Interactions of Collagen Types I and II with Chondroitin Sulfates A−C and Their Effect on Osteoblast Adhesion

Timothy Douglas,*,† Sascha Heinemann,† Carolin Mietrach,‡ Ute Hempel,‡ Susanne Bierbaum,† Dieter Scharnweber,† and Hartmut Worch†

Institute of Material Science, Max Bergmann Center of Biomaterials, Technische Universität Dresden, Budapester Strasse 27, 01069 Dresden, Germany, and Center of Theoretical Medicine, Institute of Physiological Chemistry, Technische Universität Dresden, Fiedlerstrasse 43, 01307 Dresden, Germany

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Collagen has found use as a scaffold material for tissue engineering as well as a coating material for implants. The main aim of this study was to compare the ability of the collagen types I and II to bind preparations of the chondroitin sulfate types A−C (CS A, CS B, CS C). In addition, the effect of the three CS preparations on the extent of collagen incorporated into fibrils and the morphology of collagen fibrils was investigated, as was the influence of collagen fibril coatings containing CS A−C on titanium surfaces on the adhesion of primary rat osteoblasts. Fibrils of both collagen types bound a higher mass of CS C than CS B and a greater mass of CS B than CS A per milligram of fibrils formed. Fibrils of collagen type II bound a higher mass of CS B and C than collagen I fibrils. The proportion of collagen incorporated into fibrils decreased with increasing CS A and CS C concentration but not with increasing CS B concentration. All three CS preparations caused collagen I and II fibrils to become thinner. CS A and CS B but not CS C appeared to stimulate the formation of focal adhesions by osteoblasts after incubation for 2 hours. These results could be of importance when selecting collagen type or CS type as materials for implant coatings or tissue engineering scaffolds.

Introduction

For scaffold materials in tissue engineering (TE) applications and materials for implants, surface properties which encourage cell attachment, proliferation, and differentiation along the preferred lineage are desirable. Collagen in fibrillar form has been used to coat titanium surfaces, improving osteoblast spreading, attachment, proliferation, and differentiation in vitro.[1−4] The addition of chondroitin sulfate (CS) to fibrils has enhanced osteoblast adhesion and differentiation further and has promoted the differentiation of human mesenchymal stem cells toward the osteoblastic lineage.[5,6] TE scaffolds consisting of collagen with immobilized CS have stimulated the proliferation of fibroblasts and chondrocytes in vitro while promoting cellular ingrowth and reducing foreign body response in vivo.[7−10] In addition, growth factors (GFs) have maintained their bioactivity when immobilized on scaffolds containing CS; it has been speculated that CS may exert a stabilizing influence on GFs.[11,12]

Collagens are structural proteins. Type I, the type present in mineralized bone, and type II, present in cartilage and developing bone, consist of an uninterrupted triple helix approximately 300 nm in length and 1.5 nm in diameter. Fibrils form by self-assembly of triple helices and have a cross-striated, *D*-periodic banding pattern, where $D = 67$ nm.[13,14]

Chondroitin sulfates are glycosaminoglycans (GAGs), which are practically nonimmunogenic and consist of sulfated, negatively charged, repeating dissacharide units.[15] These units contain *N*-galactosamine, which is characteristic for CS, and D-glucuronate or L-iduronate. Several types of CS have been identified. CS A, also known as chondroitin-4-sulfate, contains d-glucuronic acid and *N*-galactosamine sulfated at the C4 position. CS C, also known as chondroitin-6-sulfate, contains d-glucuronic acid and *N*-galactosamine sulfated at the C6 position. CS B, also known as dermatan sulfate, is a copolymer containing D-glucuronate-*N*-galactosamine sulfated at the C4 or C6 position and L-iduronate-*N*-galactosamine sulfated at the C4 position.[16−18] The structures of CS A−C are shown in Figure 1. CS chains are synthesized as GAG side chains of proteoglycans (PGs) and can be variably sulfated.[19] CS A and B are found in the PGs decorin and biglycan, which occur in many tissues, including bone and cartilage, and are synthesized as chains of decorin and biglycan by bone cells in vitro.[20−22]

To our best knowledge, no one has yet attempted to quantify the amounts of different CS types which bind to fibrils of collagen I and II as a result of fibrillogenesis.

This study had the following aims: (1) to compare collagen types I and II with regard to their ability to bind CS A−C during fibrillogenesis in vitro, including the influence of CS A−C on the proportion of collagen incorporated into fibrils of collagen I and II during fibrillogenesis and the effect of CS A−C on the morphology of collagen fibrils, and (2) to study the effect of fibril-bound CS A−C on osteoblast adhesion and spreading.

Materials and Methods

Unless stated otherwise, all chemicals, including the pepsin-treated collagen types I (27664, bovine skin) and II (C1188, bovine tracheal cartilage), as well as CS A (C9819, molecular weight (MW) 20−30 kD, 70% pure, 30% CS C, 15.1% sulfate), CS B (C3788, MW 40−45 kD, 90% pure, 18.5% sulfate), and CS C (C4384, MW 50−58.8 kD, 90% pure, 18.0% sulfate), were obtained from Sigma-Aldrich Chemie GmbH, Germany. Titanium for cell adhesion experiments (purity 99.6%) was obtained from Goodfellow GmbH, Germany. Titanium discs with a diameter of 9.5 mm were produced and cleaned in an

* To whom correspondence should be addressed. E-mail: Timothy.Douglas@mailbox.tu-dresden.de.
† Institute of Material Science.
‡ Institute of Physiological Chemistry.

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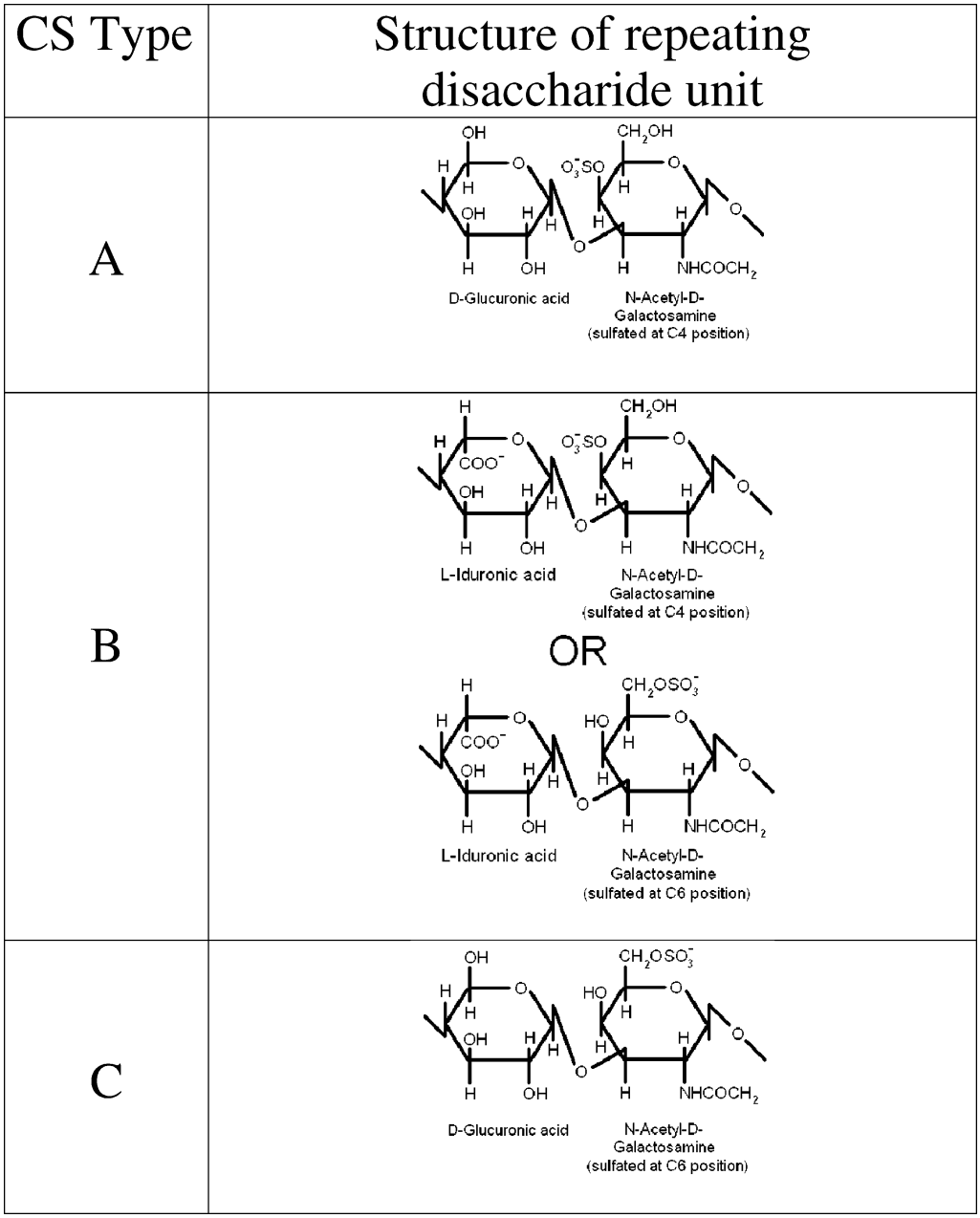

Figure 1. Structures of repeating disaccharide units of CS A−C.

ultrasound bath with 1% Triton detergent, deionized water, acetone, and ethanol for 15 min each. All chemical reagents used were reagent grade and all solutions were prepared with high-purity deionized water. Spectroscopic measurements were made using a Tecan Spectrafluor Plus spectrometer.

Preparation of Fibrils. Fibrils of collagen types I and II were prepared according to the method of Williams et al.[23] Briefly, collagen I and II were both dissolved at 1 mg/mL in 10 mM acetic acid overnight at 4 °C. Aliquots were mixed on ice with equal volumes of double-concentrated fibrillogenesis buffer (50 mM sodium dihydrogenphosphate and 10 mM potassium dihydrogenphosphate at pH 7.4 to yield 60 mM phosphate in total) in 1.5-mL microcentrifuge tubes (Brandt, Germany). The pH of the buffer was adjusted using 1 M NaOH. CS was added to reaction solutions containing either collagen I or collagen II before the start of fibrillogenesis at CS:collagen mass ratios ranging from 1:1.5 to 1:6. Controls were made in the absence of CS. Fibril formation took place at 37 °C overnight. Fibrils were subsequently separated using centrifugation for 15 min at 10 000*g*. Supernatant and pellet were retained for analysis.

Protein Determination in Supernatant and Pellet. Collagen concentration in supernatants was determined using a protocol on the basis of the method of Lowry et al.[24] Two hundred microliters of supernatant was mixed with 1 mL of a freshly prepared solution formed by diluting 2% (w/w) Na_2CO_3 in 0.1 M NaOH with 0.5% $CuSO_4 \cdot 5 H_2O$ (w/w) in 1% $Na_3C_6H_5O_7 \cdot 2 H_2O$ (w/w) by a factor of 50. Fifteen minutes after mixing, 100 μL Folin and Ciocalteu's Phenol reagent was added and the mixture was shaken vigorously. After subsequent incubation in the dark for 90 min, absorbance was measured at 700 nm. A calibration curve derived from solutions of the respective collagen type in 10 mM acetic acid ranging between 0 μg/mL and 500 μg/mL and subjected to the same procedure enabled collagen concentration to be determined. All experiments were performed six times. The mass of collagen in the pellet was calculated by subtracting the mass of collagen detected in the supernatant from the initial mass of collagen before fibrillogenesis. The reliability of this method was verified by control experiments where (1) collagen concentration was measured in fibrillogenesis solutions where fibril formation was inhibited at 4 °C and (2) fibrillogenesis took at 37 °C, the pellet was

redissolved in 10 mM acetic acid overnight, and collagen concentrations in supernatant and "dissolved fibril" solution were determined. In both cases, 100% of the collagen was detected (data not shown).

Determination of CS by Dimethylmethylene Blue (DMMB) Assay. CS was quantified using a protocol on the basis of the method of Chou et al.[12] Briefly, pellets in microcentrifuge tubes were resuspended in 500 μL of a 0.1 mg/mL papain solution in Hank's balanced salt solution (HBSS) using pulses from an ultrasound horn (UP 100H, Dr. Hieschler GmbH, Germany) at cycle = 1 and 100% amplitude for 3 s. Thereafter, digestion took place at 60 °C for 24 h. After digestion, 40 μL of the solution was transferred to a 96-well Nunc microplate and was reacted with 250 μL of a 1,9- dimethymethylene blue (DMMB) solution composed of 21 mg of DMMB, 5 mL absolute ethanol, and 2 mg sodium formate per 1 L with pH adjusted to 1.5 using 6 M HCl. The amount of CS was determined by measuring the absorbance at 590 nm and comparing it to a calibration curve consisting of CS solutions in HBSS with concentrations ranging from 0 μg/mL to 100 μg/mL. All experiments were performed three times.

Determination of CS by Hexosamine Assay. CS was quantified using a protocol on the basis of the Swann-Elson hexosamine assay as applied by Yannas et al.[25] In brief, pellets in microcentrifuge tubes were resuspended in 500 μL of 6 M HCl and were hydrolyzed at 105 °C for 6 h. HCl was removed by drying over NaOH pellets under vacuum. The residue was dissolved in 1.25-mL deionized water. One milliliter of this solution was mixed with 1 mL of solution A (1.25 M Na_2CO_3, 390 mM acetyl acetone) and was incubated at 95 °C for 1 h. After cooling, 5 mL ethanol was added, followed by 1 mL of solution B (178 mM Ehrlich's Reagent (*p*-dimethylaminobenzaldehyde), 8.46 M ethanol (50% pure ethanol by volume), 3 M HCl). The resulting mixture was left to stand for 1 h and absorbance was measured at 540 nm. Calibration samples consisting of CS masses ranging from 0 μg to 100 μg were subjected to the same treatment. All experiments were performed three times.

Examination of Morphology by Atomic Force Microscopy (AFM). Collagen coatings on titanium-sputtered glass discs were prepared by resuspending pellets after centrifugation in 300 μL fibrillogenesis buffer using an ultrasound horn as described above. The collagen fibril suspension was transferred dropwise onto smooth titanium-sputtered glass discs suitable for AFM under a laminar flow hood. After 30 min, the discs were rinsed three times with distilled water and were air-dried. Fibril morphology was assessed by AFM using a Bioscope instrument (Digital Instruments/Veeco) in tapping mode and aluminum reflex coated silicon tips. All images were obtained at a scan rate of 1.2 Hz, scanning 512 lines per image. Both height and amplitude images were captured simultaneously. For the AFM examination, fibrils were formed at CS:collagen ratios of 1:1.5 (w/w).

Preparation of Collagen-Coated Titanium Ssurfaces. Titanium discs of diameter 9.5 mm were roughened by blasting with corundum of diameter 250 μm (Finox, Germany). Blasted surfaces were then cleaned with 1% Triton X-100, acetone, and 96% ethanol, were rinsed with distilled water, and were air-dried. Fibrils formed in the step described above were resuspended in buffer solution using pulses from an ultrasound horn (UP 100H, Dr. Hieschler GmbH, Germany) to form a suspension with a "concentration" of 1 mg fibrils/mL buffer solution. One hundred microliters of this suspension was added dropwise onto the surface of each titanium disc. After being coated with fibrils by adsorption for 30 min, surfaces were washed three times with distilled water and were air-dried. Coatings were cross-linked overnight at room temperature by immersion in a solution of 19.17 mg/mL *N*-(3-dimethylaminopropyl-*N*′-ethyl-carbodiimide hydrochloride) (EDC) and 5.75 mg/mL *N*-hydroxysuccinimide (NHS) in 60% 0.1 M Na_2HPO_4 at pH 5.5 (vol %) and 40% absolute ethanol (vol %). After removal of the cross-linking solution, surfaces were immersed three times for 30 min in 0.1 M Na_2HPO_4 at pH 9.1 and then twice for 30 min in 4 M NaCl and were finally rinsed five times with double-distilled water and were air-dried. Sterilization was carried out using ethylene oxide at 45 °C. For cell experiments, fibrils of collagen I and II were formed at CS:collagen ratios of 1:1.5 or without CS for control samples.

Cell Preparation and Culture. Throughout this study, National Institutes of Health (NIH) guidelines for the care and use of laboratory animals were observed. Rat calvarial osteoblasts 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 were subcultured in Dulbecco's modified Eagle's medium (DMEM) (Biochrom) containing 10% fetal calf serum (FCS). The osteogenic differentiation and osteoblast phenotype of these rat cells (rO) was confirmed by determination of alkaline phosphatase activity, collagen type I synthesis, osteocalcin mRNA, and formation of calcium phosphate deposits. After three to eight passages, cells were used for the experiments. For adhesion experiments, osteoblasts were plated in a small volume (200-μL medium with 10% FCS for samples with a diameter of 16 mm on the top of the titanium discs at 12 500 cells/cm^2. After 2-h incubation at 37 °C the medium, which contained 10% FCS, was filled up to 1 mL.

Focal Adhesion Contacts: Immunofluorescence Staining. After incubation of the cells on the different titanium coatings, fixation was performed with 4% paraformaldehyde (w/v) in PBS for 15 min at 25 °C. The cells were then permeabilized with 0.1% Triton X-100 in PBS for 20 min. Following this, unspecific binding sites were blocked with 1% bovine serum albumin/0.05% Tween 20 (w/v) in PBS. Primary antibody binding (64 μg mouse antivinculin (hVin I clone)-IgG/mL of 1% BSA/0.05% Tween 20 in PBS, Sigma) was allowed to take place for 1 h at 25 °C. After washing with PBS, the cells were exposed to an additional incubation in blocking buffer for 10 min. Secondary antibody (66 μg goat antimouse tetramethylrhodamine isocyanate (TRITC)-labeled IgG/mL of 1% BSA/0.05% Tween 20 in PBS, DAKO) and fluorescein isothiocyanate (FITC)-phalloidine (1.5 μg/mL of 1% BSA/0.05% Tween 20 in PBS, Sigma) were used for 1 h at 25 °C. Subsequently, DAPI (0.2 μg 4′,6-diamidino-2-phenylindol/mL PBS, Sigma) for nuclei staining was applied for 15 min at 25 °C. Stained cells on coated titanium plates were then embedded in Mowiol 4-88. The staining was visualized using an AxioPhot fluorescence microscope, and digital images were acquired with an 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 exciation 450−490 nm, emission 515−565 nm; DAPI excitation 365 nm, emission 420 nm.

Statistics. For analysis of statistical significance, experiments were repeated three times. Results are presented as mean ± standard deviation. A one-way ANOVA (analysis of variance) was applied.

Results

Binding of CS to Fibrils of Collagen Types I and II. The amounts of CS A−C bound per milligram fibrils of collagen I and II are shown in Figure 2. The results of the two colormetric assays (DMMB and hexosamine) yielded similar results and indicated that, in general, collagen II bound more CS C and CS B than collagen I and that both collagen types bound different amounts of different CS types, with the amount of CS bound per unit fibril mass decreasing in the order CS C > CS B > CS A. Generally speaking, more statistically significant differences were seen at higher CS:collagen ratios.

The results in Figure 2a and 2b demonstrate that at all CS:collagen I ratios, the amount of CS C bound ranged from 1.5 to 3 times the amount of CS A bound and from 1.25 to 2 times the amount of CS B bound. At all CS:collagen I ratios except 1:6 as measured by DMMB assay, the amount of CS B bound was between about 1.25 and 1.5 times higher than the amount

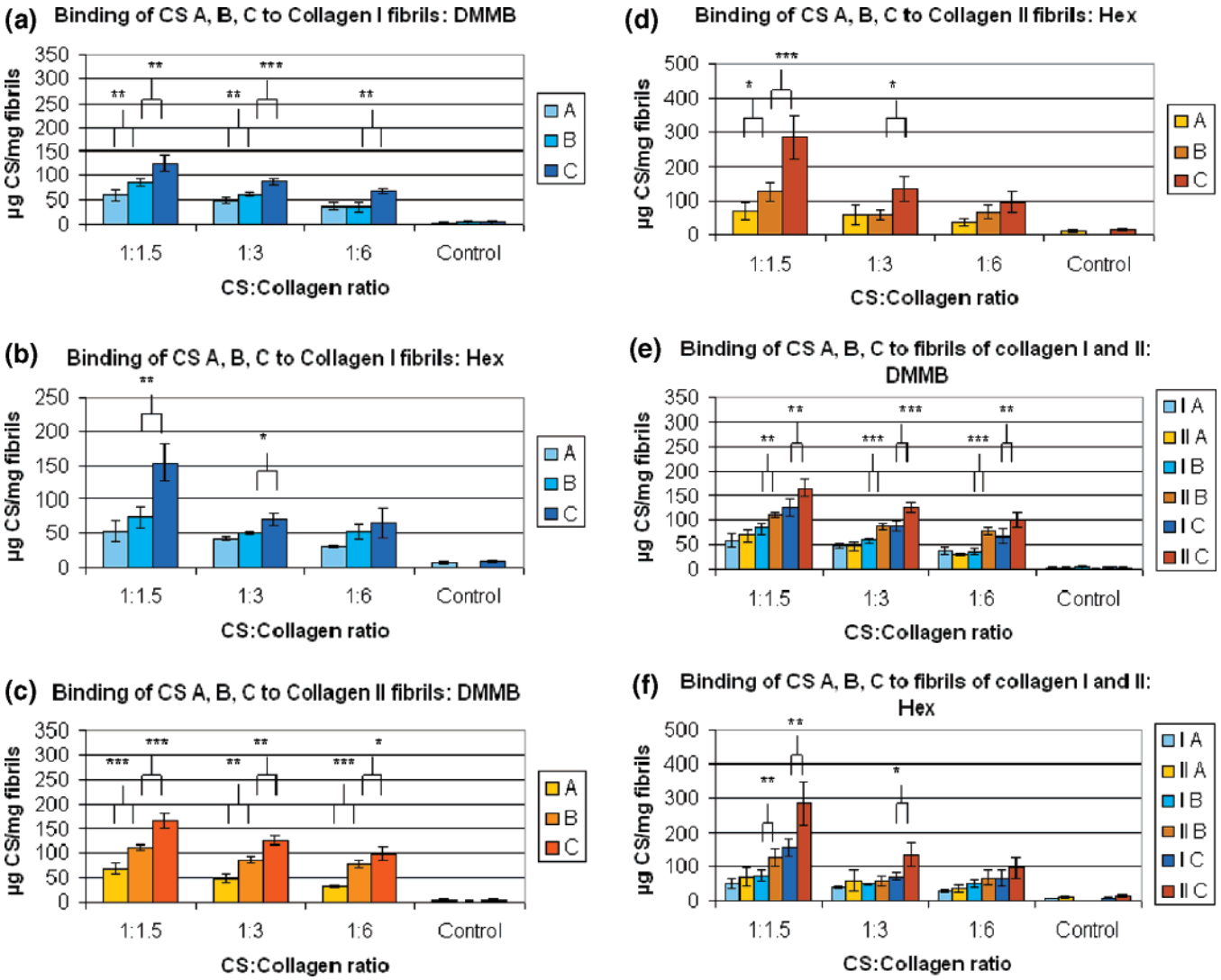

Figure 2. Comparison of amount of CS A−C bound by fibrils of collagen types I and II formed during fibrillogenesis by dimethylmethylene blue (DMMB) assay and hexosamine (hex) assay at differing CS:collagen ratios. (a, b) Amount of CS bound per mg fibrils of collagen I measured by DMMB and hex assays, respectively; (c, d) amount of CS bound per mg fibrils of collagen II measured by DMMB and hex assays, respectively; (e, f) results from a−d rearranged to aid statistical comparison of differences between collagen types I and II. Abbreviations: A, CS A; B, CS B; C, CS C; I A, collagen I + CS A; I B, collagen I + CS B; I C, collagen I + CS C; II A, collagen II + CS A; II B, collagen II + CS B; II C, collagen II + CS C. All experiments were performed three times. Error bars show standard deviation. Significances: $* = p < 0.05$, $** = p < 0.01$, $*** = p < 0.001$. The absence of stars indicates no significant difference.

of CS A bound. Significantly more CS C was bound than CS B at all three ratios according to the DMMB assay and at 1:1.5 and 1:3 according to the hexosamine assay. Significantly more CS B was bound than CS A at all 1:1.5 and 1:3 according to the DMMB assay. However, no significant differences between CS B and CS A were detected by the hexosamine assay at any ratios.

Figure 2c and 2d shows that, at all CS:collagen II ratios, the amount of CS C bound was 2.5 or more times higher than the amount of CS A and was approximately 1.5 times higher than the amount of CS B bound. Significantly more CS C was bound than CS B at all three ratios according to the DMMB assay and at 1:1.5 and 1:3 according to the hexosamine assay. Significantly more CS B was bound than CS A at all three ratios according to the DMMB assay and at 1:1.5 according to the hexosamine assay.

In Figure 2e and 2f, it can be seen that collagen II bound significantly more CS B than collagen I at all three ratios according to the results of the DMMB assay and at 1:1.5 according to the results of the hexosamine assay. In the case of CS C, significantly more was bound by collagen II at all three ratios from the results of the DMMB assay and at 1:1.5 and 1:3 according to the hexosamine assay. No statistically significant differences were observed in the amounts of CS A bound by collagen I and collagen II fibrils.

Influence of CS on Proportion of Collagen Incorporated into Collagen Fibrils. The proportion of collagen incorporated into fibrils of collagen I and II at different CS:collagen ratios can be seen in Figure 3. The percentages of both collagen I and II incorporated into fibrils decreased with increasing CS A:collagen and CS C:collagen ratios; for collagen I, the percentage collagen incorporated sank from values of 68% (CS A) and 67% (CS C) in the absence of CS to 56% (CS A) and 45% (CS C) at CS:collagen = 1:1.5; for collagen II, the percentage collagen incorporated fell from 42% (CS A) and 51% (CS C) without CS to 33% (CS A) and 30% (CS C) at a CS:collagen ratio of 1:1.5. However, increasing the CS B:collagen ratio had no appreciable effect on the proportion of collagen I or collagen II incorporated into fibrils. At 1:1.5 and 1:3, significant differences were detected between CS B and both CS A and CS C for both collagen I and collagen II.

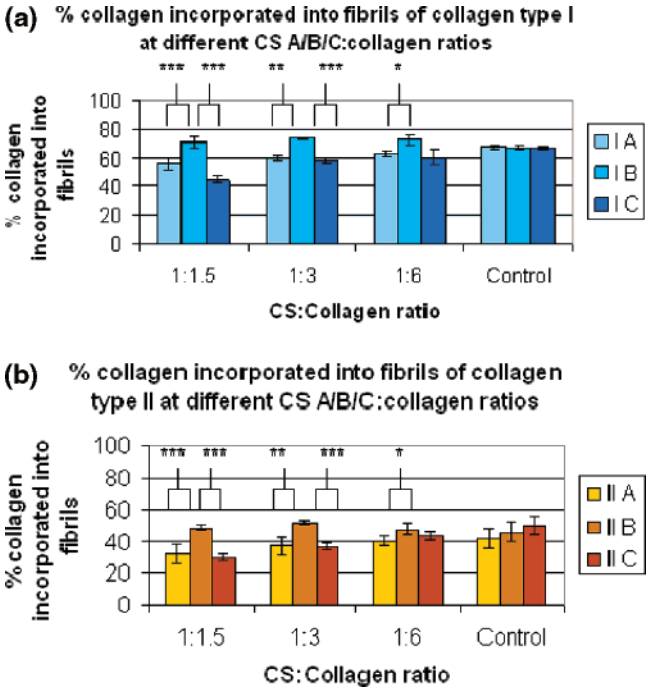

Figure 3. Influence of CS A−C on proportion of collagen I and II incorporated into fibrils during fibrillogenesis at different CS:collagen ratios. (a) Collagen I; (b) collagen II. All experiments were performed six times. Error bars show standard deviation. Significances: $* = p < 0.05$, $** = p < 0.01$, $*** = p < 0.001$. The absence of stars indicates no significant difference.

Changes in Fibril Morphology Caused by CS. Representative images of fibrils of collagen I and II formed in the presence of CS A−C are presented in Figure 4. All three CS types caused both collagen I and collagen II fibrils to become thinner. The most pronounced change was seen for collagen II fibrils formed in the presence of CS C. The smallest fibril which could be clearly identified was a collagen II fibril formed in the presence of CS C which measured approximately 250 nm in length and 100 nm in diameter.

Influence of CS on Osteoblast Adhesion and Spreading. Representative AFM images showing the formation of focal adhesions by osteoblasts cultured on coated titanium surfaces after 2 and 24 h are shown in Figure 5. Nuclei, actin, and vinculin appear blue, green, and red, respectively. A yellow color indicates the formation of vinculin−actin complexes; a yellow streak at the edge of a cell represents a focal adhesion. After 2 h, cells cultured on collagen I- and collagen II-coated surfaces had a more flattened appearance and therefore seemed to have spread better than those cultured on bare titanium. The formation of focal adhesions appeared to be stimulated by all CS types except CS C. Cells cultured on surfaces containing CS C also appeared smaller and rounder and were less well spread than cells on surfaces containing CS A and CS B. However, at 24 h, cells had adopted a spread shape and formed focal adhesions on surfaces containing CS C.

Discussion

This study had several aims. First, the ability of collagen types I and II to bind the CS A−C preparations during fibrillogenesis in vitro was compared. Second, the influence of the CS A−C preparations on the proportion of collagen incorporated into fibrils of collagen I and II during fibrillogenesis and the effect of CS on the morphology of collagen fibrils were investigated.

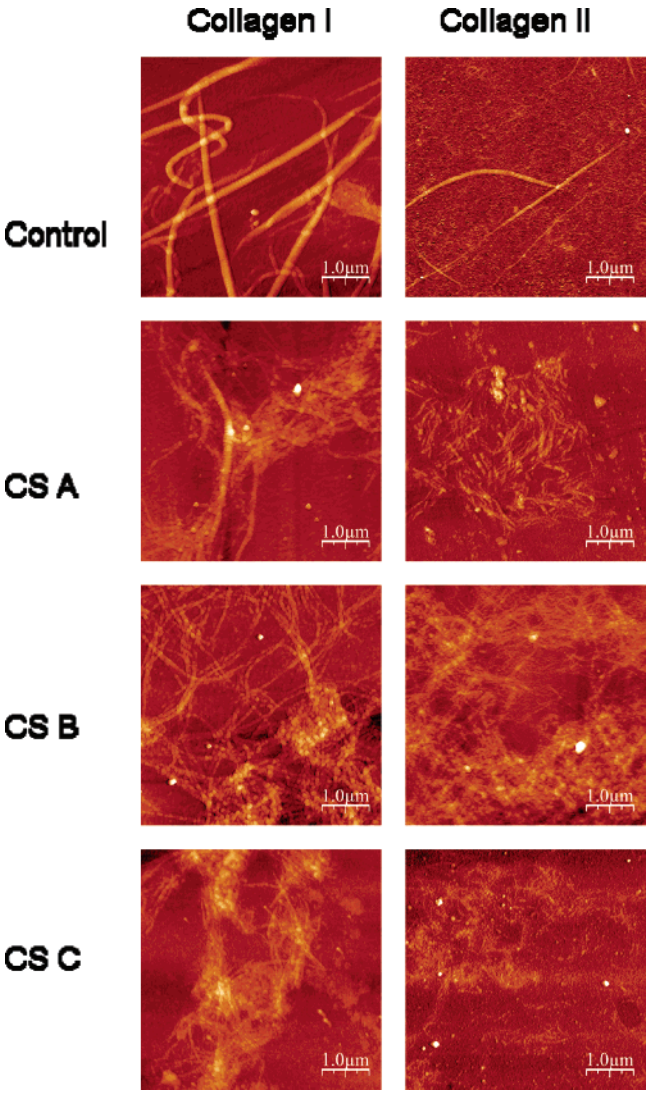

Figure 4. Influence of CS A−C on morphology of fibrils of collagen I and II. CS:collagen ratio = 1:1.5 in all cases except controls (without CS). All images have the dimensions 5.0 μm × 5.0 μm. Images were obtained using atomic force microscopy (AFM).

Third, the effect of fibril-bound CS A−C on rat osteoblast adhesion and spreading was studied.

A. Binding of CS A−C to Collagen Types I and II. It should be borne in mind that the data obtained for CS−collagen interactions during fibril formation is limited to those fibrils which can be collected by centrifugation, as tiny aggregates of collagen may escape collection.

A1. Collagen Types I and II Show Differing Affinities for CS A, B, and C. In this study, both collagen I and collagen II were shown to bind more CS C to fibrils than CS B and more CS B than CS A (Figure 2a−d). There are two differences between the CS preparations which may influence their interactions with collagen, namely, molecular weight and chemical structure. The interactions between CS and collagen are believed to be ionic since increasing ionic strength has diminished or abolished CS binding in many publications.[26−30]

Regarding differences in molecular weight, it has been suggested that GAGs might "bridge" basic, cationic regions in neighboring collagen molecules and thus exert a stabilizing effect and that a higher GAG chain length would result in a more stable interaction.[27,28,31] In this study, molecular weight decreased in the order CS C > CS B > CS A. Öbrink and co-workers examined binding of CS B and CS A preparations

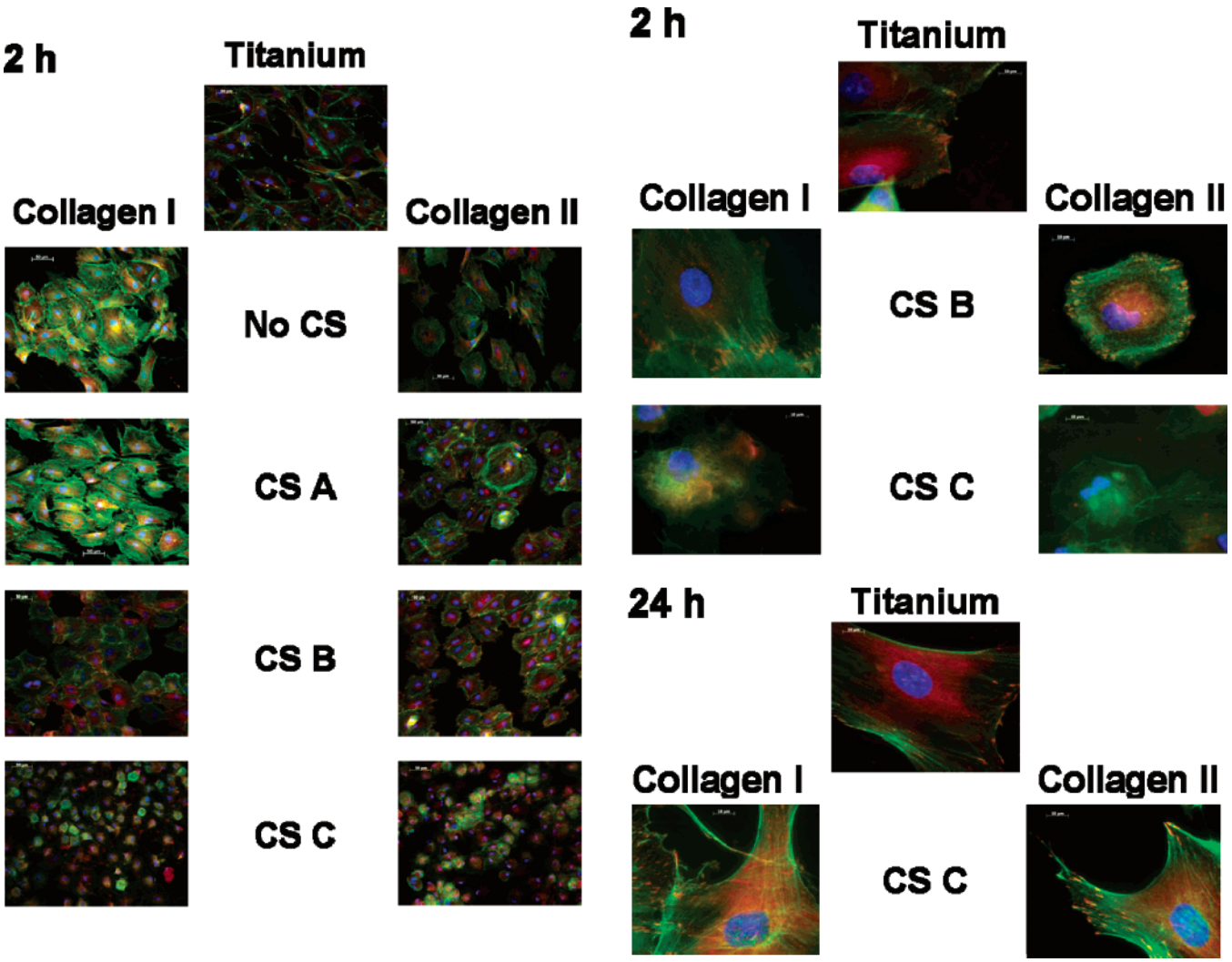

Figure 5. Effect of CS A−C on focal adhesion formation by rat calvarial osteoblasts and osteoblast spreading after 2- and 24-h incubation in medium containing 10% fetal calf serum as assessed by fluorescence staining. Titanium surfaces were coated with fibrils of collagen I or II containing CS A−C. Coatings were generated by adsorption of fibrils from a fibril suspension with a concentration of 1 mg/mL. CS:collagen ratio = 1:1.5 in all cases except controls (without CS). Red: vinculin; green: actin; blue: nuclei; yellow: vinculin−actin complex; thin yellow streaks at cell edges show focal adhesion contacts. Top: overview after 2-h incubation; middle: closeup after 2-h incubation; bottom: closeup after 24 h incubation.

with differing molecular weights to collagen molecules and found that more moles of the high molecular weight CS B and CS A was bound; furthermore, the number of binding sites on collagen for CS appeared to be higher for CS molecules of higher molecular weight.[29,30,32] This theory would explain why the affinity for collagen and mass of CS bound decreased with decreasing molecular weight (CS C > CS B > CS A).

Concerning the role that differences in the chemical structures of the CS types may play, it has been speculated that structural variations may affect the flexibility of the molecule and thereby its ability to assume certain conformations and bridge basic, cationic regions in neighboring collagen molecules; collagen has been reported to have varying affinities for different CS types with similar molecular weights in several publications.[29,32,33] In these papers, the strength of interaction between CS and collagen also decreased along the series CS C > CS B > CS A, which is in agreement with the results of this study.[34]

Both collagen I and II bound significantly more of the CS C preparation than the CS A preparation (Figure 2a−d). The fact that such a difference was seen although CS A is contaminated with 30% CS C suggests that chain length is a more important factor than chemical structure.

A2. Collagen Type II Binds More CS than Collagen Type I. More CS C and B was bound by collagen II than collagen I (Figure 2e and 2f). A stronger ionic interaction between GAGs and collagen II than between GAGs and collagen I has been suggested before. Pieper et al.[35] was unable to remove GAG from collagen II from bovine tracheal cartilage after washing with high ionic strength solution. Negroiu et al.[36] reported that collagen II precipitated from solution by addition of CS from bovine tracheal cartilage bound more CS than collagen I.

Since collagen II is found in tissues with a high PG or GAG content, such as cartilage, it can be seen that the ability to bind a larger amount of PG or GAG might be physiologically useful. Junquiera et al. reported that cartilage tissues, rich in collagen II, contain a higher mass of CS than tissues where collagen I predominates.[37]

On the basis of this evidence, it could be hypothesized that type II has an inherently higher affinity for CS than type I, leading to a higher mass of CS bound. Possibly, collagen II has binding sites for CS which are available for shorter CS chains than those on collagen I. Since CS preparations do not have a defined molecular weight, but a molecular weight range,

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the binding sites on collagen II might be available to a higher proportion of the ranges.

It is not clear why collagen II binds significantly more CS C and CS B than collagen I but not significantly more CS A. One hypothesis could be that although collagen II has binding sites available for CS C and CS B which collagen I does not have, these sites are not available for the CS A preparation used because its chain length is too short.

B. CS A−C Decrease Proportion of Collagen Incorporated into Fibrils and Reduce Fibril Length and Thickness. In this study, it was found that the proportion of collagen I and II incorporated into fibrils decreased with increasing concentration of CS A and C but not CS B. Kuijer et al. found that an increase in both CS A and CS C concentration led to a decrease in the proportion of collagen II incorporated into fibrils.[38] Bierbaum et al. reported a decrease in the proportion of collagen I incorporated into fibrils at a CS A:collagen ratio of 1:20.[5] Once bound to fibrils, CS may sterically hinder further fibril growth, resulting in less collagen being incorporated into fibrils; the more CS present, the greater the hindrance. It has been reported that sugars inhibit collagen fibrillogenesis by disrupting hydrogen-bonded water bridges between collagen helices.[39] It is not clear why a similar effect was not seen for CS B as for CS A or C.

All CS types reduced fibril thickness. Again, CS may hinder further fibril growth sterically, resulting in less collagen being incorporated into fibrils and a corresponding decrease in fibril size. The most pronounced reduction in fibril thickness was observed for collagen II fibrils containing CS C. This may be because collagen II bound considerably more CS C than CS A and CS B at the CS:collagen ratio at which fibrils formed (1:1.5). It is possible that the more CS present, the greater the hindrance of fibril growth.

C. Formation of Focal Adhesions is Promoted by CS A and CS B but Not CS C at 2 h, but by All Three CS Types at 24 h. All CS types enhanced the formation of focal adhesions except CS C after 2-h incubation. In contrast to osteoblasts cultured on surfaces coated with fibrils containing CS A and CS B, osteoblasts cultured on surfaces coated with fibrils containing CS C showed no focal adhesions formed at the cell edge after 2-h incubation and had a more rounded shape, indicating poorer adhesion. However, at 24 h, osteoblasts on surfaces containing CS C had also formed focal adhesions, suggesting that the inhibition at 2 h is only temporary. Three differences between the samples which may possibly influence focal adhesion formation are (1) differences in topography caused by differences in fibril diameter, (2) differences in the amount of CS present, and (3) differences in the chemical structure of the CS types.

Concerning topographical differences, the shape and cytoskeletal organization of several cell types seeded on collagen-coated surfaces have been influenced by organization of the collagen layer at the sub-micrometer level.[40−42] Fibrils of collagen II containing CS C were considerably smaller than those containing CS A and CS B. It could be speculated that the presence of smaller fibrils, as in the case of collagen II containing CS C, results in a topography which inhibits focal adhesion formation and causes osteoblasts to adopt a rounded, nonflattened shape at 2 h.

Regarding the influence of the amount of CS present, collagen has promoted the adhesion and spreading of osteoblasts and osteoblast-like cells in many publications[1−4] and CS has enhanced the formation of focal adhesions by osteoblasts previously.[5] However, when present in larger quantities, CS, which has been localized at the outer surface of collagen fibrils,[43] may obscure cell recognition sites on collagen, making osteoblast spreading and adhesion more difficult. The larger amount of CS C bound might lead to more recognition sites for osteoblasts on the surface of collagen fibrils being "blocked", hindering spreading and focal adhesion formation at 2 h.

It is conceivable that differences in chemical structure may play a role. However, the fact that CS A promotes focal adhesion formation despite being contaminated with 30% CS C suggests that CS chemical structure does not greatly influence focal adhesion formation after 2 h.

One possible explanation for the observation that spreading and focal adhesion formation on surfaces containing CS C improves after 24 h is that the osteoblasts secrete matrix proteins (e.g., fibronectin, collagen) which overlay the sites blocked by CS C or enable the cells to overcome the effect of the undesirable topography, allowing spreading and adhesion. Spreading and adhesion may be poor after 2 h because cells have not had sufficient time to secrete these proteins.

Summary and Outlook

Collagen types I and II showed different affinities for the CS A−C preparations used, with amount of CS bound/milligram fibrils decreasing in the order CS C > CS B > CS A. Collagen II bound more CS B and CS C than collagen I. All three CS types caused fibrils of collagen I and II to become thinner. The presence of CS A and CS B in collagen fibril coatings on titanium surfaces appeared to enhance the formation of focal adhesions by osteoblasts after 2-h incubation. These results could be important when choosing collagen or CS type for the construction of scaffolds for TE purposes or implant coatings. Further work should include more extensive cell adhesion experiments to determine the optimal CS:collagen ratios for CS A−C. In addition, the effects of coatings containing CS A−C on osteoblast proliferation and differentiation should be investigated. The presence of CS in coatings promoted osteocalcin production in a previous publication.[5] Growth factors have been immobilized onto collagen layers without losing their bioactivity.[34] Growth factors could be immobilized on layers containing CS A−C to attempt to acheive synergystic effects between the different CS types and growth factors. Interactions between growth factors and GAGs are highly sensitive to GAG composition.[19,44] The effect of a growth factor may possibly be enhanced by choice of the appropriate CS type. Also, CS preparations with more similar molecular weights should be obtained. This would aid further investigation of the effect of CS structure on affinity for collagen and osteoblast adhesion.

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