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Accelerated vascularization of tissue engineering constructs *in vivo* by preincubated co-culture of aortic fragments and osteoblasts

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ABSTRACT

There is an urgent critical need for the development of clinically relevant tissue-engineered large bone substitutes that can promote early vascularization after transplantation. To promote rapid blood vessel growth in the engineered tissue, we preincubated aortic fragments, as well as, co-cultures of aortic fragments and osteoblast-like cells in matrigel-filled PLGA scaffolds before implantation into the dorsal skinfold chambers of balb/c mice. Despite an acceptable and low inflammatory response, preincubated aortic fragments accelerate early angiogenesis of tissue-engineered constructs; the angiogenesis was found to occur faster than that observed in previous studies. Thus, the time-period for achieving a denser microvascular network could be reduced to half. In addition, co-culture with osteoblasts enhances this angiogenic effect significantly (against preincubated aortic fragments alone). During the preincubation period, aortic fragments begin to form a network of vessel-like structures additionally supported by osteoblast-like cells. After transplantation, further development of a dense microvasculature continues rapidly. Therefore, preincubation of aortic fragments, especially in co-culture with osteoblast-like cells, in 3D extracellular matrices supports the rapid vascularization of tissue-engineered constructs. This method is a promising approach to establish a dense microvascular network in these constructs.

1. Introduction

In reconstructive surgery, due to the lack of suitable alternative treatment options, autogenous bone grafts are the gold standard used for the treatment of large bone defects [1–4]. However, autografts have disadvantages with regard to donor-site morbidity and limited availability [5–7]. The concerns related to allogeneic bone grafting are the transmission of diseases and immune responses that can ultimately lead to graft failure [8]. Therefore, the development of tissue-engineered bone substitutes has become a major focus of research in biomaterial science. Appropriate three-dimensional (3D) constructs have to be histocompatible, osteoconductive, and osteoinductive. Suitable scaffolds seeded with autogenous osteoblasts fulfill these needs [9–11]. However, to avoid failure of the biomaterial after implantation, rapid vascular integration into the host organism is essential. Only sufficient blood supply by perfusion to the implanted scaffold can provide adequate oxygen and nutrients to the cells within the scaffolds, and waste disposal satisfying the metabolic requirements of the construct [12,13].

Direct application of angiogenic growth factors, such epithelial growth factor, basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF) (in particular), have been widely used to expedite vascularization [14–16]. Coating the scaffolds

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with Matrigel, a nutrient solution mainly consisting of extracellular matrix proteins such as laminin, entactin, collagen, and heparan sulfate proteoglycans, the use of which favors angiogenesis, has also been performed successfully [17]. Appropriate scaffolds have been cultured with undifferentiated multipotent bone marrow mesenchymal stem cells (bmMSCs), endothelial cells, organ specific cells, *e.g.* osteoblast-like cells (OLCs), and with co-cultures of different cell types to accelerate vascular integration of tissue-engineered constructs [16,18,19]. Recently, aortic fragments seeded in Matrigel have been shown to form a dense network of tubular vascular sprouts originating from the aortic explants when cultured in 3D extracellular matrices [20–22]. Also Shepherd et al. showed that vessel fragments cultured in a collagen I gel inosculate with the host circulation after implantation and remodel to contain morphologically identifiable arteries, arterioles, capillaries, venules, and veins assembled into a perfused, vascular bed [23]. In previous studies we have combined several of these strategies to advance the early development of vascular structures in tissue-engineered constructs [16,20,24,25]. The addition of VEGF to PLGA scaffolds seeded with mesenchymal stem cells has been shown to enhance vascularization [25]. We have proven *in vivo* the angiogenic effect of bmMSCs, OLCs and combinations of these cell types but without any additive effect on angiogenesis caused by co-cultures [16]. Seeding of Matrigel-filled PLGA scaffolds with aortic fragments and bmMSCs causes a significant enhancement in vascularization after implantation of these tissue-engineered constructs into the dorsal skinfold chambers of balb/c mice. Thereby, the angiogenic effect of co-cultured aortic fragments and bmMSCs does not differ from the amount of newly formed microvessels caused by aortic fragments or bmMSCs alone [20]. Additionally, we could show *in vivo* that early angiogenesis of tissue-engineered constructs can be accelerated significantly by the use of bmMSCs and OLCs preincubated in Matrigel for two weeks [24]. However, the effect of vessel fragments preincubated in Matrigel on the new formation of a microvascular network has not been studied yet.

Based on these findings, we continued and determined the angiogenic effect of the preincubation of aortic fragments in Matrigel for two weeks on the development of vascular structures in tissue-engineered constructs *in vivo*, expecting a clear additive effect on the vascular network because of the recently shown promising results on angiogenesis caused by aortic fragments seeded in Matrigel [20–22]. Thereby, accelerating the early vascularization of transplanted tissue-engineered constructs was the main target of the study. With regard to the long-term objective of engineering bone substitutes, we also describe the effect initiated by a co-culture with OLCs. Another reason for the application of OLCs was the expected additional effect on angiogenesis according to studies demonstrating a positive effect on vascularization in tissue-engineered constructs for co-cultures of different endothelial cell lineages and osteoblasts [26–29]. Thus, aortic fragments were preincubated in Matrigel-filled porous PLGA scaffolds with and without OLCs. After preincubation and implantation of the tissue-engineered constructs into dorsal skinfold chambers of balb/c mice, we analyzed angiogenic and inflammatory parameters using repetitive intravital fluorescence microscopy.

2. Material and methods

The current study represents a further development of a large-scale and continuous study on the early acceleration of vascularization of tissue-engineered constructs *in vivo* performed by the authors [16,20,24,25,30–32]. To guarantee the comparability of results within this experimental series, the methodology had to be similar to our previous studies. Therefore, the materials and methods are briefly summarized with references to further details.

2.1. Animals

The experiments were conducted in accordance with the German legislation for the protection of animals and the NIH Guidelines for the Care and Use of Laboratory Animals (NIH Publication No. 85-23 Rev. 1985). The experiments were approved by the local governmental animal care committee (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, reference number 33.9-42502-04-08/1437). Male balb/c mice used for the experiments were 8- to 15-weeks-old with a body weight of 18–22 g (Central Animal Laboratory, Hanover Medical School and WIGA Charles River, Sulzfeld, Germany). Keeping of animals was conducted according to a standard protocol [16,20,24].

2.2. Preparation of the dorsal skinfold chamber

Surgery, scaffold implantation and repetitive intravital fluorescence microscopy were performed under well-established anesthetic procedures [16,20,24].

The dorsal skinfold chamber allowed intravital microscopic surveillance of microcirculation in the anesthetized animal over an extended period. The surgical techniques for the preparation of the dorsal skinfold chamber and scaffold implantation have been previously described [33]. Within this experimental setup PLGA scaffolds where implanted after a minimum 48 h recovery period subsequent to surgical preparation.

2.3. Fabrication of tissue-engineered constructs

PLGA scaffolds with dimensions of $3 \times 3 \times 1$ mm were fabricated at the Freiburg Institute for Material Research and Macromolecular Chemistry as previously described [34].

For the incorporation of Matrigel (BD Biosciences, Bedford, MA, USA) into the PLGA scaffolds, the scaffolds were carefully embedded in 20 μL of Matrigel to allow the liquid Matrigel to enter all scaffold pores. To prepare PLGA scaffolds with OLCs, cells were derived from mouse calvarial bone and expanded *in vitro* as previously described [16]. OLCs were characterized by rabbit anti-mouse collagen I (Biotrend, Köln, Germany), rabbit anti-mouse osteocalcin (Acris Antibodies GmbH, Hiddenhausen, Germany) and rat anti-mouse osteonectin (SPARC, R&D Systems, Wiesbaden, Germany) [35]. For additional characterisation of OLCs, histochemical alkaline phosphatase determination was performed [36]. Cultured OLCs were gently rinsed with HBSS (PAA, Coelbe, Germany) and then incubated with Trypsin/EDTA (PAA). Cells released from the culture surface were washed two times with culture medium and then counted. Subsequently, 1×10^4 OLCs were resuspended in 20 μL of liquid Matrigel and dispensed on the scaffolds, allowing the liquid Matrigel to enter all scaffold pores. PLGA scaffolds filled with Matrigel and aortic fragments were also prepared according to an established protocol [20]. Briefly, aortas from 6-week-old male balb/c mice were isolated, dissected and minced into small fragments of approximately 200 μm diameter. A small portion, corresponding to approximately 5 μL, of the aortic fragments was mixed with 15 μL Matrigel and dispensed onto PLGA scaffolds, allowing the mixture to enter all scaffold pores. Scaffolds with OLCs and aortic fragments in Matrigel were prepared by combining 1×10^4 OLCs, approximately 5 μL aortic fragments, and 15 μL Matrigel. These components were mixed and dispensed onto PLGA scaffolds, ensuring that all scaffold pores were filled with the mixture. Each time the Matrigel was allowed to polymerize for 30 min at 37 °C and 5% CO_2.

The prepared scaffolds were either directly implanted into the dorsal skinfold chamber of the balb/c mice (no preincubation) or were preincubated for 14 days in endothelial growth medium