J Mater Sci: Mater Med (2008) 19:1653–1660
DOI 10.1007/s10856-007-3250-z

Characterization of collagen II fibrils containing biglycan and their effect as a coating on osteoblast adhesion and proliferation

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Received: 10 July 2006 / Accepted: 27 July 2007 / Published online: 13 September 2007

Abstract Collagen has been used as a coating material for titanium-based implants for bone contact and as a component of scaffolds for bone tissue engineering. In general collagen type I has been used, however very little attention has been focussed on collagen type II. Collagen-based coatings and scaffolds have been enhanced by the incorporation of the glycosaminoglycan chondroitin sulphate (CS), however the proteglycan biglycan, which is found in bone and contains glycosaminoglycan chains consisting of CS, has not been used as a biomaterial component. The study had the following aims: firstly, five different collagen II preparations were compared with regard to their ability to bind CS and biglycan and the changes in fibril morphology thereby induced. Secondly, the effects of biglycan on the adhesion of primary rat osteoblasts (rO) as well as the proliferation of rO, primary human osteoblasts (hO) and the osteoblast-like cell line 7F2 were studied by culturing the cells on surfaces coated with collagen II fibrils containing biglycan. Fibrils of the collagen II preparation which bound the most biglycan were used to coat titanium surfaces. Bare titanium, titanium coated with collagen II fibrils and titanium coated with collagen II fibrils containing biglycan were compared. It was found that different collagen II preparations showed different affinities for CS and biglycan. In four of the five preparations tested, biglycan reduced fibril diameter, however the ability of a preparation to bind more biglycan did not appear to lead to a greater reduction in fibril diameter. Fibrils containing biglycan promoted the formation of focal adhesions by rO and significantly enhanced the proliferation of hO but not of rO or 7F2 cells. These results should encourage further investigation of biglycan as a component of collagen-based scaffolds and/or coatings.

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1 Introduction

In the case of both metallic implants for bone contact and scaffolds for bone tissue engineering, surface modifications which promote osteoblast adhesion and proliferation are desirable. One approach in surface modification is to mimic the extracellular matrix (ECM) of bone by coating with collagen, its main organic component. The structural protein collagen has been widely used as a coating for titanium-based implants and tissue-engineering scaffolds. Collagens contain binding sites for osteoprogenitor cells, and in several publications, titanium or its alloys have been coated with collagen, which has improved osteoblast attachment, proliferation and differentiation in vitro, while increasing early bone remodeling, bone contact and formation of surrounding bone in vivo [1–8].

Collagens are structural proteins of which 27 types have thus far been identified [9–11]. Among the fibril-forming collagen types are I and II, whose molecules consist of an uninterrupted triple helix of approximately 300 nm in length and 1.5 nm in diameter. Fibrils are formed by self-assembly from collagen molecules and demonstrate a cross-striated, D-periodic banding pattern, where D = 67 nm [12]. Collagen type I is the most abundant type in mammals,

and is the type present in mineralized bone, but during bone development and repair, collagen type II is also expressed [13, 14].

The strategy of mimicking bone ECM can be taken a step further by incorporating the small leucine-rich proteoglycan biglycan, which is found in bone [15–17] and associates with collagen fibrils in vivo [18]. Biglycan is made up of two glycosaminoglycan (GAG) chains consisting of either chondroitin sulphate (CS) or dermatan sulphate (DS) attached to a protein core. These linear chains consist of repeating units of anionic, acidic sugars. The protein core contains 10 leucine-rich repeats flanked by cysteine-rich regions. Biglycan is synthesised by osteoblast precursors in vitro and its production is believed to be linked to their differentiation and later mineralization [19–22]. Knock-out mice unable to synthesise biglycan suffer from reduced bone mass [23].

Collagen-based coatings and scaffolds have been enhanced by the incorporation of CS. CS bound to collagen scaffolds and coatings has improved the adhesion, proliferation and differentiation of a variety of cell types in vitro [24–29].

In bone, lone CS is not naturally present, but is found as a component of the GAG-chains of biglycan and other proteoglycans. However, biglycan has not yet been used as a biomaterial component till now, although the overexpression of biglycan by smooth muscle cells led to an increase in proliferation both in vitro and in vivo [8]. Furthermore, studies in our group have shown that more biglycan can be bound than CS [29, 30]. It was hypothesized that immobilization of as much as possible would be desirable in order to maximise any possible effect.

In general, collagen type I has been used as a coating/scaffold material. Although collagen type II has been used as a scaffold for cartilage and intervertebral disc tissue engineering [31–34], to our best knowledge it has not been used before as a coating for titanium-based implants or in bone tissue engineering. Biglycan binds to fibrils of both type I and type II collagen [35, 36], however previous studies indicate that collagen II can bind more biglycan and CS than collagen I [29, 30, 37].

In addition, studies have reported that biglycan is able to bind to and modulate the activity of the growth factors TGF-beta1, which stimulates osteoblast proliferation and causes osteoblast chemotaxis, and BMP-4, which enhances osteoblast differentiation [22, 38–40]. Therefore, as well as influencing cell behaviour directly, biglycan could potentially function as a growth factor reservoir or modulator. Growth factors have retained their bioactivity after their adsorption to collagen coatings [5, 41, 42]. The use of biglycan opens the possibility of synergistic effects in combination with these or other growth factors in subsequent experiments.

It was hypothesized that biglycan, bound by fibrils during in vitro fibrillogenesis, might exert a positive influence on the adhesion and proliferation of seeded osteoblasts. Collagen II was used instead of collagen I as it has a higher affinity for biglycan and CS [29, 30, 37, 43].

This study had the following aims: firstly, various collagen II preparations were compared with regard to their ability to bind CS and biglycan and the changes in fibril morphology thereby induced. The collagen II preparations were derived from different cartilage types and purchased from different suppliers. Secondly, the effects of biglycan on the adhesion of primary rat osteoblasts (rO) as well as the proliferation of rO, primary human osteoblasts (hO) and the osteoblast-like cell line 7F2 were studied by culturing the cells on surfaces coated with collagen II fibrils containing biglycan. Fibrils of the collagen II preparation which bound the most biglycan were used to coat titanium surfaces. Bare titanium, titanium coated with collagen II fibrils and titanium coated with collagen II fibrils containing biglycan were compared. To our best knowledge, no-one has yet used collagen II as a coating material for titanium surfaces or used collagen II and biglycan in combination as a biomaterial.

2 Materials and methods

(A) *Materials*. Collagen type II preparations were obtained from different sources as shown in Table 1. Biglycan (bovine articular cartilage) and other chemicals used (all reagent grade) were obtained from Sigma-Aldrich Chemie GmbH, Germany unless stated otherwise. (B) *SDS-PAGE of collagen preparations*. Collagen preparations was subjected to SDS-PAGE on slab gels according to the method of Yang [44], using 6% (w/v) polyacrylamide for the running and 5% for the stacking gels. Lanes were loaded with 2.5 μg collagen. Silver staining after electrophoresis was carried out using a SilverSNAP® Stain Kit II (Perbio Science GmbH, Germany) according to the manufacturer's protocols. (C) *Preparation and analysis of fibrils*. Collagen (1 mg/mL in 10 mM acetic acid) was mixed at 4 °C with an equal volume of double concentrated fibrillogenesis buffer (50 mM sodium dihydrogenphosphate and 10 mM potassium dihydrogenphosphate at pH 7.4). CS and biglycan were dissolved in buffer; the CS:collagen and biglycan:collagen mass ratios were 1:1.5 and 1:7, respectively. Fibrils formed at 37 °C overnight and were subsequently separated by centrifugation for 15 min at 10000 g. Supernatant and pellet were retained. The mass of collagen in the pellet was calculated by subtracting the mass of collagen detected in the supernatant using the Lowry method [45] from the initial mass of collagen before fibrillogenesis. Biglycan in the pellet was quantified by hexosamine assay

Table 1 List of collagen II preparations investigated as well as their companies and tissues of origin

Collagen II source tissue	Company providing Collagen II preparation	Abbreviation
Bovine Tracheal Cartilage	Sigma Aldrich GmbH, Germany	Sigma
Bovine Articular Cartilage	BD Biosciences, Germany	BD
Bovine Articular Cartilage	USB Inc., USA	USB
Bovine Articular Cartilage	Elastin Products Company Inc., USA	EPC KG
Bovine Nasal Septum	Elastin Products Company Inc., USA	EPC NS

according to the protocol of Yannas [46] and CS by dimethyl-methylene blue assay according to the protocol of Chou [47]. (D) *Coating of titanium surfaces*. To modify titanium surfaces for experiments with cells, pellets were resuspended in buffer solution to form a suspension with a "concentration" of 1 mg fibrils/ml buffer solution. 100 µl of this suspension was added dropwise onto the surface of blasted titanium discs. After being coated with fibrils by adsorption for 30 min, surfaces were washed three times with distilled water and air-dried. Coatings were crosslinked overnight at room temperature (RT) with EDC/NHS according to the method of Pieper [48]. Sterilization was carried out using ethylene oxide. The same procedure was used to prepare samples for morphological examination using Atomic Force Microscopy (AFM), however very smooth titanium surfaces were used and no crosslinking was performed. (E) *Cell preparation and culture*. Throughout this study, NIH guidelines for the care and use of laboratory animals (NIH Publication #85–23 Rev. 1985) were observed. Rat calvarial osteoblasts (rO) were isolated according to Geißler et al. [3] Briefly, cells were obtained from the calvariae of newborn Wistar Kyoto rats by sequential digestion with 3.56 U collagenase P/mL (Roche) and 15 U trypsin/mL (Roche) in phosphate buffered saline (PBS) and subcultured in Dulbecco's Modified Eagle's Medium DMEM (Biochrom) containing 10% fetal calf serum (FCS). The osteogenic differentiation and osteoblast phenotype of these cells (rO) was confirmed by determination of alkaline phosphatase activity, collagen type I synthesis, osteocalcin mRNA and formation of calcium phosphate deposits. Adult human osteoblasts (hO) from a 76-year old female donor were purchased from PromoCell (Heidelberg, Germany). These cells were cultured and expanded in Osteoblast growth medium (distributed by PromoCell, Heidelberg, Germany). 7F2 osteoblast-like cells were obtained from LGC Bichochem GmbH, Germany and cultured in Alpha Medium (Biochrom, Germany) with 10% fetal calf serum (FCS). For proliferation experiments, hO, rO and 7F2 cells were plated at 7.000, 12.500 and 100.000 cells/cm^2 respectively on the top of the titanium discs. After 2 h incubation at 37 °C the medium was filled up to 1 mL. Culture medium was changed twice a week. Rat calvarial osteoblasts for adhesion experiements were isolated according to Geißler et al. [3] and subcultured in Dulbecco's Modified Eagle Medium (Biochrom) containing 10% FCS. (F) *Immunofluorescence staining of cytoskeleton to detect focal adhesion contacts.* After incubation (2h) of rO on the different titanium coatings, fixation was performed with 4% paraformaldehyde for 15 min at RT. rO were then permeabilized with 0.1% TritonX-100 in phosphate-buffered saline (PBS) for 20 min. Following this, unspecific binding was blocked by using 1% bovine serum albumin (BSA)/0.05% Tween 20 in PBS. Primary antibody binding (mouse-anti-Vinculin hVin I, 1:150 in 1% BSA/0.05% Tween 20 in PBS) took place for 1 h at RT. After washing with PBS, rO were subjected to an additional incubation in blocking buffer for 10 min. Secondary antibody (goat-anti-mouse-TRITC, DAKO, 1:50; Phalloidin-FITC, 1:30 in 1% BSA/0.05% Tween 20 in PBS) incubation was performed for 1 h at RT. Subsequently DAPI (4′,6-Diamidino-2-phenylindol) was used for nuclei staining for 15 min at RT (1:5000). Stained cells on coated titanium plates were then embedded in Mowiol 4–88. The staining was visualized using the AxioPhot fluorescence microscope; digital images were acquired with a AxioCam MRm camera (Zeiss) working with the AxioVision software version 4.4. The fluorescence signals were detected with the following optics: TRITC excitation 546 nm, emission 590 nm; FITC excitation 450–490 nm, emission 515–565 nm; DAPI excitation 365 nm, emission 420 nm. (G) *Proliferation rate*. Proliferation of hO and rO was monitored by quantifying DNA synthesis as described previously [2] after 2 days of culture. Proliferation of 7F2 cells was assed using a lactate dehydrogenase (LDH) Cytotoxicity Detection Kit (Takara Bio Inc., Manual v.03.01 Cat# MK401) according to the manufacturer's protocols after 1 and 6 days of culture. (H) *Statistics*. For analysis of statistical significance, experiments were repeated 3 times. A one-way ANOVA (analysis of variance) was applied.

3 Results

3.1 SDS-PAGE analysis, binding of CS and biglycan and changes in morphology

After SDS-PAGE all collagen II preparations showed the same banding pattern on the gel, with the banding

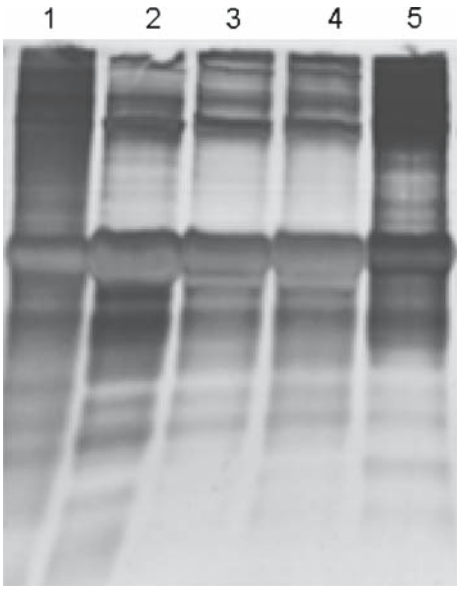

Fig. 1 SDS-PAGE and silver staining of Collagen II preparations. Each lane contained 2.5 μg of collagen

Key to lanes in Figure 1

Lane No.	Collagen type	Collagen II source tissue	Company providing Collagen II preparation
1	II	Bovine Tracheal Cartilage	Sigma Aldrich GmbH, Germany
2	II	Bovine Articular Cartilage	BD Biosciences, Germany
3	II	Bovine Articular Cartilage	USB Inc., USA
4	II	Bovine Articular Cartilage	Elastin Products Company Inc., USA
5	II	Bovine Nasal Septum	Elastin Products Company Inc., USA

intensities of the preparations from bovine articular cartilage (BD, USB and EPC-KG) appearing to be more similar to each other than to those of Sigma and EPC-NS preparations, from bovine tracheal cartilage and bovine nasal septum, respectively (Fig. 1).

Differences were seen in the amounts of both CS (Fig. 2) and biglycan (Fig. 3) bound by different collagen II preparations. However in both cases a similar trend was seen. In general, preparations which bound more CS also bound more biglycan. Collagen from tracheal cartilage (Sigma) (79.0 ± 4.3 μg CS/mg fibrils) and collagen derived from articular cartilage (BD) (70.4 ± 11.2 μg CS/mg fibrils) bound approximately 1.5 to 2 times as much CS as the other collagens derived from articular cartilage, (USB, EPC-KG) and collagen derived from nasal septum (EPC-NS) (44.7 ± 8.1, 37.1 ± 5.2 and 48.1 ± 3.5 μg CS/mg fibrils, respectively). In the case of biglycan, collagen from tracheal cartilage (Sigma) (177.3 ± 4.3 μg biglycan/mg fibrils) and collagen derived from articular cartilage (BD) (163.6 ± 11.2 μg biglycan/mg fibrils) bound more than twice as much as collagen derived from articular cartilage and nasal septum from EPC (50.0 ± 11.4 and 77.3 ± 13.6 μg biglycan/mg fibrils, respectively) and significantly more than collagen derived from articular cartilage from USB (122.4 ± 11.8 μg biglycan/mg fibrils). Although the articular collagen from USB did not bind

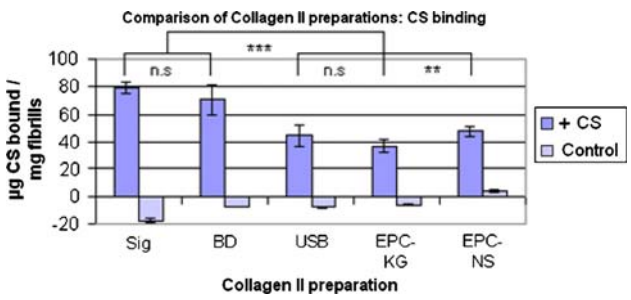

Fig. 2 Quantification of CS bound per mg Collagen II fibrils: different preparations. CS:collagen ratio = 1:1.5 (w/w). Experiments were performed in triplicate; error bars show standard deviation. Abbreviations of preparations are given in Table 1. Significances: *** = <0.001, ** = <0.01, n.s. = not significant

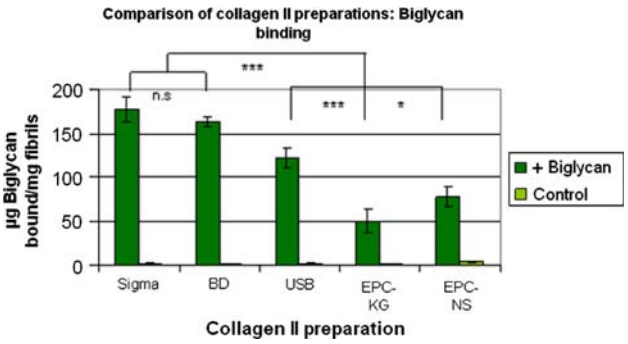

Fig. 3 Quantification of biglycan bound per mg collagen II fibrils: different preparations. Biglycan:collagen ratio = 1:7 (w/w). Experiments were performed in triplicate; error bars show standard deviation. Abbreviations of preparations are given in Table 1. Significances: *** = <0.001, * = <0.05, n.s. = not significant

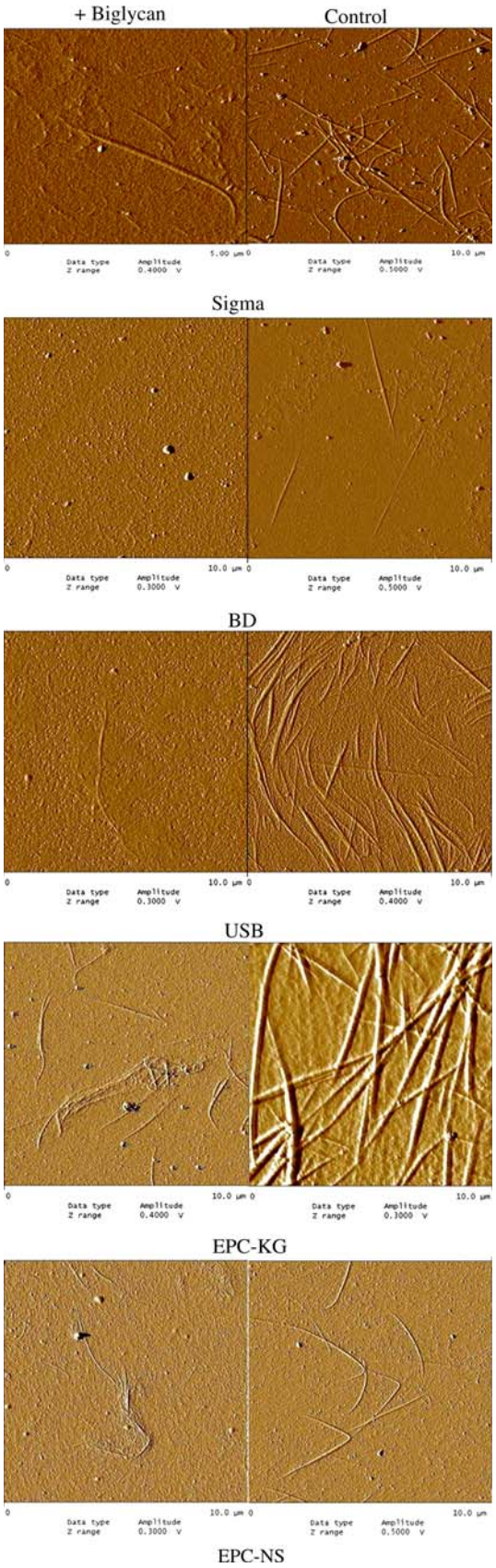

Fig. 4 Effect of biglycan on fibril morphology of different collagen II preparations. Left: with biglycan (biglycan:collagen ratio = 1:7 (w/w)); right: without biglycan (controls). Abbreviations of preparations are given in Table 1. All images have dimensions of 10 μm × 10 μm

more CS than the collagen derived from articular and nasal cartilage from EPC, it bound more biglycan.

The control fibrils of the collagen II preparations, without biglycan, showed very different thicknesses, with two collagens from articular cartilage (EPC-KG and USB) having the highest widths (Fig. 4). Biglycan reduced the thicknesses of fibrils of all collagen preparations with the exception of that derived from tracheal cartilage (Sigma). The greatest reduction was seen for the articular cartilage-derived collagens from EPC and BD. Greater reduction in fibril diameter did not correlate with increased binding of biglycan. Fibrils of collagen derived from articular cartilage obtained from BD containing biglycan were small and difficult to detect using AFM.

It was considered desirable to bind as much biglycan as possible to maximize potential biglycan-osteoblast interactions. The fibrils formed from tracheal cartilage-derived collagen (Sigma) in the presence of biglycan were larger than those of collagen from articular cartilage obtained from BD. Previous work in our group has shown that collagen fibrils, after adsorption to titanium substrates, are less readily desorbed than collagen molecules [49]. The greater resistance to desorption of fibrils is believed to be thanks to the larger size of fibrils which allows more contact points to be made with the titanium substrate. We therefore hypothesized that the larger Sigma fibrils would likely be more strongly adsorbed to titanium surfaces. Hence the Sigma collagen preparation was chosen for use in cell adhesion and proliferation experiments.

3.2 Cell experiments

Considerably more focal adhesions were seen on surfaces coated with collagen II fibrils containing biglycan than on surfaces coated with collagen II fibrils without biglycan and on bare titanium (Fig. 5). Proliferation of hO did not increase after coating with collagen II fibrils, but was significantly enhanced by the presence of biglycan in fibrils (Fig. 6). Proliferation of rO was not significantly affected by the presence of biglycan and was increased by a collagen II layer but not significantly (Fig. 7). Proliferation of osteoblast-like 7F2 cells did not increase in the presence of a collagen fibril layer or a collagen fibril layer containing biglycan (Fig. 8).

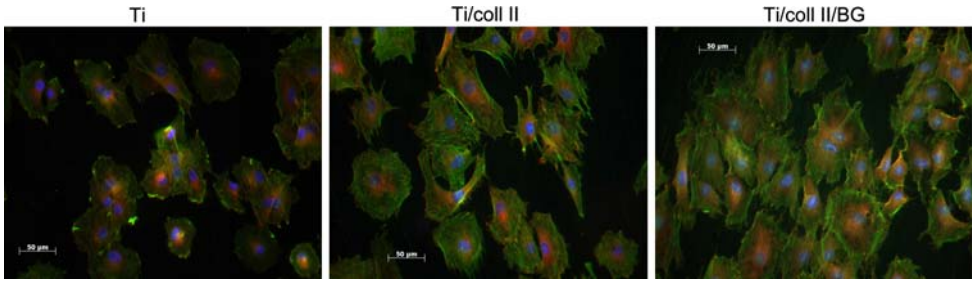

Fig. 5 Effect of biglycan on focal adhesion formation of rat calvarial osteoblasts to coated surfaces as assessed by cytoskeletal staining after 2 hours. Red: vinculin; green: actin; blue: nuclei; yellow: focal adhesions. Collagen II from Sigma was used. Coatings were generated by adsorption of fibrils from a fibril suspension with a "concentration" of 1 mg/ml. Ti: uncoated titanium; Ti/coll II: titanium coated with collagen II fibrils; Ti/coll II/BG: titanium coated with collagen II fibrils containing biglycan

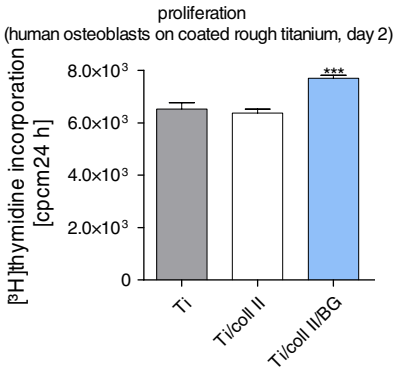

Fig. 6 Effect of biglycan on proliferation of primary human osteoblasts (hO) after 2 days. Collagen II from Sigma was used. Coatings were generated by adsorption of fibrils from a fibril suspension with a "concentration" of 1 mg/ml. Ti: uncoated titanium; Ti/coll II: titanium coated with collagen II fibrils; Ti/coll II/BG: titanium coated with collagen II fibrils containing biglycan. Experiments were performed in triplicate; error bars show standard error from mean. Significances: *** = <0.001

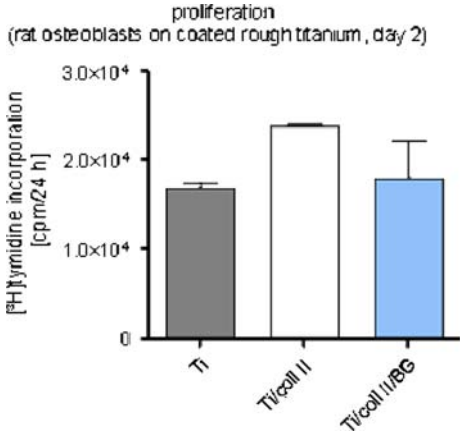

Fig. 7 Effect of biglycan on proliferation of primary rat osteoblasts (rO) after 2 days. Collagen II from Sigma was used. Coatings were generated by adsorption of fibrils from a fibril suspension with a "concentration" of 1 mg/ml. Ti: uncoated titanium; Ti/coll II: titanium coated with collagen II fibrils; Ti/coll II/BG: titanium coated with Collagen II fibrils containing biglycan. Experiments were performed in triplicate; error bars show standard error from mean. No significant differences were seen

4 Discussion

This study had two main aims. Firstly, fibrils of different collagen II preparations were characterized according to the amount of CS and biglycan bound and the changes in their morphology caused by biglycan. Secondly, the effects of fibril-bound biglycan on primary osteoblast adhesion and the proliferation of primary rat and human osteoblasts and an osteoblastic cell line were investigated.

4.1 Binding of CS and biglycan and morphological changes

Biglycan is believed to interact with collagen both via the GAG chains [35] and the core protein [36]. The results suggest that different collagen II preparations from different sources have different affinities for biglycan core protein or GAG chains or both. This may partially be due to the different cartilage types the collagens originate from (bovine tracheal cartilage, bovine articular cartilage and bovine nasal septum). Although three preparations (BD, USB and EPC KG) came from the same tissue, bovine articular cartilage, and no differences in structure could be detected by SDS-PAGE analysis, they bound very different amounts of biglycan. It can be speculated that differences in preparation methods may play a major role. Three collagen preparations were produced using the same method according to information provided by the suppliers; two were derived from articular cartilage (USB, EPC-KG) and one from nasal septum (EPC-NS). The similarity of the preparation methods may possibly explain why these three preparations bound significantly less CS and biglycan than collagen from Sigma and BD. However it would not explain why USB fibrils bind more biglycan than those of EPC-KG.

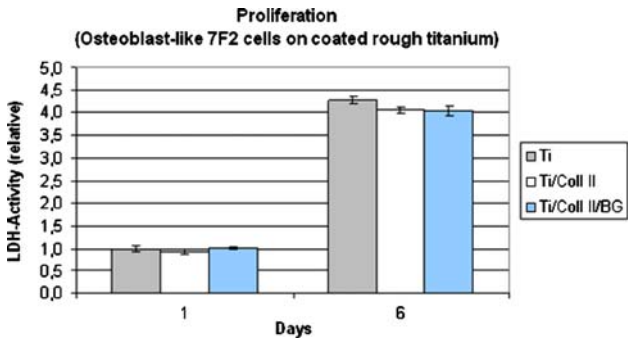

Fig. 8 Effect of biglycan on proliferation of 7F2 osteoblast-like cells after 1 and 6 days. Collagen II from Sigma was used. Coatings were generated by adsorption of fibrils from a fibril suspension with a "concentration" of 1 mg/ml. Values shown are relative LDH activities relative to uncoated titanium. Ti: uncoated titanium; Ti/coll II: titanium coated with collagen II fibrils; Ti/coll II/BG: titanium coated with collagen II fibrils containing biglycan. Experiments were performed in triplicate; error bars show standard error from mean. No significant differences were seen

Preparations which bound more biglycan also bound more CS. The higher affinity for biglycan may be due in part to a higher affinity for CS or DS in the biglycan GAG chains; both CS and DS bind to collagen [50].

Biglycan caused the fibrils of four out of the five preparations tested (the exception being the trachea- derived collagen (Sigma)) to become thinner. The extent of biglycan's effect on the fibril morphology of preparations appears to be independent of the preparation's ability to bind biglycan; a greater decrease in fibril diameter does not correlate with a greater increase in biglycan bound. Based on this observation, it could be speculated that the surface area available for biglycan binding, which would increase with decreasing fibril diameter, is not a significant influence on the ability of the preparation to bind biglycan. Differences in the preparation methods may possibly influence morphological changes. However, as collagen from articular cartilage from the firms USB and EPC are prepared according to the same method, the differences between the fibril morphologies of these two preparations cannot be ascribed to differences in preparation.

4.2 Cell experiments

Biglycan bound to collagen II fibrils promoted the formation of focal adhesions. A possible explanation is that negative charges of CS and DS may bind cations in the medium, which are required for focal adhesion formation; this would be in agreement with previous work reporting enhanced osteoblast adhesion to surfaces coated with collagen I fibrils containing CS and the proteoglycan decorin, which possesses a GAG chain consisting of CS and DS [28, 29].

Biglycan stimulated the proliferation of hO, which could be due to its GAG chains; as mentioned in the introduction, CS bound to collagen I has improved proliferation of fibroblasts [25] and chondrocytes [24, 26]. Also, CS and DS carry negative charges; negative surface charges have enhanced osteoblast-like cell proliferation [51]. Alternatively, biglycan may bind growth factors, both exogenous and cell-secreted, increasing their bioavailability to the cells. Binding of growth factors to CS and DS [52] and biglycan core protein [39] has been reported. Biglycan did not enhance the proliferation of rO, suggesting that biglycan's effect may depend on the source of the primary osteoblasts. Biglycan may conceivably play different roles in primary rat and human osteoblast cultures; publications suggest that the temporal expression pattern of biglycan by rat and human osteoblasts in vitro is different [20, 21]. Biglycan did not increase the proliferation of 7F2 cells. Cell lines are known to differ from normal bone cells in functional activities and response to osteogenic agents [53].

5 Summary and outlook

Different commercially available collagen II preparations from different bovine tissues demonstrate different abilities to bind biglycan and CS. Preparations binding higher amounts of CS also bound higher amounts of biglycan, perhaps due to a higher affinity for the GAG chains of biglycan. Biglycan reduced fibril diameter in four out of the five preparations investigated, however the ability of a preparation to bind more biglycan did not appear to lead to a larger reduction in fibril diameter. Biglycan bound to collagen II fibrils is able to promote primary human osteoblast proliferation and the adhesion of primary rat osteoblasts. Further research is necessary to compare fibrils of different collagen types containing biglycan, and to investigate the effect of exogenous growth factors on collagen fibril-coated surfaces containing biglycan on osteoblast behaviour. These results may be of significance when choosing a source of collagen for use as a coating or a tissue engineering scaffold.

Acknowledgements The authors thank the German Ministry for Education and Research (BMBF) for financial support and and M. Schuhmann, H. Zimmermann and Ch. Kupke for technical assistance.

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