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Sulfated hyaluronan improves bone regeneration of diabetic rats by binding sclerostin and enhancing osteoblast function

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ABSTRACT

Bone fractures in patients with diabetes mellitus heal poorly and require innovative therapies to support bone regeneration. Here, we assessed whether sulfated hyaluronan included in collagen-based scaffold coatings can improve fracture healing in diabetic rats. Macroporous thermopolymerized lactide-based scaffolds were coated with collagen including non-sulfated or sulfated hyaluronan (HA/sHA3) and inserted into 3 mm femoral defects of non-diabetic and diabetic ZDF rats. After 12 weeks, scaffolds coated with collagen/HA or collagen/sHA3 accelerated bone defect regeneration in diabetic, but not in non-diabetic rats as compared to their non-coated controls. At the tissue level, collagen/sHA3 promoted bone mineralization and decreased the amount of non-mineralized bone matrix. Moreover, collagen/sHA3-coated scaffolds from diabetic rats bound more sclerostin *in vivo* than the respective controls. Binding assays confirmed a high binding affinity of sHA3 to sclerostin. *In vitro*, sHA3 induced BMP-2 and lowered the RANKL/OPG expression ratio, regardless of the glucose concentration in osteoblastic cells. Both sHA3 and high glucose concentrations decreased the differentiation of osteoclastic cells. In summary, scaffolds coated with collagen/sHA3 represent a potentially suitable biomaterial to improve bone defect regeneration in diabetic conditions. The underlying mechanism involves improved osteoblast function and binding sclerostin, a potent inhibitor of Wnt signaling and osteoblast function.

1. Introduction

In 2015, almost 415 million people were affected by diabetes mellitus and the prevalence is increasing due to the aging population, high caloric intake, and low physical activity [1]. Type 2 diabetes mellitus (T2DM) is the most common form affecting 90% of all diabetes patients [1]. Besides macro- and micro-vascular complications, T2DM also alters bone metabolism, resulting in reduced bone quality aspects and an increased fracture risk despite higher bone mineral density [1–6]. In addition, fracture healing is delayed and partially incomplete leading to pain and a prolonged immobility [6]. The underlying mechanisms for the diminished bone healing in diabetics are still poorly defined, but may include increased circulatory levels of the Wnt inhibitor sclerostin, an

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elevated level of advanced glycation end products (AGE) and reactive oxygen species (ROS), and altered dynamics of inflammation and callus formation [6–12]. Therefore, it is critical to gain more insights into fracture healing under diabetic conditions as there is a high demand for novel and innovative treatment options to improve fracture healing in diabetics.

In biomaterial research, several studies have focused on generating biomaterials that release osteoinductive factors to promote bone formation and osseointegration, especially for patients with a decreased bone formation rate such as found in T2DM. Thus, a FGF-2 release system using polyglycolate:polylactide membranes, a BMP-2-enriched fibrin-based matrix for constant delivery of BMP-2, and platelet-rich plasma have already been tested with regard to bone healing in diabetic animals and enhanced bone regeneration in compromised bone [13–15]. Poly(lactic-*co*-glycolic acid) (PLGA) scaffolds also promote bone healing in normal rats [16], but were not well tolerated by diabetic rats (A.-K. Picke and L.C. Hofbauer, unpublished data). Thermopolymerized lactide-based (TriLA) scaffolds represent a novel porous material [17] that is amenable to chemical modifications such as surface chemistries, mechanical properties and degradation profiles. Growth factors or other molecules can be stably bound. The pore size is between 300 and 500 μm which appears to be optimal for *de novo* formation of bone tissue in PLGA bone implant material [16]. In addition, the mechanical properties of the scaffold are variable and can be decreased for the use in skin wounds or increased for use in bone defects.

Recently, the functionality of scaffold materials has been enhanced by evoking cellular responses via coatings with collagen and glycosaminoglycans (GAG). Besides collagen, the GAG hyaluronan (HA) is a key component of the native extracellular matrix (ECM) which can be modified via controlled increased sulfation (sHA3) to alter its effects on cells [18]. Recently, we reported the marginal effects of non-sulfated HA on bone cells while high sulfation degree of HA inhibits osteoclast differentiation and function, while at the same time, it supports osteoblast differentiation and osteocyte viability suggesting a positive effect on bone regeneration [19–23]. In addition, sHA3 has a high binding affinity to sclerostin which when bound to its receptor LRP5/6 inhibits Wnt signaling in osteoblasts and decreases their differentiation potential [24]. Therefore, scaffolds coated with sHA3 may have a great potential as innovative functional biomaterials for bone tissue engineering and regenerative medicine [25].

In this study, we tested the hypothesis that a TriLA scaffold coated with collagen and sHA3 can improve bone defect regeneration in type 2 diabetic rats. We did not elect to use native low and unsulfated GAGs as sHA3 demonstrated superior biological effects in our previous *in vitro* studies. For this purpose, we used an established rodent model of T2DM, the male Zucker Diabetic Fatty (ZDF) rat [26–28]. These rats are characterized by a reduced bone mass and delayed bone regeneration and are suitable to study bone defect healing under diabetic conditions [28]. In addition, we investigated the cellular and molecular mechanisms of action of sHA3 using bone cell cultures and an array of specific binding assays. Here, we show that bone defect regeneration was improved by collagen/sHA3-coated scaffolds in diabetes by supporting osteoblast function, possibly via prolonged binding of sclerostin.

2. Materials and methods

2.1. Materials

Native hyaluronan (from *Streptococcus*, $M_W = 1.1 \times 10^6$ g mol^{-1}, PD = 4.8) was obtained from Aqua Biochem (Dessau, Germany), sulfur trioxide/dimethylformamide complex (SO_3-DMF, purum, ≥97%, active $SO_3 \geq 48\%$) from Fluka Chemie (Buchs, Switzerland). Rat tail collagen type I was available from Corning (Kaiserslautern, Germany). Collagenase from Clostridium histolyticum, Direct Red 80, Stains-All and Toluidine Blue and other chemicals if not stated otherwise were acquired from Sigma-Aldrich, Schnelldorf, Germany. Fluoraldehyde™ *o*-phthaldialdehyde reagent solution was purchased from Thermo Fisher Scientific (Schwerte, Germany). Recombinant human sclerostin was obtained from Creative BioMart (SOST-870H, New York, USA) and D-(+) glucose from Sigma-Aldrich (St. Louis, USA). The Cell Death Detection ELISA and Cell Proliferation ELISA, BrdU were acquired from Roche (Basel, Switzerland) and the Caspase-Glo® 3/7 Assay from Promega (Madison, USA). Series S Sensor Chips C1 and HBS-EP were obtained from GE Healthcare Europe GmbH (Freiburg, Germany).

2.2. Animals

Male ZDF rats (ZDF *fa/fa*) and Zucker lean rats (ZDF +/+, Charles River Laboratories) housed in Makrolon type IV living in a light/dark cycle of 12:12 h at RT and were fed with high-fat, high-carbohydrate chow (Purina 5008) and water ad libitum. At the age of nine to eleven weeks male ZDF (*fa/fa*) rats spontaneously develop T2DM caused by a homozygous mutation of the leptin receptor [26,27]. Non-diabetic ZDF (+/+) rats served as controls. All invasive procedures were approved by the local Institutional Animal Care Committee and the local authorities (DD24-5131/207/19).

2.3. Subcritical size defect

A subcritical 3-mm cross-sectional mid-shaft defect was created in the left femur of eleven-week-old rats as described previously and stabilized by internal fixation of the femur by using a 4-hole plate (Stryker, Kalamazoo, USA) [28–31]. Prior to wound closure, the positive control or TriLA scaffolds (3 × 5 mm, size of bone gap defect x diameter of femur) were inserted into the bone gap like a press-fit material. The rats were randomly allocated to six groups per genotype which resulted in 12 groups in total: (1) the negative control where the defect remained unfilled, (2) the positive control where the defect was filled with a collagen sponge (Spongostan®, Ethicon, Somerville, New Jersey, USA) and bone chips from the resected part of the femur, followed by the test groups where the defect was filled with TriLA scaffolds that were either (3) uncoated, (4) coated with collagen, (5) collagen and HA, or (6) collagen and sHA3. After monitoring blood glucose levels every second week for 12 weeks, the ZDF rats were sacrificed under general anaesthesia and blood samples and bones were collected (see Table 1).

2.4. TriLA scaffold

The macroporous, biodegradable DL-lactide-based TriLA/polyethylene glycol (PEG) scaffolds were cross-copolymerized from three-armed methacrylate-terminated oligomers (Tri134LA6) synthesized from trimethylolpropane (TMP, MW: 134 Da) and lactide (9 mol/mol TMP, i.e. 6 lactic acid units per OH group of TMP) and PEG-monomethacrylate (MW: 1000 Da) in a weight ratio of 83.33% Tri134LA6 and 16.67% PEG1000-monomethacrylate as shown previously [17]. Macroporosity was introduced by co-polymerization in presence of solid lipid microspheres (300–500 μm) that were leached out with *n*-hexane and isopropanol after thermopolymerization of the TriLA/PEG mix at 50 °C. The macroporous cylinders were trimmed to the desired size (3 × 5 mm) using biopsy punches and razor blades. After vacuum-drying the scaffolds were sterilized by gamma irradiation using 15 kGy (Synergy Health Radeberg GmbH, Radeberg, Germany).