Recombinant human vascular endothelial growth factor enhances bone healing in an experimental nonunion model

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The re-establishment of vascularity is an early event in fracture healing; upregulation of angiogenesis may therefore promote the formation of bone. We have investigated the capacity of vascular endothelial growth factor (VEGF) to stimulate the formation of bone in an experimental atrophic nonunion model.

Three groups of eight rabbits underwent a standard nonunion operation. This was followed by interfragmentary deposition of 100 µg VEGF, carrier alone or autograft.

After seven weeks, torsional failure tests and callus size confirmed that VEGF-treated osteotomies had united whereas the carrier-treated osteotomies failed to unite. The biomechanical properties of the groups treated with VEGF and autograft were identical. There was no difference in bone blood flow.

We considered that VEGF stimulated the formation of competent bone in an environment deprived of its normal vascularisation and osteoprogenitor cell supply. It could be used to enhance the healing of fractures predisposed to nonunion.

During the healing of fractures signal peptides are released from the initial haematoma; the temporal and spatial expression patterns of these growth factors suggest that they have a pivotal role in bone repair. High levels of angiogenic growth factors have been recorded in the fracture haematoma early in the healing sequence which, probably, stimulate interfragmentary vascular invasion. Since vascular invasion precedes the formation of bone, there is growing interest in angiogenic growth factors and their ability to promote bone repair.

Vascular endothelial growth factor (VEGF), the most potent angiogenic growth factor, stimulates the formation of new blood vessels. It is used clinically to increase intramyocardial blood flow in patients suffering from ischaemic heart disease, and it also seems to be directly involved in the formation of bone. In vitro studies suggest that VEGF couples angiogenesis to the formation of bone through an intimate interplay with bone morphogenetic proteins (BMPs) and direct activation of osteoblasts. VEGF deposition at the site of bone damage has been shown to enhance the formation of bone in burr hole defects, murine femoral fractures and critical-sized defects of the radius in rabbits. These findings suggest that application of VEGF could enhance bone repair in orthopaedic conditions. However, studies using clinically relevant animal models are needed to assess the potential of this treatment.

In this study, we investigated the potential of exogenous VEGF to enhance bone healing in a clinically-relevant model of compromised healing that mimics the scenario of open tibial fractures. We used a validated rabbit nonunion model, in which tibial nonunion develops after mid-tibial osteotomy, excision of periosteum and endosteum at the fracture site and rigid fracture fixation.

We hypothesised that application of VEGF to a fracture site that is predisposed to nonunion may prevent its development and that the effect of the application of VEGF is comparable with autologous bone-grafting.

Materials and Methods

Design. Skeletally mature New Zealand White rabbits, five to six months old, randomly allocated to three groups of eight, underwent a unilateral standard operation for nonunion.

In one group, recombinant human (rh) VEGF was instilled into the osteotomy gap in a hyaluronic acid carrier during the operation. The second group received hyaluronic acid carrier alone and the third received an autograft. The mechanical properties and blood flow at the osteotomy site were assessed after seven weeks. The experiment was approved by the Danish Committee on Animal Experimentation.
**Growth factor, hyaluronic acid, autograft.** In order to calculate the optimal rhVEGF dose, we performed a pharmacokinetic pilot study on three rabbits using radiolabelled rhVEGF and a hand-held scintillation counter positioned over the osteotomy site. The elimination of an interfragmentary injection of radiolabelled rhVEGF delivered in hyaluronic acid followed first order kinetics, with $T_{1/2} = 24$ hr, after an initial burst at one hour during which the injected dose was reduced to 10%. Assuming homogenous distribution of the 100 µg rhVEGF injectate in a volume equal to the interfragmentary gap, we predicted that 1000 ng rhVEGF would remain in the gap at day six after injection, and 7 ng after 13 days, corresponding to the ED$_{max}$ and ED$_{50}$ of rhVEGF. In contrast, free rhVEGF is cleared from the circulation in four hours. We used VEGF 165 (rhVEGF, 293-VE; R&D Systems; Minneapolis, Minnesota), a 42 kDa peptide with 97% homology to rabbit VEGF. We suspended 100 µg lyophilised, carrier-free VEGF in 0.2 ml saline, added 0.2 ml hyaluronan gel (1.4% sodium hyaluronate, I-Visc Plus, I-med Pharma Inc., Montreal, Canada) and mixed the suspension in a syringe for several minutes. The control group received saline and hyaluronan gel only. Autograft was collected from the left iliac crest with two cancellocortical grafts being harvested using a cylindrical drill. The harvested bone cylinder was crushed, cleared of cortical adhesions and used immediately without further processing.

**Anaesthesia.** Balanced anaesthesia was achieved with a mixture of 20 ml ketamine (50 mg/ml, Pfizer, Ballerup, Denmark), 2.5 ml lidocaine (20 mg/ml, Xylacaine, Astra, Albertslund, Denmark) and 1 ml acepromazine (10 mg/ml, Plegicid, Pharmacia & Upjohn, Albertslund, Denmark) administered as 23.5 ml/kg subcutaneous injections repeated every 30 minutes during the operation. Buprenorphine (0.1 mg/kg, Temgesic, Reckitt & Coleman, Hull, England) was used for post-operative pain relief for two to three days.

**Surgical procedure.** A validated nonunion model was modified by the use of plating instead of external fixation. Using an anteromedial approach in anaesthetised rabbits, we osteotomised the right distal tibial diaphysis, stripped the periosteum and endosteum 15 mm proximal and distal to the osteotomy, and secured a 5-hole 3/8 Orthofix plate to the anterolateral aspect of the tibia leaving a 2 mm gap between the osteotomised bone ends. The osteotomy was placed between holes 3 and 4 counting from proximal to distal. After saline irrigation, rhVEGF, hyaluronic acid carrier alone or autograft was deposited in the bone gap. The wound was sutured in two layers and the rabbits were allowed free movement. After seven weeks bone blood flow measurement, radiographic evaluation, bone dissection, micro CT-scanning, and torsional testing were performed sequentially. An intracardial pentobarbital injection (Mebumal, 200 mg/ml, Sygehus Apotekerne, Aarhus, Denmark) of anaesthetised rabbits ensured pain-free death.

**Blood flow measurement and bone dissection.** Blood flow measurements were performed according to the technique of Hales. In brief, 2 x 10$^6$ strontium-85 labelled 15-µm microspheres, suspended in 10% Dextran and 0.01% Tween (New England Nuclear; Boston, Massachusetts) were mixed for five minutes by vigorous shaking and then injected into the left ventricle of the heart through a catheter via the left common carotid artery, the catheter was positioned under fluoroscopic control. Another catheter was positioned in the right common carotid artery, without obstructing the flow, and was connected to a reversed Terumo syringe pump that acted as the reference organ. The microspheres were injected over 30 seconds, reference blood samples were taken over two minutes at a rate of 2 ml/min and the rabbits were killed. Mean arterial blood pressure, heart rate, rectal temperature and blood saturation were monitored to document cardiovascular stability. The soft tissue was removed from the bones and lateral and anteroposterior radiographs of the operated tibiae were taken. The plates and screws were removed and tibiae were cut into six sections; proximal epiphysis, proximal metaphysis, proximal diaphysis, distal diaphysis, distal metaphysis and distal epiphysis. The screwholes provided landmarks for cutting thus producing identically-sized sections. The distal diaphysis, which included the osteotomy site, was cut between screwhole 2 and 3 proximally and between screwhole 4 and 5 distally. After symmetrical sampling from the contralateral leg, all samples were counted in pre-weighed vials in a gamma counter along with the reference blood samples, which were collected in similar tubes (Packard Cobra 5000; GMI, inc.; Clearwater, Massachusetts). The counts in each channel were corrected for background radiation. The samples containing the osteotomy site were then frozen.

**Micro CT.** The frozen samples containing the osteotomy gap were thawed and scanned with a high-resolution microtomographic system (µ-CT 20, Scanco Medical AG, Zurich, Switzerland). The bone was scanned between 1 to 2 mm proximal and 1 to 2 mm distal to the gap and a 3D image reconstructed using 250 horizontal micro CT slide images 16 µm thick giving a resolution of 20 µm. Bone was isolated using an automatic thresholding procedure and all specimens were segmented using a common optimal threshold. The callus cross-sectional area was measured in an interfragmentary horizontal slice.

**Torsional testing.** After micro CT-scanning both ends of the specimens were embedded in bone cement so that the screwholes adjacent to the gap were embedded. The construct was then fastened to the torsion device of an 858 Bionix hydraulic material-testing machine, computer-controlled by a TestStar II Operating System (MTS Systems Corporation, Minneapolis, Minnesota). The specimen was tested to failure in torsion at a rate of 2°/sec. The torsional failure moment, stiffness and failure angle were recorded from the torque angular displacement curve.
Statistical analysis. In view of the small sample size, non-parametric tests were used. Groups were compared using the Mann-Whitney U test for two unpaired samples. Values for p < 0.05 were regarded as significant.

Results
All rabbits tolerated the experimental procedure, none had infections and they moved freely a few days after surgery. There were no significant inter-group differences in pre-operative bodyweight or in weight gain during the study. Two rhVEGF-treated rabbits and one autografted rabbit were excluded from the study due to fracture through the screw hole distal to the osteotomy.

Macroscopic evaluation. In the rhVEGF and autograft-treated rabbits, interfragmentary bone filled the posterior half of the osteotomy and posterolateral callus bridged the gap, uniting the osteotomy solidly. In the control group, only small amounts of interfragmentary bone had formed; in four of the eight rabbits there was a fibrous, mobile union and in the remaining four a bony union with external callus. Sparse formation of bone was seen on the anterior aspect of the tibia; the plate and screws were not covered by callus, removal was therefore easy.

Blood flow measurements. The cardiovascular function remained stable during microsphere injection. Three rabbits in the control group, one in the rhVEGF group and one in the autograft group were excluded due to technical difficulties during the microsphere injection. Table I shows the regional blood flow on the operated leg, normalised to the non-operated contralateral leg. There was no difference between groups, but regional blood flow at the osteotomy site was higher than in the contralateral diaphysis or the rest of the ipsilateral tibia.

Radiographic examination. Radiographic examination showed interfragmentary radio-opacity in the rhVEGF- and autograft-treated groups; the continuity between bone ends was almost fully restored. In contrast, the control group demonstrated a radiolucent gap without restoration of continuity.

Micro CT. An interfragmentary 3D-reconstruction from each group is shown in Figure 1. Abundant callus was seen

Table I. Ratio of regional blood flow on the operated side (normalised to non-operated side) presented as the median with 25th and 75th centiles in brackets

<table>
<thead>
<tr>
<th>Region</th>
<th>rhVEGF (n = 5)</th>
<th>Control (n = 5)</th>
<th>Autograft (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tibia</td>
<td>1.5 (1.2 to 1.8)</td>
<td>1.4 (1.1 to 1.7)</td>
<td>1.5 (1.2 to 1.6)</td>
</tr>
<tr>
<td>Proximal epiphysis</td>
<td>0.9 (0.8 to 1.2)</td>
<td>0.9 (0.7 to 1.2)</td>
<td>1.0 (0.9 to 1.0)</td>
</tr>
<tr>
<td>Proximal metaphysis</td>
<td>1.5 (0.8 to 1.6)</td>
<td>1.0 (0.8 to 1.4)</td>
<td>1.0 (0.7 to 1.4)</td>
</tr>
<tr>
<td>Proximal diaphysis</td>
<td>3.0 (2.0 to 3.5)</td>
<td>2.8 (1.4 to 6.3)</td>
<td>2.2 (1.5 to 2.6)</td>
</tr>
<tr>
<td>Distal diaphysis</td>
<td>4.9 (4.1 to 7.1)</td>
<td>3.6 (2.1 to 9.8)</td>
<td>4.8 (4.2 to 6.7)</td>
</tr>
<tr>
<td>Distal metaphysis</td>
<td>2.9 (1.9 to 4.4)</td>
<td>2.2 (2.0 to 4.4)</td>
<td>2.6 (2.4 to 3.0)</td>
</tr>
<tr>
<td>Distal epiphysis</td>
<td>0.5 (0.4 to 0.9)</td>
<td>0.7 (0.3 to 0.7)</td>
<td>0.8 (0.4 to 0.8)</td>
</tr>
</tbody>
</table>

* rhVEGF, recombinant human vascular endothelial growth factor

Table II. Mechanical results and area of callus. Median followed by first and third quartile in brackets. The Mann-Whitney U test was used to calculate the p value

<table>
<thead>
<tr>
<th></th>
<th>rhVEGF (n = 6)</th>
<th>Control (n = 8)</th>
<th>Autograft (n = 7)</th>
<th>p value (rhVEGF vs control)</th>
<th>p value (rhVEGF vs autograft)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Failure torque (Nmm)</td>
<td>1500 (1100 to 1850)</td>
<td>400 (0 to 1000)</td>
<td>1300 (1200 to 1400)</td>
<td>0.009</td>
<td>0.4</td>
</tr>
<tr>
<td>Failure angle (°)</td>
<td>9 (8 to 9)</td>
<td>2 (0 to 7)</td>
<td>11 (6 to 16)</td>
<td>0.02</td>
<td>0.5</td>
</tr>
<tr>
<td>Stiffness (Nmm x °⁻¹)</td>
<td>250 (110 to 300)</td>
<td>30 (0 to 200)</td>
<td>200 (180 to 320)</td>
<td>0.05</td>
<td>0.9</td>
</tr>
<tr>
<td>Cross-sectional area of callus (mm²)</td>
<td>54 (43 to 61)</td>
<td>39 (8 to 48)</td>
<td>55 (52 to 60)</td>
<td>0.06</td>
<td>1</td>
</tr>
</tbody>
</table>

* rhVEGF, recombinant human vascular endothelial growth factor

Interfragmentary sections from reconstructed micro CT scans. Note the abundant posterior callus in the recombinant human vascular endothelial growth factor (rhVEGF) and autograft group and lack of bone formation in the control group.
in the rhVEGF and autograft groups and the cross-sectional area of the interfragmentary callus was higher in these groups than in the control group but did not reach significance (p = 0.06).

**Torsional testing.** The torque angular displacement curve comprised a linear loading segment reflecting the stiffness, then an inflection. The curve peaked at a point representing the maximum torsional moment and the corresponding angular displacement at failure. Stiffness, median failure moment and angular displacement at failure were higher in the rhVEGF group than in the control group, but not different from the autograft group (Table II). Failure occurred as a spiral fracture.

**Discussion**

We found that all of the tibiae treated with rhVEGF united whereas only half of the osteotomies treated with vehicle only went on to union. The mean failure torque, failure angle and stiffness were significantly higher in rhVEGF-treated tibiae than in the vehicle-treated group, but were not different from the autograft group. Moreover, the cross-sectional area of the callus was larger in the rabbits treated with rhVEGF and autograft than in the rabbits treated with vehicle only. The blood flow in the rhVEGF group was 5 times higher, and in vehicle-treated osteotomies 3.5 times higher, than in the contralateral diaphyses. The difference between rhVEGF- and vehicle-treated groups was not significant (p = 0.7). Previous experiments showed that the interfragmentary gap in the comminuted rabbit tibia contained fibrous tissue, fibrocartilage, hyaline cartilage, bony islands and many arterioles and capillaries; essentially similar findings were seen in biopsies from human atrophic and hypertrophic nonunions. The rabbit nonunion model thus mimics the clinical scenario of an infection associated with the use of bovine collagen carriers. It has recently become possible in rabbits.

Our biomechanical data showed an unequivocal difference between rhVEGF treatment and vehicle treatment, implying that rhVEGF can prevent nonunion in a clinically relevant model of compromised healing, and that it induces competent formation of bone in an environment deprived of its normal osteoprogenitor cell supply.

At the end of the seven-week healing period we found that blood flow at the osteotomy site rose to 3.5 to 5 times that of the contralateral non-operated diaphysis. There was a trend towards increased blood flow in the rabbits treated with rhVEGF compared with those treated with vehicle only. Technical difficulties during microsphere injection reduced the number of rabbits available for analysis so that we were unable to draw conclusions about the angiogenic effect of rhVEGF delivery. Serial measurements of bone blood flow at several time points during healing would have been valuable, but this is very demanding and has only recently become possible in rabbits.

The hyaluronic acid carrier that we used was 100% synthetic, eliminating the hypothetical risk of disease transmission associated with the use of bovine collagen carriers. It has been used for ophthalmic surgery for years, it is non-immunogenic, it stimulates osteoinduction by itself, it provides the osteoprogenitor cells with a scaffold for migration and prolongs the effects of growth factors at the site of deposition. Endogenous VEGF is secreted from endothelial cells, fibroblasts, osteoblasts and other cell types and its expression, and the expression of its receptor, are upregulated by hypoxia and ischaemia. VEGF binds to endothelial cell surface receptors, resulting in their growth, proliferation and migration. Its physiological effects include increased angiogenesis, increased permeability of vessels and vasodilation. Its clinical potential in the treatment of myocardial ischaemia has attracted attention, but its role in osteogenesis is not clear. BMPs stimulate angiogenesis through the production of VEGF by osteoblasts. However, VEGF produced by this mechanism does not seem to be directly involved in osteoblast differentiation, but couples angiogenesis to bone formation. VEGF enhanced formation of endochondral bone in bone defects, but failed to increase the formation of intramembranous bone in an experimental distraction osteogenesis model, although blood flow in bone was increased. In the current study, all rhVEGF-treated osteotomies united through a bulky callus, indicating that formation of endochondral bone was enhanced. Thus, vessels induced by VEGF repopulated the osteotomy gap and provided the growth factors and bone-forming cells necessary for competent formation of endochondral bone. As VEGF itself has no osteoinductive capacity, bone-forming cells were probably recruited from pericytes, circulating cytokines and cells, the blood clot and the fractured bone ends. VEGF potentiates the actions of several cytokines, and it mediates the angiogenic actions of most growth factors. It could therefore complement other cytokines such as basic fibroblast growth factor or bone morphogenetic proteins in enhancing fracture healing. The findings of this study constitute a promising demonstration of enhanced formation of bone after rhVEGF stimulation, but the biomechanical properties of the osteotomies stimulated by exogenous rhVEGF were not superior to those that had received peri-operative autografts.

In summary, interfragmentary deposition of rhVEGF used with a hyaluronic acid carrier prevented the development of fibrous nonunion in an experimental model. The bone formed was competent and its mechanical properties were no different from those of autografted osteotomies.

**References**


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