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Fracture vascularity and bone healing: A systematic review of the role of VEGF

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Summary Fracture healing constitutes a complex and delicate physiological process. Local vascularity at the site of the fracture has been identified as one of the most significant parameters influencing the healing procedure. VEGF is the most important component of the regeneration of the vascular system at the fracture site. The aim of this review is to determine the evidence supporting the direct role of VEGF in the enhancement of fracture healing and the possible clinical use of VEGF for non-unions. The literature search was performed via the internet using the Medline. The key words which were searched in the abstracts were the terms "VEGF", "angiogenesis", "fracture", "bone" and "healing". Twenty-five articles were relevant to the topic of interest. A total of 11 articles were excluded from our research due to non conformity of their content to the inclusion criteria. Evidence retrieved suggests that VEGF could be extremely valuable for the treatment of critical size bone defects and that VEGF could have a direct effect on osteoprogenitor cells, mainly by promoting the differentiation of osteoblasts and by increasing the mineralisation of the regenerated bone. The former observation could have very interesting repercussions for the field of non-unions and the latter for the field of osteoporosis.

Introduction

Bone healing constitutes a unique regenerative procedure in the human body.[1-3] Although it can be completed without any scarring, a significant percentage of fractures fail to heal adequately.[4-8] Amongst others, angiogenesis occupies a central role in the whole process of bone regeneration after fracture.[9,10] The two main hormonal pathways controlling angiogenesis are the VEGF pathway and the angiopoietin pathway.[3,11,12] By far the most important of the two is the VEGF pathway.

VEGF was first described as VPF (tumour Vascular Permeability Factor) in 1983,[13] but was finally discovered and characterised in 1989.[14] The most important isomers of human VEGF are VEGF121, VEGF165, VEGF189 and VEGF206. Various in vitro studies have documented the capability of VEGF to promote the development of vascular endothelial cells derived from arteries, veins, and lymphatic

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vessels.[15-18] Angiogenesis has a cornerstone role in endochondral ossification, during which the avascular cartilagenous tissue is gradually transformed into vascular osseous tissue.[15] Hypertrophic chondrocytes in the epiphyseal growth plate express VEGF, which promotes the invasion of the cartilage by metaphyseal vessels resulting in new bone formation. This was pointed out in a study where blocked VEGF receptors resulted in the suppression of vascular invasion of the cartilage and of bone formation.[19] Termination of the anti-VEGF treatment was followed by capillary invasion, resorption of the hypertrophic cartilage, re-establishment of bone development and normal growth plate architecture. All these point out that VEGF mediates the capillary invasion that constitutes a "sine qua non" pre-requisite for the complex process of endochondral ossification.[19]

It has been also suggested that VEGF can influence the process of fracture healing by other pathways.[20] For instance, the use of an anti-angiogenic agent in an experimental model of fracture created an "atrophic non-union" resembling tissue, both in endochondral and intramembraneous ossification.[1,21] The anti-angiogenic agent used in this study was TNP-470, an inhibitor of the potent vascular growth factor VEGF, as it has been also demonstrated by another study using the same substance for VEGF inhibition.[22] These findings could imply that VEGF is not only involved in endochondral ossification, but also in intramembraneous ossification. The important role of VEGF in coupling bone formation and angiogenesis has not been fully understood and the clinical consequences of the use of recombinant VEGF for fracture healing remain unexplored.

This systematic review aspires to identify the best available evidence concerning the role of VEGF in fracture healing, clarify certain aspects of the patho-physiology of delayed or atrophic non-union, point out the questions remaining unanswered and finally, formulate recommendations for the clinical use of VEGF (or similar pharmaceutical substances) for fracture healing and atrophic non-unions.

Materials and Methods

The literature search was performed via the internet using the Pubmed search engine. The key words which were searched in the abstracts were the terms "VEGF", "angiogenesis", "fracture", "bone" and "healing". The search was limited to articles concerning controlled in vivo trials that used VEGF (or VEGF inhibitors) in order to modify fracture healing. This way VEGF was considered as a therapeutic intervention in prospective cohort studies using models linked (directly or indirectly) with non-unions and not as a diagnostic or prognostic tool for prospective or retrospective studies. In other words, all the selected studies evaluated the direct use of VEGF (or its inhibitor), administered by various ways, for fracture healing alone or combined with other substances or growth factors.

As exclusion criteria were set the following: articles using language other than English, articles that were not experimental controlled trials (reviews, letters and expert opinion publications) or articles without available full text. Furthermore, this search focused on conventional bone healing and excluded other mechanisms of bone metabolism such as distraction osteogenesis, osteoporosis and osteonecrosis. Articles that conformed to our criteria were retrieved and then all the articles related to these were searched. The level of evidence of each study was also documented.

Results

In total 25 articles met the inclusion criteria.[23-47] A sum of 11 articles was excluded from our research due to nonconformity of their content to the inclusion criteria.[24,26,30,31,33,36,37,41,43,45,46] Three articles were excluded because they were focused on distraction osteogenesis.[24,26,45] Two further articles were excluded because they described osteonecrosis models,[33,43] three described an ectopic ossification model,[31,37,41] two described a bone development (not fracture healing) inhibition model[36,46] and one an osteoporosis model (not osteoporotic fracture healing).[30] It is also worth mentioning that all the papers retrieved during the search involved animal in vivo protocols. This could be explained by the innovative character of this therapeutic approach as VEGF treatment still remains at the level of preclinical research. The fourteen papers[23,25,27-29,32,34,35,38-40,42,44,47] which fulfilled the inclusion criteria could be distributed in four main categories:

(a) the first category (Table 1) comprised 4 studies concerning the use of VEGF signaling inhibitors for the assessment of the role of this growth factor on fracture healing;[29,39,40,42]
(b) the second category (Table 2) included 4 studies using gene transfer and therapy techniques for the enhancement of VEGF activity at the site of the fracture;[27,28,44,47]
(c) the third category (Table 3) comprised 4 studies focussed on scaffold use for VEGF administration at the fracture site;[23,32,35,38]

(d) the fourth category (Table 4) included 2 studies comparing the local use of VEGF for bone healing.[25,34] These distributions were rather rough and a lot of overlapping could be observed, but finally a prioritisation of the endpoints of each study defined the final outcome. For example, two articles[39,40] could be attributed to both the first and the second category, but the use of VEGF receptor inhibitor and the complexity of endpoints superseded the use of gene therapy techniques.

Use of VEGF signaling inhibitors for the assessment of the role of this growth factor on fracture healing[29,39,40,42] (Table 1)

Hausman et al.[29] investigated the VEGF inhibitor, TNP-470 and the side effects of the use of this substance in oncology as anti-VEGF to inhibit neo-vascularisation. This study showed that TNP-470 can totally prevent osteogenesis in a model of closed femoral fracture, creating an atrophic non-union like situation (inhibiting both endochondral and intramembraneous ossification). It is common knowledge that osteolytic lesions, leading to fractures, are frequent complications of skeletal tumors.[48-50] The fact that the use of this anti-angiogenic drug for cancer chemotherapy increases the risk of impaired fracture healing is, in this context, of particular interest. Apart from that, we can deduce very important messages, even through an indirect pathway, for the crucial role of VEGF in the revascularisation of healing bone.[29]

Street et al.[42] designed a study which comprised 175 mice (used for midshaft, fixed femur fractures with periosteum stripping simulating endochondral ossification or for full thickness unicortical defects of the tibia simulating intramembraneous ossification) and 30 rabbits (used for a critical gap of the radius created by bone and periosteum removal, simulating atrophic non-union, where local administration of VEGF was performed). In the mice with femoral fracture, the local administration of exogenous VEGF enhanced blood vessel formation, ossification, and new bone (callus) maturation. In the rabbit subgroup, the local administration of exogenous VEGF promoted bony bridging in radial defects predisposing to atrophic non-unions. These observations were consistent with the theory of a direct autocrine role for VEGF in osteoblast differentiation formulated by the authors. Inhibition of VEGF receptors dramatically inhibited healing of tibial cortical bone defects in mice. All these results indicate that slow-release VEGF used locally could be proven to be an effective therapeutic weapon in the armentarium of orthopaedic trauma surgeons dealing with large bone defects[51] and/or extensive vasculature and soft tissue damage predisposing to non-unions.[6,52-54] In these patients with relatively poor prognosis concerning their bone healing, the role of VEGF could be proven to be crucial not only for the vascularity of the fracture site, but also for the enhancement of osteoblast growth per se.[42]

The third article of the first category is characterised by a small sample of mice, but this disadvantage was compensated by the innovative aims and methods of the study.[40] This study consisted of an experimental gene therapy using muscle-derived stem cells (MDSCs),[11,55] which were genetically engineered to express human Bone Morphogenic Protein-4 (BMP4), human VEGF165, or the VEGF receptor antagonist, the soluble Flt1 (sFlt1). The results revealed that VEGF alone did not promote bone regeneration, but it acted synergistically with BMP4 for the amplification of recruitment of mesenchymal stem cells, for the enhancement of cell survival and for the increase of cartilage formation in the early stages of endochondral bone formation. VEGF promotes endochondral bone formation, mainly produced by the influence of BMP4,[56] not only by affecting steps after cartilage formation, but also by interfering with early stages of the bone tissue formation cascade. This interesting influence of VEGF coupled with the observation that cranial defects were unexpectedly healed via endochondral ossification instead of the standard intramembraneous ossification of the skull, supported the authors' opinion about the direct (not only via neo-vascularisation) involvement of VEGF in the early stages of bone healing. This opinion was further consolidated by the finding that VEGF-specific antagonist (sFlt1) could inhibit bone formation by reducing cartilage formation and delaying cartilage resorption in the endochondral ossification pathway. Another stimulating observation of this study is that the beneficial effect of VEGF on bone healing is directly linked with its ratio to BMP4. When this balance (1:5 ratio of MSCs expressing VEGF and MSCs expressing BMP4) is altered, the result can be detrimental for the healing process, but could also be linked with side effects such as haemangioma formation. Excessive VEGF in relation to BMP4 leads to an impairment in bone formation, possibly by promoting mesenchymal stem cell differentiation toward an endothelial lineage, consequently reducing the availability of MSCs

Table 1
Articles concerning VEGF signaling inhibitors

Author (year)	Level of evidence	Sample	Type of intervention	Type of VEGF treatment	Conclusions
Hausman MR et al., 2001[29]	II (not actively randomised, small sample)	20 Sprague-Dawley rats(not very clear)	Unilateral closed femoral fractures were created	10 animals received TNP-470 (VEGF inhibitor) sc and 10 received placebo sc	Creation of non-unions at the animals receiving TNP-470 due to the vascular impairment
Street J, Bao M et al., 2002[42]	II (not actively randomised)	A. 175 male C57BL6 mice, B. 30 male NZW rabbits	A1.midshaft, fixed femur fracture with periosteum stripping (endochondral ossification), A2. a full thickness unicortical defect of tibia (intramembraneous ossification), B. critical gap of radius by bone and periosteum removal (atrophic non-union)	A1. local use of VEGF, A2. IP administration of VEGF receptor inhibitor, B. local use of VEGF	A1. exogenous VEGF enhanced blood vessel formation, ossification, and new bone (callus) maturation in mouse femur fractures, A2. Inhibition of VEGF also dramatically inhibited healing of a tibial cortical bone defect, B. exogenous VEGF promoted bony bridging in radial defects
Peng H, Wright V et al., 2002[40]	II (not actively randomised, small sample per group)	C57BL/6J mice	Skull defect in 8 animals	Gene therapy using muscle-derived stem cells (MDSCs) genetically engineered to express human BMP4, human VEGF165, or the VEGF antagonist soluble Flt1 (sFlt1)	VEGF activity is required for optimal bone formation elicited by MDSCs expressing BMP4. VEGF and BMP4 act synergistically. The synergistic effect between VEGF and BMP4 should be more prominent in individuals suffering from compromised circulation, such as patients with diabetes or atherosclerosis. The beneficial effect of VEGF on bone healing depends critically on its ratio to BMP4.
Peng H, Usas A et al., 2005[39]	II (not actively randomised, small sample per group)	C57BL/6J mice	Skull defect in 8 animals	Gene therapy using muscle-derived stem cells (MDSCs) genetically engineered to express human BMP2, human VEGF165, or the VEGF antagonist soluble Flt1 (sFlt1)	Similar to the relationship between VEGF and BMP4, VEGF and BMP2 act synergistically for bone healing and the ratio between the two hormones is crucial for bone regeneration. Some differences between BMP2 and BMP4 were observed.

for chondrogenic and osteogenic differentiation. This theory was supported by the observation that in new bone tissue filling the defects, a much greater confluence of endothelial cells (i.e., CD31-positive cells) was demonstrated when the optimal VEGF/BMP4 ratio was disrupted in favor of VEGF expressing cells. Alternatively, excessive VEGF may increase recruitment of osteoclasts into the bone regeneration sites and lead to excessive bone resorption. This very intriguing paper outlines the synergistic role of BMP4, VEGF and osteoprogenitor cells for the successful healing of critical size defects, but also underscores that the synergistic effect between angiogenic factor VEGF and osteogenic factor BMP4 could produce even more surprising results in patients with predisposing factors for atrophic non-union linked to compromised circulation, such as diabetes or atherosclerosis.[40]

The fourth article of the first category was originated by the same team as the previous one, so it is not unexpected that the two papers present several similarities.[39] This study is rather an evolution of the previous, with the most important difference being the use of BMP2 instead of BMP4. The design of the study was quite similar (same sample, same methodology and pharmacological interventions). This study reported some interesting findings: angiogenesis occurring during endochondral bone healing elicited by MSCs expressing BMP2 could be distinguished in two phases; the early phase of angiogenesis takes place before the appearance of hypertrophic cartilage that secretes VEGF, so this could be attributed to the possible presence of VEGF at the haematoma of the fracture site. It has been shown that VEGF interacts synergistically with BMP2 (as expected from the authors' previous findings on BMP4) in order to enhance bone healing by acceleration of angiogenesis and Metaloproteinase-9 (MMP9) expression, leading to increased cartilage resorption and associated mineralized bone formation. But some differences between BMP2 and BMP4 were also demonstrated. BMP2 was less influenced by exaggerated addition of VEGF, which could be attributed to the more angiogenic intrinsic properties of BMP2 than BMP4. As a consequence, the disruption of the optimum BMP2/VEGF ratio has less negative effects on bone formation. Both the two papers concluded that the direct pro-osteogenic effect of VEGF could not sufficiently address critical size defects without the added osteogenic effect of BMPs.[39]

Gene transfer and therapy techniques for the enhancement of VEGF activity at the site of the fracture[27,28,44,47] (Table 2)

The first study in this group is a rather simple paper and probably one of the first attempts of using VEGF for gene therapy strategies concerning the enhancement of fracture healing.[44] A cylindrical 3 mm osteoperiosteal defect was drilled in both distal femurs of the rats. Then the muscle of one femur was injected with adenoviral vector encoding VEGF and the collateral served as control. The defect was healed in both groups (control and VEGF), but the process of healing was promoted in the VEGF-treated animals that completed the endochondral phase earlier than control. Bone mineral content was enhanced in the VEGF-treated femurs. If it is taken into account that finally both defects were healed, these findings could support the theory that VEGF enhances mineral density, but not osteoid formation. The feasibility of a first-generation adenoviral vector to deliver VEGF locally at the site of the fracture was proven in this study.[44]

The second paper used 60 New Zealand white rabbits in order to verify the efficacy of VEGF in critical size bone defects.[27] The defects were unilateral, 15 mm-long and located at the radial diaphysis. The form of VEGF protein delivery was dictated by the observation that VEGF is a very unstable, short-lived protein in vivo and very costly to produce. In this context it is only natural to explore different alternatives for delivering low doses of VEGF over a period of several days from an actively expressing transgene rather than using boluses of recombinant protein. Apart from the adenovirus model which is linked with incidents of immunogenicity, used in the previous study, an interesting alternative consists of the gene-activated matrix (GAM) technology carrying, in this model, a plasmid coding for human VEGF165. This GAM may exhibit many advantages compared to the use of adenovirus in vivo transfection or ex vivo transfected cells. The most interesting advantage of this technology could be the fact that this carrier could deliver various growth factors, but only after the arrival of repair cells at the region of the fracture. This could be very important in the light of the aforementioned findings that the synergistic effects of angiogenic (i.e. VEGF), osteogenic (i.e. BMPs) and osteoprogenitor cells have additive positive effects on the rate of fracture repair. The 60 rabbits were divided in five groups (three of them were different kinds of control and the last two contained the VEGF plasmid).

Table 2
Articles concerning VEGF gene therapy techniques

Author, (year)	Level of evidence	Sample	Type of intervenetion	Type of VEGF treatment	Conclusions
Tarkka T et al., 2003[44]	II-III (not actively randomised, sample non mentioned)	Male Sprague-Dawley rats	A cylindrical 3 mm osteoperiosteal defect was drilled in both distal femurs of the animals	Adenoviral vector encoding VEGF were injected into the muscle of one femur and the collateral served as control	Healing was promoted in the VEGF-treated animals that however completed the endochondral phase earlier compared with the control. Bone mineral content was enhanced in the VEGF-treated femurs
Geiger F, Wang Z et al., 2005[27]	II (not actively randomised, small sample per group)	60 female New Zealand white rabbits	Unilateral 15 mm-long critical size defects were prepared in the radial diaphysis	5 treatment groups: A. group not loaded, B1.group loaded with 0.1 or B2 group with 1 mg of the empty control plasmid or C1-C2 grouops loaded with the plasmid containing the VEGF gene	Scaffold (group A) and control plasmid (groups B) showed no defect healing, at the contrary the VEGF groups showed defect healing, net bone mass increase and enhancement of the vasculature. This study supports the use of VEGF in this plasmid version for atrophic non-union healing
Zhao D et al., 2007[47]	II (not actively randomised)	30 New Zealand white rabbits	1 cm Osteoperiostal defects from the middle of the bilateral radii	At one foreleg plasmid injection codifying VEGF, at the collateral control	Enhancement of fracture healing and vasculature development at the VEGF group. Local gene therapy is a new, cheap, feasible, and effective method for repairing bone defects, speeding up bone union, and decreasing complications
Geiger F, Lorenz H et al., 2007[28]	II (not actively randomised, small sample per group)	24 female New Zealand White rabbits	non-union model (unilateral 15 mm long critical size defects in the radial diaphysis of the animals)	4 Groups: The scaffold was (A) coated with a control-plasmid DNA (group 1), (B) coated with VEGF-plasmid DNA (group 2), (C) loaded with mesenchymal stem cells (BMSC) with control plasmid (group 3), (D) with both stem cells and the VEGF plasmid (group 4)	The highest degree of osteogenesis was observed in the BMSCs group, both VEGF groups (2&4) were superior to control (group 1) for vascularisation, osteogenesis, but VEGF trans-fected BMSCs (group 4) showed the highest performance on vascularisation and scaffold resorption

The results showing the success of GAM VEGF treatment for critical size defects and the inability of control groups to heal the defects provide further evidence for the role of VEGF in fracture and especially atrophic non-union healing. The innovation lies in the demonstration of the efficacy of new gene therapy techniques for optimisation of VEGF (and other growth factors) delivery locally.[18,27]

The third article described a gene therapy technique using a coralline scaffold for local administration of VEGF via VEGF plasmid.[28] The experiment was quite similar in methodology (similar sample, similar critical defect, similar endpoints). The groups of rabbits were quite different; the scaffold was coated: (a) with a control-plasmid DNA (group 1), (b) coated with VEGF-plasmid DNA (group 2), (c) loaded with mesenchymal stem cells (BMSC) with control plasmid (group 3), (d) with both stem cells and the VEGF plasmid (group 4). This study investigates the influence of VEGF on a coralline bone substitute and the first one, which describes a negative effect on bone regeneration compared to administration of BMSC on a carrier. The results from the solitary VEGF- and VEGF-transfected cells (groups 2 and 4) demonstrated significantly enhanced vascularization, osteogenesis and resorption of the carrier when compared to the control group. The highest degree of osteogenesis was found when the carrier was loaded with BMSC (group 3), whereas VEGF-transfected cells (group 4) led to the highest vascularization and fastest resorption of the bone substitute. Additionally, VEGF-transfected BMSC led to a more homogenous vascularization of the defect. Optimal bone growth and scaffold resorption relies on proper reabsorption rate of the material. An exaggerated stimulus for vascularisation would reabsorb the bone scaffold too soon, resulting in non-union. Without an angiogenic stimulus, there would be incomplete vascularisation and bone regeneration at the defect site.[28]

The fourth study presented another alternative for local delivery of the VEGF gene through the use of a gelatine sponge that contained a plasmid encoding VEGF165.[47] The 30 rabbits underwent an operation resulting in a 1 cm osteo-periosteal defect at the middle of the bilateral radii. The defects were repaired with absorbable gelatine sponge, which was injected at the one forearm with the VEGF plasmid and at the other forearm with normal saline. The results were significantly better in the VEGF group both concerning bone healing and angiogenesis. The contribution of this study is the demonstration of the use of local gene therapy,[9] which can accelerate bone induction, as a new, cheap, feasible, and effective method for treating bone defects and atrophic non-unions.[47]

Scaffold use for VEGF protein administration at the fracture site[23,32,35,38] (Table 3)

The first paper of the section presents a different approach for the administration of VEGF.[38] The rats, that had a cranial critical bone defect, were divided into three different groups: non bio-mineralised scaffold, bio-mineralised scaffold and bio-mineralised scaffold with VEGF. The addition of VEGF to a mineralized substrate significantly increased the generation of mineralized tissue compared with mineralized scaffold alone. This might be linked to a significant increase in vascularization throughout VEGF-releasing scaffolds compared with mineralized scaffolds without VEGF. Not very surprisingly, there was no significant difference in total osteoid formation between the two samples, suggesting that increased vascularization enhances mineralized tissue generation, but not necessarily osteoid formation. This implies that angiogenesis speeds the differentiation and/or maturation of infiltrating osteoblasts and osteoblast precursor cells during neo-bone development, perhaps by providing a conduit for delivery of osteoinductive soluble signals. These observations could support the theories mentioned already elsewhere for the collaboration of angiogenesis and osteoinductive and osteoconductive factors. It is worth mentioning that this study could not clarify whether there is a direct influence of VEGF on osteogenic cells, if we consider the fact that the lack of difference in total osteoid formation between the two bio-mineralised groups could imply that the osteoid growth could be attributed to the bio-mineralised scaffold and not to VEGF. But the possible utility of this bio-mineral scaffold has been clearly outlined in this paper.[38]

The second article is also quite similar to two previous, since all three studies come roughly from the same laboratories.[32] The innovation of this study is based on the use of bioglass bio-mineralised material with or without VEGF for the healing of critical cranial bone defects caused by irradiation, simulating the necrotising effects of radiotherapy on bone. It is interesting to note again the observation that no great difference was reported concerning the quantity of bone produced when comparing the VEGF and the non VEGF biomaterial groups, but the difference was evident at the level of bone quality as measured by BMD between the same groups. The present

Table 3
Articles concerning scaffold use for VEGF protein local release

Author, (year)	Level of evidence	Sample	Type of intervenetion	Type of VEGF treatment	Conclusions
Murphy WL et al., 2004[38]	II–III (not actively randomised, sample non explicitly mentioned, small number of animals)	possibly17 Lewis rats	A full thickness (1.5–2 mm in depth) and circular (9 mm diameter) defect was created in the cranium	3 groups: A. non-biomineralised scaffold (group 1), B. Bio-mineralised scaffold (group 2), C. Bio-mineralised scaffold with VEGF (group 3)	Bio-mineralised scaffold was superior to control scaffold and VEGF bio-mineralised scaffold was the best of all groups. There was no significant difference in total osteoid between groups 2 and 3, suggesting that increased vascularisation enhances mineralised tissue generation, but not necessarily osteoid formation. This suggests that angiogenesis promotes the differentiation and/or maturation of infiltrating osteoblasts and osteoblast precursor cells during bone healing, perhaps by providing a conduit for delivery of osteoinductive soluble signals
Kaigler D et al., 2006[32]	II (not actively randomised, small sample)	12 Fisher rats (not very clear)	Irradiation of the animals skull, then creation of osseous defects at the irradiated region, followed by placement of scaffolds	6 animals receiving VEGF scaffolds and 6 animals receiving control scaffolds	VEGF scaffolds have the potential to promote neovascularisation and bone regeneration in irradiated osseous defects, outlining a novel approach for engineering tissues in hypovascular environments
Leach JK, Kaigler D et al., 2006[35]	II (not actively randomised, small sample per group)	10 Lewis rats	Scaffolds were implanted into a critical-sized defect created in the crania of Lewis rats	2 groups of 5 animals: A. group of BG-coated scaffold + VEGF, B. group of BG coated control scaffold	Enhanced growth of endothelial cells with both scaffolds, but mainly with VEGF. Bone mineral density was significantly increased in VEGF scaffolds versus coated controls, whereas the increase in bone volume fraction was rather insignificant
Clarke SA, Hoskins NL et al., 2007[23]	II (not actively randomised, small sample per group)	24 male New Zealand White rabbits	bilateral 1.5 cm ulnar defects	6 groups of Groups of 4 animals and 8 defects: A. JAX™ alone, B. JAX™ + Autologous BMSCs, C. JAX™ + autologous BMSCs cultured on granules, D. JAX™ + fresh bone marrow, E. Cortical autograft, F. JAX™ + VEGF	More new bone in the BMA and VEGF groups compared to JAX™ alone. Groups with autologous BMSCs had slightly more quantity of bone than JAX™ alone, but BMSCs did improve the healing of the defect. Significant increase in bone volume in the BMA group compared to JAX™ alone. Formulation of the theory that provision of osteogenic precursor cells alone is insufficient to promote the bridging of critical defects and combination of factors is important

study, along with previous evidence, supports the theory that administration of exogenous VEGF does not have a profound impact on the amount of bone formed, but instead contributes to the rate of bone maturation in a rat critical-sized defect. The conclusion of this study was, once again, the importance of coordination of the three (angiogenic, osteogenic and osteoconductive) parameters of bone healing for optimum results, especially in difficult situations like irradiation defects that create serious impairment of the vasculature of the defect. It is important to remember that VEGF cannot be used without caution in patients receiving radiotherapy for tumors, because premature administration of VEGF can lead to tumour recurrence.[32]

The third study extends further more the concept of the previous study.[35] The methodology is rather similar, but instead of using biomineral film, this study tested the validity of Bioglass®. The new material was evaluated first in vivo using MSCs. The differentiation of MSCs was not significantly enhanced, but the bioglass increased the mitogenic activity of the endothelial cells, especially when VEGF was added. When the new biomaterial was used in rats, it was observed that the bone mineral density was significantly increased in VEGF scaffolds versus coated controls, whereas the increase in bone volume fraction was rather insignificant concerning the same comparison. But as shown in the previous study, similar results, although of less magnitude, were observed when bioglass scaffolds without VEGF were compared to simple control scaffolds. These results point out once again the need for close collaboration of osteogenic and angiogenic factors supported by scaffolds.[35]

The last study of this section could be differentiated from the previous three.[23] This study used a different scaffold and had a complex methodology comparing various groups: the scaffold alone, the scaffold loaded with MSCs, the scaffold loaded with VEGF, the scaffold loaded with fresh bone marrow autograft or cortical autograft. Scaffold with VEGF showed better results than scaffold alone. From the point of view of bone quality, the best results were reported in the bone marrow autograft (BMA) group. The groups in which the scaffold was loaded with MSCs exhibited worse results than BMA, reflecting the lack of growth factors probably present in the BMA. All these findings could be underscoring the fact that the success of the healing process relies on combination of osteoprogenitor cells and osteoinductive and angiogenetic factors.[23]

Use of VEGF protein for bone healing[25,34] (Table 4)

The first article describes a rather simple model of VEGF administration to an experimental fracture non-union model.[25] The rabbits of the VEGF and autograft group both showed statistically significant differences concerning the radiological evaluation and the bio-mechanical testing, when compared to control, but without any differences between VEGF and autograft. The blood flow and the vascularity were not shown to be statistically different in the VEGF group when compared to the other two groups. The authors support the opinion that BMPs can promote VEGF secretion through osteoblast stimulation and in general tend to ignore any direct effect of VEGF on osteoprogenitor cells. But on the other hand, their explanation of the phenomenon of healing of a critical size defect seems a little speculative when compared with the theories that were aforementioned. Despite this, the conclusion that VEGF can exhibit similar results to autograft for the prevention or treatment of atrophic non-unions adds more data supporting the positive role of VEGF in bone healing.[25]

The innovation of the last study in this group lies in the use of mandibles for creation of the (non critical) bone defect.[34] Once more, it has been observed that there was no difference in the quantity of new bone produced, but increased density of the mineralised bone was observed in the VEGF group. The vessel formation was intense in all the groups, but in the VEGF group, the development of the vascularity was sustained for a longer period than in the two other groups. This paper concludes that VEGF can be very useful for improving angiogenesis and, through that, for stimulating bone healing especially when the vasculature is compromised locally or systemically.[34]

Discussion

In this review, solid and valid data confirming the cornerstone role of VEGF in the angiogenesis component of the complex process of fracture healing has been reported. The interaction between the different growth factors affecting bone regeneration also became evident.[51] The complex collaboration of different biological factors (osteoinductive, osteoconductive and angiogenic) for the enhancement of fracture healing was documented and the relationship of biological and bio-mechanical factors influencing the bone regeneration phenomenon was also presented.

Table 4
General articles concerning use of VEGF protein

Author, (year)	Level of evidence	Sample	Type of intervenetion	Type of VEGF treatment	Conclusions
Eckardt H et al., 2005[25]	II (not actively randomised, small sample per group)	24 Skeletally mature New Zealand White rabbits	Osteotomy of the right distal tibial diaphysis, stripped by the periosteum and endosteum creating a non-union model	3 treatment Groups (8 animals each): A. Autograft, B. hyaluronic acid, C. hyaluronic acid + (rh)VEGF	The biomechanical properties of the groups treated with VEGF and autograft were identical, but biomechanical properties and callus size established that VEGF group defects had united whereas both the control and hyaluronic acid groups failed to unite.
Kleinheinz J et al., 2005[34]	II (not actively randomised)	56 adult and skeletally mature male White New Zealand rabbits	4 bicortical defects of 5 mm diameter were drilled in the mandible	2 groups: A. Defects were left empty or filled with pure type I collagen (Control), B. Defects were filled with collagen matrix and rhVEGF165	No difference in the quantity of new bone, but increased bone density at the VEGF group. Increased vessel formation at the VEGF group

All the papers included in this systematic review underlined the impact of the role of VEGF on the process of bone healing. Additionally, these papers showed that VEGF could be extremely valuable for the treatment of critical size bone defects, mimicking the non-union model. This became very clear by the articles indicating that inhibitors of VEGF could induce atrophic non-union of the fractures.[29,39,40,42]The therapeutic utility of VEGF is quite evident, but there are some limitations that we should be aware of. For example, it has been shown that the VEGF should be administered in very accurate and elaborated dosology, because there is always the risk of haemangiomas or recurrence of tumour, when it is administered after radiotherapy or local excision of tumour.[32] It is very important to recall that the therapeutic range of VEGF is quite narrow and the ratio between VEGF and BMPs has to be respected in order to circumvent possible adverse effects on fracture healing.[40] This difficult balance between VEGF and BMPs recapitulates the complexity of the fracture healing mechanism and the close collaboration of the different factors for this mechanism.[51,57]

Another message that one can derive from the herein study is the finding, confirmed by various studies[23,28,35,38-40] that VEGF could play a very important role in fracture healing having even possible direct effects on osteoprogenitor cells. It has been also clarified that the best results, when treating critical size defects, were obtained when there was collaboration between osteoinductive, osteoconductive and angiogenic factors. The direct effect on osteoprogenitor cells was supported by the observation, repeated in several studies, that VEGF could promote the mineralization of the bone and increase the bone density.[27,34,35,38,44] The repercussions of this finding could be very intriguing in the domain of post-menopausal osteoporosis,[11,58] since it has been reported that after menopause there is a decrease of the levels of VEGF.[59] If this decrease of VEGF could be linked with a decrease in bone mineral density, then the improvement of the levels of VEGF could be proven very important. It is worth mentioning that hormone replacement therapy could increase VEGF in postmenopausal women.[60,61]

In the area of angiogenesis it is important to notice that an interesting alternative to the use of VEGF is the use of Erythropoietin (Epo), a drug used quite widely for haemoglobin increase not only in medical patients suffering from anaemia (i.e. oncology, nephrology, chronic diseases), but also in surgery and trauma (i.e. peri-operative blood loss, extensive trauma blood loss).[62] It has been reported that Epo and VEGF could exhibit an equal angiogenic potential.[63] A rather recent paper showed that Epo could express receptors at the chondrocyte level, but also induce better bio-mechanical strength, callus formation, histomophometric image and increased bone density in treated with Epo animals when compared with control animals.[64]

In conclusion, it appears that the currently existing evidence on the use of VEGF for the enhancement of fracture healing and bone regeneration is positive. However, all the available studies are experimental in nature and have been performed in different animal models. Future research based on clinical studies would provide the evidence required in terms of efficacy and safety before VEGF could be used in the clinical setting, as an agent for bone regeneration procedures.

Conflict of interest statement

All authors declare that no benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article. No funds were received in support of this study.

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