Influence of osteogenic stimulation and VEGF treatment on in vivo bone formation in hMSC-seeded cancellous bone scaffolds

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Abstract

Background: Tissue engineering approaches for reconstruction of large bone defects are still technically immature, especially in regard to sufficient blood supply. Therefore, the aim of the present study was to investigate the influence of osteogenic stimulation and treatment with VEGF on new bone formation and neovascularization in hMSC-loaded cancellous bone scaffolds in vivo.

Methods: Cubic scaffolds were seeded with hMSC and either cultured in stem cell medium or osteogenic stimulation medium. One osteogenically stimulated group was additionally treated with 0.8 μg VEGF prior to subcutaneous implantation in athymic mice. After 2 and 12 weeks in vivo, constructs and selected organs were harvested for histological and molecular analysis.

Results: Histological analysis revealed similar vascularization of the constructs with and without VEGF treatment and absence of new bone formation in any group. Human DNA was detected in all inoculated scaffolds, but a significant decrease in cells was observed after 2 weeks with no further decrease after 12 weeks in vivo.

Conclusion: Under the chosen conditions, osteogenic stimulation and treatment with VEGF does not have any influence on the new bone formation and neovascularization in hMSC-seeded cancellous bone scaffolds.

Keywords: hMSC, Tissue engineering, VEGF, Vascularization, Osteogenic stimulation

Background

Large bone defects caused by trauma, tumor or infection still represent a major problem in reconstructive surgery. Tissue engineering approaches for reconstruction of such bone defects are appealing, but to date technically insufficient. In this context, one major problem – the blood supply during the first days in vivo – remains unsolved. Since the rate of vessel ingrowth into a cell-loaded scaffold is hypothesized to be less than one millimeter per day, a considerable period is needed to provide sufficient blood supply and delivery of substrates such as glucose and oxygen to inner parts of the engineered scaffold [1,2]. Likewise, removal of waste products such as carbon dioxide and lactate via the bloodstream is delayed until a mature vascular system is established. Since bone represents a metabolically active tissue, the insufficient blood supply during the first days in vivo might compromise cell survival and hence lead to failure of the graft integration [2]. Therefore, various tissue engineering strategies have been developed to address this problem.

One promising approach is the use of growth factors such as vascular endothelial growth factor (VEGF), which plays an important role in inducing neovascularization and bone healing [3]. Kaigler et al. reported on significantly increased vascular perfusion and bone formation in irradiated osseous defects using VEGF-releasing polymer scaffolds [4]. Beside the improved blood supply, this phenomenon could also be ascribed to the VEGF-triggered bilateral communication between endothelial and osteogenic cell lineages leading to a broad proliferation of endothelial cells and...
differentiation of osteogenic progenitor cells into osteoblasts [5,6].

The ability of hMSCs for differentiation along several cell lineages is well known and depends on the tissue source, whereas a substantial loss of their multi-potent properties was observed during culture in vitro [7]. Although many preclinical animal models showed ectopic as well as orthotopic bone formation following implantation of 3D cell-loaded constructs, questions regarding the optimal osteogenic differentiation method for hMSCs as well as an effective initiation of osteogenesis in vivo have not been finally clarified.

Therefore, the aim of this study was to determine if VEGF of osteogenically stimulated hMSCs loaded on cancellous bone scaffolds is capable of enhancing neovascularization and bone formation in an ectopic mouse model.

Methods

Scaffold loading and cultivation of human mesenchymal stem cells

Human mesenchymal stem cells (hMSC) (Cambrex, East Rutherford, USA), which were harvested and purified from bone marrow aspirates of one healthy donor were purchased (Lonza, Basel, Switzerland) and cultivated in mesenchymal stem cell growth medium (MSCGM) (Lonza, Basel, Switzerland). Fresh medium was supplied three times per week. When cell layers approached confluence, cells were detached using trypsin-EDTA. Cultures were maintained in a humidified atmosphere of 95% air with 5% CO2 at 37°C.

The day before loading, cubic solvent-preserved and irradiated bovine cancellous bone scaffolds (Tutobone, Tutogen Medical, Neunkirchen am Brand, Germany) with an edge length of 3 mm were preincubated for 24 hours in MSCGM [8]. A suspension of 1.1 × 106 hMSCs (passage 4) in 660 μl medium was evenly applied to each scaffold. For maximum seeding efficiency, scaffolds were turned and the cell suspension was resuspended onto each construct every 20 minutes. After 6 hours, all scaffolds were transferred to well-plates for further incubation. The seeding efficiency was assessed by measuring the number of remaining cells within the supernatant suspension in each well.

Following seeding, the scaffolds were cultured for 14 days either in MSCGM or in case of osteogenic stimulation in Dulbecco’s Modified Eagle Medium (DMEM, Gibco, Invitrogen, Carlsbad, USA) containing 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, USA), 4 mM L-Glutamine, 100 nM Dexamethasone, 10 mM β-Glycerophosphate and 50 mM L-Ascorbic acid 2-phosphate. Medium was changed every third day. Sections of 12 μm thick sections were collected from each scaffold and used for molecular analysis (Figure 1b). In addition, selected organs (blood, brain, heart, lung, liver, kidneys, spleen, testes, scaffold surrounding skin and muscle tissue) were harvested, snap frozen in liquid nitrogen and stored at −80°C until further use.

This study adhered to the ARRIVE guidelines, was approved by the “Government of Upper Bavaria” and all animals were handled according to the LMU guidelines (Ludwig-Maximilians-University of Munich) for the care and use of laboratory animals.

Histological analysis

Scaffolds fixed in the formaldehyde/methanol solution were dehydrated in a graded series of ethanol over 5 days and afterwards embedded into methyl methacrylate (MMA). Next, 4 μm thick sections were collected from each scaffold by using a Polycut microtome (Reichert-Jung, Heidelberg, Germany). Sections were stained with Goldner’s Trichrome and Toluidine Blue.

Scaffolds fixed in methanol were decalcified with 5% EDTA/PBS solution over 4 weeks. The solution was changed every third day. Sections of 12 μm thickness were obtained by using a cryomicrotome (Microm, Walldorf, Germany) and then stained with Hematoxylin and Eosine.

All sections were investigated using a light microscope (Axioskop, Zeiss, Jena, Germany) and graded by 3 independent observers for the presence and extent of: i)
granulation tissue, ii) necrosis, iii) fat cells, iv) neovascularization and v) foreign body giant cells. The grading scale ranged from 1 (tissue extent 1-20% of the scaffold/single cells in the border areas) to 5 (tissue extent 80-100%/lots of cells in all areas) according to the established method by van Gaalen [9].

Extraction of genomic DNA
The isolation of DNA from the scaffolds was performed with DNeasy blood and tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. In brief, harvested tissue samples were lysed with proteinase K (>600 mAU/ml). The DNA in the lysed samples was then absorbed on a silica matrix and cell debris was removed by washing with DNeasy washing buffer. Finally, the DNA was collected by elution in AE buffer (Qiagen, Hilden, Germany) and photometrically quantified.

Semiquantitative polymerase chain reaction (PCR)
Detection of human DNA within the scaffolds and various organs was performed by genomic PCR. An 850 bp fragment of the α-satellite region of the human chromosome 17 was amplified using primers as previously described in Becker et al. [10].

Each PCR reaction (end volume of 50 μl) contained 250 ng of human genomic DNA, 200 μM of nucleotide mixture, 250 μM of each primer, 2.5 U Taq Polymerase and Q-solution (Qiagen, Hilden, Germany) supplemented with 15 mM MgCl₂.

An initial denaturation of 3 min. at 94°C was followed by 40 cycles comprised of 30 sec. at 94°C, annealing at 58°C for 1 min. and for another 1 min. a polymerase reaction at 72°C. Finally, 10 minutes of polymerisation and an interruption of the reaction by cooling down to 4°C completed the cycles. Amplified DNA fragments (850 bp) were transferred onto 1.75% agarose gels and after the electrophoresis, they were stained with ethidium bromide and analysed using ultraviolet light.

Isolated DNA from hMSCs served as positive control while DNA extracted from murine fibroblasts served as negative control in each PCR run. To prove the PCR sensitivity, genomic DNA mixture containing DNA from murine fibroblasts and hMSCs in different ratios (1 × 10⁶ murine fibroblasts + 10 hMSCs and 1 × 10⁶ mouse fibroblasts + 1 hMSC) was also used in each PCR run.

Quantitative PCR
Quantitative PCR was performed by using LightCycler technology (Roche, Mannheim, Germany). A specific human primer set was purchased from Search-LC (Search-LC, Heidelberg, Germany). The final volume of each PCR-reaction was 20 μl containing 10 μl of 5 ng genomic DNA, 10 μl of a PCR-Mix (6 μl PCR grade water, 2 μl of primers and 2 μl of LightCycler Fast Start Master SYBR Green I; Roche).

Each PCR cycle consisted of a 10 second denaturation at 95°C followed by annealing at 68°C for 10 sec. with a transition to 58°C in steps of 0.5°C per cycle. Elongation was performed at 72°C for 16 seconds during which fluorescence intensity was measured in each cycle. The temperature transition rate of all steps was 20°C per second. Serial dilution steps of human genomic DNA in 10 μl distilled water (60 ng, 6 ng, 0.6 ng, 0.06 ng, 0.006 ng, 0.0006 ng DNA) were used to generate standard curves. In every PCR, DNA
from hMSCs was used as a positive control, while DNA from murine fibroblasts served as a negative control.

Finally, the number of cells on the scaffold after explantation was calculated by the measured amount of human DNA and the common presumption of a genomic DNA content of 6 pg in eukaryotic cells.

Statistical analysis
Comparison analysis was performed with the quantitative PCR data using SPSS software (IBM, Armonk, USA) and the unpaired t-test. The results are shown as mean ± standard deviation. A p-value of ≤0.05 was considered statistically significant.

Results
Seeding efficiency
From the original amount of 1.1 × 10^6 hMSCs applied onto the scaffolds, a mean of 7.1 × 10^5 cells (median 7.0 × 10^5 cells, range 4.1 × 10^5 – 9.1 × 10^5 cells) adhered to the scaffold (Figure 2). The mean seeding efficiency of all scaffolds was 64.8% (median 64.1%, range 41.7% – 83.3%). The statistical evaluation of seeding efficiencies did not show significant differences between the four experimental groups.

Animal experiments
All animals showed good general conditions, dry wounds with no sign of irritation as well as appropriate behavior at the time of explantation. No animals died or suffered from diseases during the whole study duration prior to euthanasia.

Histological findings
After an implantation period of 2 weeks, an incipient ingrowth of fibrous- and granulation tissue as well as small vessels was observed in each scaffold. In all groups (1. unstimulated empty scaffolds, 2. inoculated with hMSCs, 3. inoculated with osteogenic stimulated hMSCs, 4. inoculated with osteogenically stimulated hMSCs and treated with VEGF), newly formed capillaries and small vessels were detected, lying evenly distributed mainly in the outer regions of the scaffold (Figure 3). Granulation tissue was also observed in all groups (Figure 4). Inner parts of the constructs contained scattered fat cells, inflammatory cells, and sometimes necrotic areas. Multinucleated giant cells were found in scaffolds from all groups. These cells were mostly aligned in groups of 2–4 along the edge of the scaffolds. Fat cells were observed within loose fibrous tissue, especially in outlying areas covering more than 20% of the scaffold surface. No apparent differences in the occurrence and distribution of necrotic areas were detected. Newly formed osteoid, bone- or cartilage tissue was not apparent after an implantation period of 2 weeks.

In the 12 weeks group, further ingrowth of the surrounding tissue into the constructs occurred. Especially, a change in the vascularization and the appearance of bigger fat cell aggregations was noticeable in all scaffolds (Figure 5). Compared to the histological results after 2 weeks of implantation, a clear increase in size and amount of vessels was detected after 12 weeks in all groups. Capillaries, venules and arterioles were evenly distributed over all parts of the scaffolds. Furthermore, an increased expansion of fat cells was found in all groups, covering up to 60% of the scaffold surface in some cases (Figure 6). The highest extent of fatty tissue was observed in unstimulated empty (group 1) and inoculated scaffolds (group 2), while the lowest extent was found in osteogenically stimulated constructs (group 3). Next to the fatty tissue, flanking necrotic areas were detected. Multinucleated giant cells showed an inhomogeneous distribution pattern and were found in the scaffold periphery but also in central areas. After an implantation period of 12 weeks, neither newly formed osteoid nor bone or cartilage tissue was obtained.

No differences regarding vascularization or tissue reaction and formation were observed between the groups independent from the different culture and stimulation conditions.

Semi-quantitative PCR
Human DNA was found in all inoculated scaffolds after explantation at both points in time. In blank scaffolds, no human DNA was detected. Moreover, we did not detect human DNA in any of the explanted organs as well as in the soft tissue surrounding the implanted scaffolds. Cell suspensions with hMSCs and murine cells served as controls allowing a detection of even 1 hMSC in 10^6 murine cells.

Quantitative PCR
With real time PCR, a significant decrease (p <0.001) of human DNA and accordingly the directly proportional cell numbers on all cell-seeded constructs (2 and 12 weeks in vivo) could be measured compared to the amount of initially seeded cells (Figure 7). After 12 weeks, a further
decrease was detected in group 2 (inoculated) and 4 (inoculated with osteogenically stimulated hMSCs and VEGF treatment). Only for group 3 (seeded with osteogenically stimulated hMSCs), a slight increase of the cell number was observed after 12 weeks compared to 2 weeks (Figure 8). We identified higher cell numbers in the scaffolds with additional VEGF (group 4) after 2 weeks in comparison to just osteogenically stimulated scaffolds (group 3). After an implantation time of 12 weeks, group 4 showed inferior cell numbers. However, the above-mentioned differences were statistically not significant.

Discussion
In this study, we aimed to evaluate the influence of osteogenic stimulation and application of vascular endothelial growth factor (VEGF) on bone formation in hMSC-seeded cancellous bone scaffolds after 2 and 12 weeks of subcutaneous implantation. During the implantation period, an ingrowth of surrounding host tissue was observed in all groups. Migration of human cells to host organs after xeno- genetic implantation was not seen in any of the animals, thus confirming previous results of our group [8]. Human DNA was detected in explanted scaffolds at all points in time, but a significant decrease in cells within the scaffolds could be measured in vivo compared to the amount of initially seeded cells. Neither bone formation nor higher amounts of neovascularization was observed. According to the journal guidelines ARRIVE check list for this study has been provided as Additional file 1.

hMSC cultivation
The high amount of mesenchymal stem cells (hMSCs) within the commercially available cells used in this study was proven and described previously [11]. However, authors reporting on successful in vivo regeneration of bone often used BMSCs (Bone Marrow Stromal Cells) gained from bone marrow containing quantitatively more pre-differentiated progenitor cells than hMSCs [12,13]. The presence of such progenitor cells, which can be expanded in vitro by osteogenic stimulation, is crucial for ectopic bone formation [14,15]. In this context, additional contribution of BMP-2 and FGF-2 should be taken into account since an increased osteoblastic differentiation in vitro might have positive effects on ectopic bone formation in vivo [16].

The lack of newly formed bone could also be attributed to the static cultivation procedure. Since an ideal nutrient supply plays a major role during the initiation and
maintenance of cell differentiation processes in vitro, a
dynamic cultivation could be beneficial. Dynamic com-
pared to static cultivation of osteogenically stimulated
load matrix constructs was shown to enhance the forma-
tion of an extracellular matrix in vitro which facilitates
more new bone formation in vivo [17]. Additionally, the
differentiation of osteogenically stimulated cells along
the osteoblastic cascade is positively influenced by cyto-
mechanical forces actuated within dynamic culture
tanks [18]. In this context, various methods to stimulate
load cells with mechanical forces were reported including
tensile stress, compressive stress, shear stress, vibration
and magnetic stimuli [19].

A decrease of oxygen concentration towards the cen-
ter of the scaffold was shown under static cultivation,
whereas the oxygen concentration of dynamically cul-
tured constructs did not fall below 4% after 5 days [20].
These data reinforce the assumption that a considerable
number of cells probably died during static cultivation
in vitro due to a decreasing oxygen gradient towards the
inner parts of the construct. This could be one reason
for our results which show a significant decrease of cell
numbers after 2 weeks in vivo but no further significant
decrease after 12 weeks.

Vasculature
Taking into consideration that capillaries were predom-
nantly located in the outer scaffold areas after 2 weeks
in vivo, a further reason for the considerable decrease of
hMSCs might be the lack of a sufficient vascular supply
[8]. In our opinion, physical prerequisites regarding the
diffusion threshold of 200 μm – 3.5 mm were met by the
use of small cubic scaffolds with an edge length of 3 mm
[21,22]. However, considering the obtained results, cell
death must be assumed at least for inner cell layers
within the scaffold. Likewise, Potier et al. reported on
broad cell death in vitro after a maximum of 5 days
when the cells were exposed to hypoxia and deprivation
of serum [23]. In contrast, maintaining constant or pre-
conditioning levels of hypoxia was shown to improve
the osteogenic potential of hMSCs and possibly preserve
the stem cell character of hMSCs in vitro [24]. The fact
that even in the VEGF group sufficient vascular supply
was not initiated and cell survival was not achieved
could be attributed to the biological instability of VEGF
in vivo as well as the short stimulation time [3]. This
shortcoming could be overcome by the use of novel scaf-
dolds binding VEGF by heparin crosslinking and thus,
achieving localized and sustained delivery of this growth

Figure 4 Tissue after 2 weeks. Granulation tissue (*) was observed in all groups after 2 weeks in vivo. (Gp = Group, os-hMSCs = osteogenically
stimulated hMSCs) Hematoxylin-Eosin: scale bars: 200 μms.
factor [25]. Consistently, improved angiogenesis after subcutaneous implantation of heparinized scaffolds loaded with VEGF was observed [3]. Further improvements could be achieved by developing prevascularized cell-matrix-constructs providing a sufficient nutrient supply at early stages after implantation [26]. Prevascularized constructs, allowing an early functioning metabolic cycle, were shown to improve the survival rate of osteoblasts in vivo compared to non-prevascularized constructs [27].

**Tissue**

The initiation of inflammatory processes by implantation of biological or synthetic materials is evident [8,28,29]. Essential parameters for the character and value of biological reaction are material properties and quality of the implanted foreign body [30]. Therefore, surface enlargement by means of interconnecting pores could be responsible for an increased invasion of reactive cells leading to foreign body reactions even in immunodeficient, athymic mice [31]. This might affect the survival of cells as a decrease of pH and release of degradation enzymes lead to changes in homeostasis [30]. If and to which extent phagocytosis of human mesenchymal stem cells could take place in the course of such foreign body reactions remains unclear [32,33].

Development of granulation tissue was observable in all groups after 2 weeks in vivo. We suggest that this phenomenon is to be explained by the physiological tissue chronology during foreign body reactions [30]. The production of e.g. TNFx or EGF by macrophages is responsible for the initiation of neovascularization and proliferation of fibroblasts resulting in the formation of granulation tissue, which is replaced by permanent tissue afterwards. Consistent with previous results, a regression of granulation tissue was observed after 12 weeks in all groups [8].

No bone formation was observed in any of the scaffolds indicating an insufficient amount of osteoprogenitor cells as well as vascular supply. Low osteogenic capacity of the ectopic implantation site as well as missing osteoinductive properties of the scaffold must be assumed as possible reasons for our results [15]. Therefore, implantation of the scaffolds in orthotopic sites might improve bone formation by chemotactic agents. However, Kruyt et al. have already demonstrated no significant difference in cell-based bone formation between ectopic and orthotopic implantation [34].

The study is limited, beside the abovementioned shortcomings, especially by a small sample size. Therefore,
further studies are needed to clarify if VEGF is capable of stimulating murine cells. Alternatively, the use of co-cultures such as osteoblasts and endothelial cells could help to induce a sufficient blood supply in vivo and hence the new formation of bone.

**Conclusion**

Under the chosen prerequisites, VEGF treatment of osteogenically stimulated hMSCs loaded on cancellous bone scaffolds is not sufficient to enhance neovascularization, bone formation and/or improved cell survival. Human DNA can be found in inoculated scaffolds even...
after 12 weeks in vivo but not in organs or the soft tissue surrounding following xenotransplantation.

**Additional file**

**Additional file 1: ARRIVE check list.**

**Abbreviations**

VEGF: Vascular Endothelial Growth Factor; hMSC: human Mesenchymal Stem Cells; EDTA: Ethylenediaminetetraacetic acid; CO2: Carbon dioxide; MSCGM: Mesenchymal Stem Cells Growth Medium; DiWEM: Dubecco’s Modified Eagle Medium; PBS: Fetal Bovine Serum; MMA: Methyl Methacrylate; PBS: Phosphate Buffered Saline; DNA: Deoxyribonucleic acid; PCR: Polymerase Chain Reaction; SPSS: Statistical Package for the Social Sciences; BMSCs: Bone Marrow Stromal Cells; BMP-2: Bone Morphogenetic Protein-2; FGF-2: Fibroblast Growth Factor-2; pH: potential of hydrogen; TNFα: Tumor Necrosis Factor α; EGF: Epidermal Growth Factor.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

UL and FP were in charge of data acquisition, drafting and writing the manuscript and contributed to the conception of the study. SS, CE, SM and DD participated in the conception and design of the study, helped to draft the manuscript and gave substantive intellectual feedback on the manuscript. DD and MS contributed to the conception and the design of the manuscript and contributed to the conception of the study. SS, CE, SM and DD participated in the conception and design of the study, helped to draft the manuscript and gave substantive intellectual feedback on the manuscript. All authors read and approved the final manuscript.

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