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Collagen/glycosaminoglycan coatings enhance new bone formation in a critical size bone defect — A pilot study in rats

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ABSTRACT

Bone regeneration in critical size bone defects still represents an important but unsolved clinical problem. Glycosaminoglycans (GAGs) like chondroitin sulfate (CS) or hyaluronan (HA) are important multifunctional components of the extracellular matrix (ECM) in bone and may stimulate bone healing by recruitment of mesenchymal stromal cells and by supporting their differentiation. Sulfation of GAGs affects their biological activity and thus their interactions with growth factors and/or cells involved in the bone healing process.

The aim of this pilot study was to evaluate the osteogenic capacity of chemically high-sulfated chondroitin sulfate (sCS3) and hyaluronan (sHA3) with an average degree of sulfation DS ≈ 3 on bone healing. Titanium-coated polyetheretherketone (Ti-PEEK) plates were coated with collagen type I (col), collagen-based artificial ECMs containing CS or HA and compared to col/sCS3 and col/sHA3 coatings bridging a critical size bone defect in rat femur. After 4 weeks the gap size of 5.1 mm ± 0.1 mm following surgery was significantly reduced to 1.4 mm ± 0.9 mm for col/sHA3 and to 0.9 mm ± 0.7 mm for col/CS. The highest amount of newly formed bone was detected for col/CS (79% ± 30%) and col/sHA3 (36% ± 20%) compared to uncoated plates (13% ± 3%) or col-coated plates (18% ± 16%). Enchondral ossification could be confirmed for col/CS, col/HA, and col/sHA3 by positive staining for Alcian blue and collagen type II.

These results suggest that an artificial ECM has osteogenic effects and is able to enhance bone healing in critical situations.

1. Introduction

Bone exhibits a high natural capacity for self-repair. However, large bone defects resulting from trauma, tumor resection or debridement after infection or osteonecrosis fail to heal spontaneously. Still autologous bone grafting is the golden standard because autologous bone provides optimal osteoconductive, osteoinductive and osteogenic properties [1,2]. Disadvantages of autologous bone grafting include limited availability, donor site morbidity, an additional surgery for harvesting and thus increased risk of infection or other complications [3,4]. The development of new therapies that promote the healing of large bone defects is therefore an important area of research.

Coating of bone implants with components of the extracellular matrix (ECM) appears to be one promising approach to enhance bone healing [5,6]. Collagen type I (col) is the most abundant organic matrix component in bone. Several studies showed that coatings with col improve and accelerate implant fixation and osseointegration [7,8,9,10]. Glycosaminoglycans (GAGs) like hyaluronan (HA) and chondroitin sulfate (CS) are other important multifunctional components of the ECM. They are known to bind growth factors and cytokines and thus modulate their availability [11]. HA is an unbranched GAG that is involved in regulating cell migration, adhesion and differentiation [12]. In bone, HA plays an important role for the recruitment of osteoblast precursor cells from the bone marrow and is involved in the osteogenic differentiation of mesenchymal stromal cells [12,13]. In addition, HA enhances the interaction between osteoblasts and osteoclasts [14]. The potential of HA in bone healing was shown in a rabbit femur model where HA-coating increased bone ingrowth, mechanical strength of implant fixation, and bone maturation suggesting enhanced bone remodeling [15].

CS is a naturally sulfated GAG found in cartilage as well as in cancellous and cortical bone [16]. It is a promising coating material as it can mediate the binding of osteoblasts and osteoclasts to the matrix and capture soluble mediators like growth factors onto the matrix and at cell surfaces [17]. *In vitro* col/CS coating of titanium surfaces or textile

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scaffolds enhanced adhesion, spreading and differentiation of mesenchymal stromal cells [18,19]. In a standardized rat tibia model the bone-implant contact around col/CS-coated titanium pins was significantly higher compared to uncoated and col-coated pins implicating a significant impact of CS on bone healing [5]. The positive effect of CS on bone formation was confirmed in several other studies employing various small and large animal models [20,21,22]. The combination of col and GAG has frequently been used to prepare an artificial ECM (aECM) to further improve the properties of bone implants [23,24].

Furthermore, the chemical properties of GAGs can be modified by sulfation. It is well known, that the interactions of GAGs with growth factors depend on the number and position of sulfate groups [25,26,27]. aECMs composed of col and sulfated CS and HA are known to increase the osteogenic differentiation of human mesenchymal stromal cells [28], even in the absence of dexamethasone [29] and influence their cell-matrix interactions, signal transduction pathways and endocytosis [30,31]. Sulfated HA (sHA) also showed enhanced interaction with human BMP-4 or recombinant TGF-β1 [32,33]. *In vivo* the coating of dental implants with low-sulfated HA increased the bone-implant contact and the bone volume density after 4 weeks in the maxilla of minipigs [34]. In addition, col/CS and col/sHA coatings seem to enhance early bone remodeling around these implants [35].

There is evidence that bone healing in critical size bone defects cannot be achieved without osteogenic or osteoinductive materials [36,37,38,39]. Therefore, we wanted to evaluate the osteogenic capacity of aECM coated on polymer (Ti-coated polyetheretherketone, Ti-PEEK) plates on new bone formation without any osteoconductive material in a critical size bone defect model in rat femora to compare chemically high-sulfated GAGs to the physiologically sulfated CS and HA.

2. Materials and methods

2.1. Materials

Hyaluronan (HA; from Streptococcus, M_w 1.1×10^6 g/mol) was purchased from Aqua Biochem (Dessau, Germany). Chondroitin sulfate (CS; from bovine tracheal, a mixture of 70% chondroitin-4-sulfate and 30% chondroitin-6-sulfate) was purchased from Sigma–Aldrich (Schnelldorf, Germany). The sulfating agent sulfur trioxide/dimethylformamide complex (SO_3-DMF, purum, ≥ 97%, active SO_3 ≥ 48%) was obtained from Fluka Chemie (Buchs, Switzerland). Rat tail tendon collagen type I was available from BD Bioscience (Heidelberg, Germany). Direct Red, Toluidine blue, 3,3′-Diaminobenzidine and other chemicals if not stated otherwise were acquired from Sigma-Aldrich (Schnelldorf, Germany). Ketamine was purchased from Riemser Arzneimittel (Greifswald, Germany). Xylazine was obtained from Pharma-Partner (Hamburg, Germany). PEEK plates (RatFix) were acquired from AO Foundation (Davos, Switzerland) and coated with 20 nm Titanium by pfm medical titanium (Nuremberg, Germany). Carprofen (Rimadyl) was purchased from Pfizer (Berlin, Germany). Ethylenediaminetetraacetic acid (Osteosoft) was obtained from Merck (Darmstadt, Germany). Alcian blue was available from Clin-Tech (Guildford, UK), Goldner-Masson trichrome staining kit by Merck (Darmstadt, Germany). Collagen type II mouse monoclonal antibody was acquired from Thermo Fisher Scientific (Braunschweig, Germany), Vectastain® ABC kit from Vector Laboratories (Burlingame, USA). Smooth muscle actin mouse monoclonal antibody was purchased from DAKO (Glostrup, Denmark).

2.2. Preparation of GAG derivatives

High-sulfated derivatives of HA (sHA3) and CS (sCS3) were synthesized and characterized as previously described [32,40]. The characteristics of the prepared GAGs are summarized in Table 1, the chemical structures are shown in Fig. 1.

Table 1
Characteristics of synthesized HA and CS derivatives (degree of sulfation (DS)), number-average (M_n) and weight-average (M_w) molecular weights as determined by Laser Light Scattering (LLS) detection and Refraction (RI) detection (in brackets), molecular weight distributions (polydispersity index: PD) based on values calculated from RI detection.

Sample	HA	sHA3	CS	sCS3
DS	–	3.4	0.9	3.1
M_n [g/mol]	1,019,625 (394,940)	29,605 (51,420)	16,270 (38,970)	17,675 (28,500)
M_w [g/mol]	1,174,868 (1,894,675)	51,145 (88,555)	19,763 (60,860)	19,915 (41,565)
PD	4.8	1.7	1.6	1.5

2.3. Coating of Ti-PEEK plates with different aECMs

Ti-PEEK plates (8 hole, 23 mm length, Fig. 2A) were cleaned consecutively with 1% Triton X100, 100% acetone and 100% ethanol for 15 min in an ultrasound bath each. In between, samples were washed with deionized, sterile water and finally dried.

A collagen stock solution was diluted to 1 mg/ml with ice-cold 10 mM acetic acid and mixed either with 60 mM ice-cold phosphate buffer (50 mM disodium hydrogen phosphate and 10 mM potassium dihydrogen phosphate, pH 7.4). For the preparation of the aECM, the collagen stock solution was mixed with an equal volume of GAG derivative (CS, 1.37 mg/ml; HA, 1.11 mg/ml; sCS3, 1.99 mg/ml; sHA3, 2.04 mg/ml) dissolved in the 60 mM ice-cold phosphate buffer. *In vitro* fibrillogenesis of collagen was allowed to take place for 16–18 h at 37 °C. The solutions were centrifuged at 3000 ×*g* for 10 min, the resulting supernatants (pre-coating solutions) collected, pellets washed with 30 mM phosphate buffer pH 7.4 and centrifuged again. Pellets were then solubilized to 3 mg/ml collagen (coating solutions) in the same buffer. The Ti-PEEK plates were placed in 2 ml vials hanging on a thread and briefly pre-coated with the supernatants of the centrifugation steps, followed by 30 min incubation in the respective coating solutions and dried afterwards. The coating step was repeated three times. Plates were then briefly washed two times in deionized water and incubated for 1 h in PBS at 37 °C. After a drying step the plates were subjected to gamma sterilization with 15 kGy.

2.4. Quantification of the collagen content of the collagen coating on Ti-PEEK plates

The collagen content of the collagen coating on Ti-PEEK plates was analyzed by Sirius Red staining [18]. Samples were incubated for 1 h in 0.1% Direct Red (Sirius Red) in saturated picric acid and then washed with 0.01 M hydrochloric acid. The remaining bound dye was solubilized with 0.1 M sodium hydroxide, which was incubated with samples and standard for 1 h. Absorbance was measured at 540 nm. A calibration curve of collagen coated 24 well plates served as control. The collagen coatings contained 120 ± 9 μg of the fibrillar protein.

2.5. Characterization of the GAG distribution

To visualize the distribution of the GAGs and sulfated GAGs, the Ti-PEEK plates were incubated in Toluidine blue solution (0.4 mg/ml Toluidine blue in 0.1 M HCl, 2 mg/ml NaCl) for 4 h at room temperature with gentle shaking. Ti-PEEK plates were washed several times with water and dried (Fig. 2D–F).

2.6. Animal surgery

The study was approved by the local animal care committee and the respective authorities (permission no. 24-9168.11-1/2013-66). All animals were housed according to the European guidelines for the care and use of laboratory animals. Thirty-six male Wistar rats with an