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Effects of locally applied adipose tissue-derived microvascular fragments by thermoresponsive hydrogel on bone healing

M. Orth [a,b,*], M.A.B. Altmeyer [a,b], C. Scheuer [b], B.J. Braun [a,b], J.H. Holstein [a,b], D. Eglin [c], M. D'Este [c], T. Histing [a,b], M.W. Laschke [b], T. Pohlemann [a], M.D. Menger [b]

[a] Department of Trauma, Hand and Reconstructive Surgery, Saarland University, 66421 Homburg, Germany
[b] Institute for Clinical and Experimental Surgery, Saarland University, 66421 Homburg, Germany
[c] AO Research Institute Davos, Davos, Switzerland

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ABSTRACT

Insufficient vascularization is a major cause for the development of non-unions. To overcome this problem, adipose tissue-derived microvascular fragments (MVF) may serve as vascularization units. However, their application into bone defects needs a carrier system. Herein, we analyzed whether this is achieved by a thermoresponsive hydrogel (TRH). MVF were isolated from CD-1 mice and cultivated after incorporation into TRH, while non-incorporated MVF served as controls. Viability of MVF was assessed immunohistochemically over a 7-day period. Moreover, osteotomies were induced in femurs of CD-1 mice. The osteotomy gaps were filled with MVF-loaded TRH (TRH + MVF), unloaded TRH (TRH) or no material (control). Bone healing was evaluated 14 and 35 days postoperatively. MVF incorporated into TRH exhibited less apoptotic cells and showed a stable vessel morphology compared to controls. Micro-computed tomography revealed a reduced bone volume in TRH + MVF femurs. Histomorphometry showed less bone and more fibrous tissue after 35 days in TRH + MVF femurs compared to controls. Accordingly, TRH + MVF femurs exhibited a lower osseous bridging score and a reduced bending stiffness. Histology and Western blot analysis revealed an increased vascularization and CD31 expression, whereas vascular endothelial growth factor (VEGF) expression was reduced in TRH + MVF femurs. Furthermore, the callus of TRH + MVF femurs showed increased receptor activator of NF-κB ligand expression and higher numbers of osteoclasts. These findings indicate that TRH is an appropriate carrier system for MVF. Application of TRH + MVF increases the vascularization of bone defects. However, this impairs bone healing, most likely due to lower VEGF expression during the early course of bone healing.

Statement of significance

In the present study we analyzed for the first time the in vivo performance of a thermoresponsive hydrogel (TRH) as a delivery system for bioactive microvascular fragments (MVF). We found that TRH represents an appropriate carrier for MVF as vascularization units and maintains their viability. Application of MVF-loaded TRH impaired bone formation in an established murine model of bone healing, although vascularization was improved. This unexpected outcome was most likely due to a reduced VEGF expression in the early phase bone healing.

1. Introduction

Delayed bone healing and non-unions are significant clinical problems. Their efficacious and rapid treatment is of pivotal importance to the patient, physician and the healthcare system [1,2]. A major cause of impaired or even failed bone healing is an insufficient vascularization between the bone fragments [2,3].

In order to improve bone repair, multiple approaches have been developed. To date, the transplantation of autologous bone is still considered to be the 'gold-standard' for bone grafting [4]. However, its use is restricted due to limited supply and donor-site morbidity as e.g. pain, injury, hematoma and fracture [5]. Given the fact that

* Corresponding author at: Department of Trauma, Hand and Reconstructive Surgery, Saarland University, Kirrberger Strasse 1, D-66421 Homburg, Germany.
E-mail address: marcel.orth@uks.eu (M. Orth).

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tissue regeneration crucially depends on an adequate vascularization, adipose tissue-derived microvascular fragments (MVF) have been introduced as vascularization units in regenerative medicine [6]. MVF can be isolated in large amounts from fat samples, represent a rich source of mesenchymal stem cells and release proangiogenic growth factors [6,7]. Moreover, transplanted MVF interconnect rapidly with each other and with the surrounding microvasculature to form blood-perfused microvascular networks [6,8]. Accordingly, the application of MVF into bone defects may represent a promising novel strategy to improve bone healing.

The application of MVF to a defect site requires a biocompatible carrier system. Various drug delivery systems have recently been developed. Among others, hydrogels are promising carriers for in vivo delivery [9]. D'Este et al. [10] synthesized a thermoreversible hyaluronan-poly(*N*-isopropylacrylamide) hydrogel (TRH) that is liquid at room temperature and exhibits a gel-like consistency at body temperature. Thus, TRH can be formed according to the needed shape making it favorable for the use in bone defects compared to other carrier systems such as rigid scaffolds. Moreover, hyaluronan is ubiquitous, non-immunogenic and a natural component of the extracellular matrix of various connective tissues, ensuring a good biocompatibility of TRH [10]. TRH shows no change in volume during the gelation process, while the gelling is quick and reversible. It is injectable and has previously been applied in vivo to osteochondral defects in rabbits, showing no adverse effects on vascularization and no interference with the intrinsic healing response while remaining in the osteochondral defect within a moving synovial joint [11]. Thus, TRH may provide some distinct advantages as carrier for cells and micro-tissues in bone healing. The gentle physical gelation of TRH allows the encapsulation of cells and micro-tissues without catalysts, chemical reactions or exposure to harmful radiations [12,13]. The rapid gelation upon the change of temperature enables the exact positioning of TRH in bone defects.

Based on these findings, we herein hypothesized that i) TRH is an adequate carrier system for the local application of MVF to a bone defect and that ii) MVF improve vascularization and bone repair in a standardized murine bone healing model.

2. Materials and methods

2.1. Animals

CD-1 mice with an age of 9 to 14 weeks and a body weight of 35 ± 5 g were used. The animals were bred at the Institute for Clinical and Experimental Surgery, Saarland University, Germany, and kept at a 12 h (h)/12 h light/dark cycle with free access to tap water and standard pellet food (Altromin, Lage, Germany). The study was conducted in accordance with the German legislation on protection of animals and the NIH Guidelines for the Care and Use of Laboratory Animals. It has been approved by the local governmental animal care committee.

2.2. Isolation of MVF

MVF were isolated from epididymal fat pads of male CD-1 mice (n = 69; mean adipose tissue content: 0.83 mL/animal) as previously described in detail [7]. Briefly, the donor mice were anesthetized by intraperitoneal injection of ketamine (75 mg/kg body weight; Pharmacia GmbH, Erlangen, Germany) and xylazine (25 mg/kg body weight; Rompun; Bayer, Leverkusen, Germany). After midline laparotomy, the epididymal fat pads were harvested and transferred into Dulbecco's modified Eagle medium (DMEM; 10% fetal calf serum (FCS), 100 U/mL penicillin, 0.1 mg/mL streptomycin; PAA, Cölbe, Germany). Then, the fat pads were washed three times in phosphate-buffered saline (PBS), finely minced with micro-scissors and digested in collagenase NB4G (0.5 U/mL; Serva, Heidelberg, Germany) for 5–7 min under vigorous stirring at 37 °C in a humidified atmosphere with 5% CO_2. After neutralizing the collagenase with PBS containing 20% FCS, the cell suspension was incubated for 5 min at 37 °C and the fat supernatant was removed. The incubation period and removal of fat supernatants was repeated multiple times. The remaining cell suspension contained MVF, which were enriched in a small volume by centrifugation for 10 min at 40×*g*.

2.3. Preparation of TRH

For the present study we used a thermoresponsive hydrogel, which is liquid at room temperature and of gel-like consistency at 37 °C body temperature. At a temperature of 30 °C the hydrogel loses its flowability and viscoelastic shear moduli rise by 2–3 orders of magnitude in a temperature range of less than 2 °C [10]. The TRH was synthesized as described previously in detail [10,11]. Briefly, TRH was prepared by grafting a thermoreversible segment of poly(*N*-isopropylacrylamide) with a molecular weight of 38×10^3 g/mol on a hyaluronan backbone via amidation at the carboxy group. This composition was selected because of its volumetric stability upon gelation, which facilitates the encapsulation of viable cells. The polymer solution was lyophilized and sterilized with ethylene oxide. We selected this method to avoid the inevitable heat-induced degradation of hyaluronic acid and the impact on the thermal gelation properties induced by conventional steam sterilization. In previous studies we could verify that ethylene oxide treatment does not affect the rheological properties of TRH (Eglin et al., unpublished data). The hydrogel was then reconstituted in PBS (pH = 7.4) at a concentration of 10% wt/vol and stored at 4 °C until further use. In a pilot-study, this concentration represented the best compromise between gel stability and intraoperative use. Lower concentrations resulted in a reduced viscosity, and hence, in a lower stability of MVF-loaded hydrogel at 37 °C, most likely because of insufficient physical cross-linking to warrant the cohesion of this hydrogel composition. Higher concentrations were not suitable for intraoperative injection through a syringe to apply the MVF-loaded hydrogel in vivo, most likely because of its increased viscosity.

2.4. Morphology and viability of cultivated MVF

For the in vitro evaluation of the morphology and viability of MVF, specimens containing half of the MVF of one donor mouse were generated (n = 24). In the group TRH + MVF (n = 12) MVF were mixed with 10 μL of liquid TRH at room temperature. The TRH was then resuspended in 1 mL preheated Endothelial Cell Growth Medium (ECGM) MV (PromoCell, Heidelberg, Germany) in a 24-well plate, resulting in solid drops of TRH incorporating MVF. The specimens were kept under humidified atmospheric conditions (37 °C, 5% CO_2). In the control group (n = 12) MVF were resuspended in 1 mL preheated ECGM MV (PromoCell) in a 24-well plate without TRH.

The morphology and viability of the TRH-incorporated MVF and non-incorporated controls was analyzed immediately after the isolation procedure (n = 4) as well as after 3 days (n = 4) and 7 days (n = 4) of cultivation (including regular exchange of cultivation medium every 48 h). For this purpose, the samples were transferred at 37 °C from cultivation medium into 2% low melting agarose gel (Sigma-Aldrich, Munich, Germany) and from there into 2% high melting agarose gel (Sigma-Aldrich) before fixing the specimens in 4% phosphate buffered formalin. The use of low melting agarose gel allowed for embedding of MVF at a constant temperature due to the low gel point, while the use of high melting agarose gel

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