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Observations on the microvasculature of bone defects filled with biodegradable nanoparticulate hydroxyapatite

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ABSTRACT

The microvascularization of metaphyseal bone defects filled with nanoparticulate, biodegradable hydroxyapatite biomaterial with and without platelet factors enrichment was investigated in a minipig model. Results from morphological analysis and PECAM-1 immunohistochemistry showed the formation of new blood vessels into the bone defects by sprouting and intussusception of pre-existing ones. However, no significant differences were observed in the microvascularization of the different biomaterials applied (pure versus platelet factors-enriched hydroxyapatite), concerning the number of vessels and their morphological structure at day 20 after operation. The appearance of VEGFR-2 positive endothelial progenitor cells in the connective tissue between hydroxyapatite particles was also found to be independent from platelet factors enrichment of the hydroxyapatite bone substitute. In both groups formation of lymphatic vessels was detected with a podoplanin antibody. No differences were noted between HA/PLF− and HA/PLF+ implants with respect to the podoplanin expression level, the staining pattern or number of lymphatic vessels. In conclusion, the present study demonstrates different mechanisms of blood and lymphatic vessel formation in hydroxyapatite implants in minipigs.

1. Introduction

The integration of bone graft material during ossification of bone defects depends on the implants' microvascularization [1–3]. Investigations of the embryogenesis, oncogenesis and wound healing have shown various possible angiogenetic mechanisms that result in neoformation of vessels [4–7].

Angiogenesis is defined as the process of capillary formation by intussusception or invagination in pre-existing vessels [8–10]. Intussusception is commenced by the proteolysis of the vessels intima which results in vasodilatation and fenestration of the vessels' basal lamina. The induced increase in permeability leads to extravascularisation of plasma proteins like fibrinogen and plasminogen, both necessary as matrix for endothelial cell migration [11]. Lumina of vessels are formed by proliferation and adhesion of endothelial cells. The maturation of newly formed vessels is characterized by the completion of the basal lamina. Their stabilization is induced by extracellular matrix proteins, e.g. fibronectin [12], and the adhesion of α-smooth muscle actin-positive pericytes [13]. During the process of intussusception, invagination of opposing capillary walls extends into the lumen and thereby building a bilayer. The perforated bilayer allows endothelial cells, fibroblasts or pericytes to penetrate into the lumen which provides an extracellular matrix and finally split the single vessel into two [9,10,14]. To date, intussusceptive newly vessel formations have been observed only in the pulmonary circulation [15] and skeletal muscles [16].

Vasculogenesis can be defined as the process of differentiation of endothelial cells derived from haemotopoietic precursor cells called angioblasts, which are positive for VEGFR-2 (Flk-1) [17–20]. Angioblasts migrate and merge close to the blood clots and finally differentiate into endothelial cells to develop a primitive vessel tube. There is a final maturation of the vascular tree by pruning and extension through angiogenesis, and by completion of the basal lamina and adhesion of pericytes.

During embryogenesis both mechanisms of vessel neoformation exist. The postnatal formation of new vessels is also achieved by

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vasculogenesis in endodermal tissue of the lung [21], while angiogenesis dominates in ectodermal and mesodermal development of the kidneys [6]. Previous reports suggest that endothelial cells are capable of forming a system of veno-lymphatic vessels [22–24]. However, knowledge on the molecular mechanisms during lymphangiogenesis is not available to date. Edwards et al. [25] could only detect lymphatic vessels in connective tissue overlying the periosteum, but not in cortical or cancellous bone.

To our knowledge, the role of biomaterial-associated lymphatic vessel formation has not been well investigated. Formation of new vessels is regulated by cell adhesion molecules, e.g. PECAM-1, and cytokines, e.g. VEGF, bFGF, and PDGF [9,17,26–29]. By application of an excess of growth factors, the process can also be overstimulated leading to uncontrolled vessel formation [9]. Earlier investigations of the microvascularization during fracture healing in diaphyseal bone have demonstrated blood vessel formation by sprouting and vasculogenetic processes [20].

Thus, our study aims were to investigate the molecular mechanisms and morphology of the microvasculature, including lymphangiogenesis of metaphyseal bone defects filled with biodegradable nanoparticles of hydroxyapatite implants in miniature pigs. Further, additional effects of autologous platelet factors on microvascularization of implants were tested.

2. Materials and methods

2.1. Nanoparticulate biodegradable hydroxyapatite

The hydroxyapatite (HA) used in the present study is the fully synthetic nanoparticulate paste Ostim® (Coripharm, Obernburg, Germany) and consists of a suspension of pure hydroxyapatite in water prepared by a wet chemical reaction. The needle shaped hydroxyapatite crystals with a size of 21 nm in a-direction and of 36 nm in c-direction form agglomerates. Phase purity of the hydroxyapatite was determined by X-ray-diffraction which showed conformity with pure HA and an average crystallite size of 18 nm. The atomic ratio of calcium:phosphorus is 1.67. Ostim® paste does not harden after application into the bone and is free of endothermal heating in contrast to calcium phosphate bone cements [30,31].

2.2. Animal models

The study protocol was approved by an independent institutional review board prior to surgery. A total of 28, 9-month-old male Lewe miniature pigs (SBMF Laboratories, Dresden, Germany) were used for the current randomized study in which a cylindrical defect with a diameter of 8.9 mm and a depth of 10 mm was created in the subchondral region of the right femur condyle using a saline cooled diamond bone-cutting system (DBCS, Stryker, Duisburg, Germany). Miniature pigs were divided into two groups. The defects in pigs of group 1 were filled with pure hydroxyapatite (HA/PLF−). The animals of group 2 received composites of hydroxyapatite, enriched with platelet factors (HA/PLF+) in a 10:1 ratio of HA to PLF (1.8 ml Ostim® 33% + 0.3 ml platelet factors). After 10 or 20 days under general anaesthesia, operated animals were euthanasized by intravenous application of embutramid mebezonium iodide tetracaine (T 61®, CliniPharm, Zürich, Switzerland). For morphological investigations three animals per group were perfused through the femoral artery with 600 ml of heparinized Ringer solution, followed by 600 ml 4% paraformaldehyde (PFA) in 1% phosphate buffered saline (PBS). After euthanasia distal femora of all animals containing the defect areas was removed, dissected and stripped free of all soft tissues. The implant with neighbouring original bone of each femur was transversally cut into four specimens which were subjected to different fixation methods for further investigation. For light microscopical, immunohistochemistry and immunofluorescence preparations, all samples were additionally fixed in 4% PFA overnight. Thereafter, the samples were carefully washed with phosphate buffered saline (PBS), decalcified for 6 weeks in EDTA and embedded in paraffin. For electron microscopic investigations the samples were additionally fixed overnight in Yellow-Fix (4% paraformaldehyde, 2% glutaraldehyde, 0.04% picric acid) and embedded in Epon. Snap-frozen unfixed samples were used for PCR and Western blots.

2.3. Autologous platelet factor (PLF)

One hundred and fifty ml of peripheral blood of the external jugular vein was centrifuged at 100*g* for 10 min at RT. Platelet-rich plasma was separated and again centrifuged at 300*g* for 10 min, followed by withdrawal of the plasma and another centrifugation step at 1000*g* for 30 min. The platelet-rich plasma supernatant was carefully transferred into a preconnected bag for further preparation of the growth factors. One thousand IU thrombin (Gentrac Inc., Middleton, Massachusetts, USA) and 10 ml of 8.4% calcium-gluconate (Braun Melsungen AG, Melsungen, Germany) were added for aggregation and degranulation of the platelets. After degranulation, the liquid supernatant was filled into tubes and stored at −20 °C. The plasma was heat-inactivated at 65 °C for 30 min and again centrifuged at 1000*g* for 30 min at RT. The liquid supernatant was then transferred into tubes and stored at −20 °C.

For measurements of platelet cytokines PDGFs (AA, BB, AB), TGF-β_1, VEGF, IGFs (I, II), EGF and bFGF, Quantikine™ ELISA-kits (R&D Systems Minneapolis, Minnesota, USA) were used (Table 1).

2.4. Isolation of RNA

Total RNA isolation from snap-frozen granulation tissue of implants (HA/PLF− and HA/PLF+) was performed according to the manufacturers' guidelines with Trizol isolation reagent (Invitrogen, Karlsruhe, Germany). The yield of total RNA was determined in a BioPhotometer (Eppendorf, Hamburg, Germany) using UV measurement. DNase digestion was done by incubation with RNasefree DNase at 37 °C for 30 min. The yield of total RNA was finally determined spectroscopically at a wavelength of 280 nm.

2.5. Reverse transcription

Reverse transcription was carried out in a T3 Thermocycler (Biometra, Göttingen, Germany) using the method described by Welter et al. [32], the reaction condition was as follows: 2 μg of total RNA, 2.5 μM random hexamers (Invitrogen, Karlsruhe, Germany), 25 μM of each dNTP (Roche, Mannheim, Germany), 1 × RT buffer and M-MLV reverse transcriptase (Promega, Madison, Wisconsin, USA), incubation at 37 °C for 10 min and 75 °C for 5 min. The reverse transcription reaction was stopped by heating to 92 °C for 2 min.

2.6. PCR reaction

Commercially synthezised primers (MWG, Ebersberg, Munich, Germany) used to amplify specific porcine mRNA-transcripts [32] are described in Table 2.

The annealing temperature of each primer pair was optimized in a T3 Thermocycler (Biometra, Göttingen, Germany). A volume of 2 μl of each transcribed cDNA-preparation and 5 U of AmpliTaq Gold 1000 kit (Biosystems, Middletown, Massachusetts, USA) were used for PCR reaction. Amplicons were generated with an initial denaturation step (99 °C, 2 min) and 35 cycles of denaturation at 94 °C for 45 s, annealing at 60 °C for 45 s, extension at 72 °C for 45 s, followed by a final extension step of 2 min at 72 °C. The PCR products were analyzed by gel electrophoresis in a 2% agarose gel with 1 μg/ml ethidium bromide. Water was used instead of cDNA as a negative control.

2.7. Western blot

The specificity of the anti-VEGFR-2 (Flk-1) antibody was tested by Western blotting. Total proteins of homogenates were extracted from snap-frozen specimens of the distal minipig femora with Trizol (Invitrogen, Karlsruhe, Germany) and dissolved in Laemmli sample buffer (Sigma–Aldrich, Munich, Germany). Proteins were separated by means of SDS-PAGE by using a 10% acrylamide gel (Invitrogen, Karlsruhe, Germany). As marker, the dual color standard marker (BioRad Lab., Hercules, California, USA) with specific weight of 250 kDa was applied. The separated proteins were transferred by electroblotting with a Hybond PVDF membranes (Amersham Biosciences, Piscataway, New Jersey, USA). Non-specific protein binding sites were blocked with PBS containing 5% BSA buffer (Merck, Darmstadt, Germany) and were incubated with mouse anti-VEGFR-2 (Flk-1) antibody (Santa Cruz, Heidelberg, Germany, diluted 1:100) overnight at 4 °C. After several washes in PBS, containing 1% BSA and 1% Tween 20 (Sigma, Taufkirchen, Germany), the membranes were incubated with the secondary antibody (mouse anti-rabbit IgG, DAKO, Hamburg, Germany, diluted 1:500) for 45 min, before careful washing in TRIS-buffer. For visualization of the antigen–antibody complexes the APAAP method was used.

Table 1
Concentration of platelets (pt/ml) and various growth factors (pg/ml) in the applied mixture of platelet factors

Platelets	1,021,900 ± 345,956
TGF-β_1	69,000 ± 20,500
PDGF-AA	639 ± 423
PDGF-BB	578 ± 486
PDGF-AB	162,000 ± 68,500
VEGF	<10
bFGF	110 ± 85
EGF	311 ± 220
IGF-I	57,000
IGF-II	284