total RNA was isolated (Trizol reagent, Gibco BRL, Grand Island, NY, USA), treated with DNAse I (Promega, Madison, WI, USA), quantitated and reverse transcripted (random hexamers, Perkin-Elmer). Non-competitive amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was employed to demonstrate mRNA integrity. To discard false-positive results, RT-PCR of isolated RNA from p$VEGF_{165}$-treated hearts was performed omitting the RT reaction.

Western blot: tissue samples weighing 0.1–0.2 g were homogenized in 1 ml lysis buffer, containing 10 mmol/l Tris HCl, pH 7.6, 100 mmol/l NaCl, 0.1% sodium dodecyl sulfate (SDS), 1 mmol/l EDTA, pH 8, 1 μg/ml aprotinin, 100 μg/ml phenylmethylsulfonyl fluoride. Samples were centrifuged (10 000 *g* for 20 min) and the supernatants analyzed for protein concentration using a Bradford assay (Bio-Rad, CA, USA). Sample proteins (100 μg) were resolved under non-reducing conditions on a 12% SDS-polyacrylamide gel. Immunoblotting was performed using a monoclonal antibody against human $VEGF_{165}$ (MAB 293, R&D Systems, MN, USA) at a dilution of 1:100 in non-fat milk/Tris buffer. The membrane was subsequently probed with a secondary anti-mouse antibody conjugated to horseradish peroxidase (P260 Dako, CA, USA) at a dilution of 1:1000 and developed with chemiluminescence (ECL, RPN 2106, Amersham, IL, USA). The membrane was then exposed to X-ray film (BioMax ML, Kodax, NY, USA). A standard of human purified $VEGF_{165}$ (293-VE, R&D Systems) was used as positive control. To exclude the influence of fibrosis on myocyte proteins, immunoblotting for sarcomeric α-actin was performed as an internal loading control.

Statistics

Left ventricular perfusion, infarct size and morphometric results (except capillary density) were analyzed with Student's *t*-test for unpaired data. For capillary density, a non-parametric procedure (Mann–Whitney rank-sum test) was employed due to the small sample size ($n=4$ per group). Left ventricular function was analyzed with non-parametric two-way ANOVA (Friedman's test).

$P<0.05$ was considered to indicate statistical significance. Results are expressed as mean $\pm$ s.e.m.

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