Effect of Modified Pectin Molecules on the Growth of Bone Cells

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The aim of this study was to investigate molecular candidates for bone implant nanocoatings, which could improve biocompatibility of implant materials. Primary rat bone cells and murine preosteoblastic MC3T3-E1 cells were cultured on enzymatically modified hairy regions (MHR-A and MHR-B) of apple pectins. MHRs were covalently attached to tissue culture polystyrene (TCPS) or glass. Uncoated substrata or bone slices were used as controls. Cell attachment, proliferation, and differentiation were investigated with fluorescence and confocal microscopy. Bone cells seem to prefer MHR-B coating to MHR-A coating. On MHR-A samples, the overall numbers as well as proportions of active osteoclasts were diminished compared to those on MHR-B, TCPS, or bone. Focal adhesions indicating attachment of the osteoblastic cells were detected on MHR-B and uncoated controls but not on MHR-A. These results demonstrate the possibility to modify surfaces with pectin nanocoatings.

Introduction

Bone is a dynamic tissue, which is continuously remodelled by two bone cell types: bone-resorbing osteoclasts and bone-forming osteoblasts. The operative areas of the coupled functions of these bone cells are called bone remodeling units, in which osteoblasts produce new bone after osteoclastic resorption of the old bone tissue. Osteoclasts differentiate from bone marrow hematopoietic stem cells by fusion of mononuclear progenitors into large multinuclear bone-resorbing cells.[1] Osteoclasts can be detected as multinuclear tartrate-resistant acid phosphatase (TRACP)-positive cells. Bone resorption occurs via tight attachment of osteoclasts onto the target bone area, in which both organic (mainly collagen) and inorganic (hydroxyapatite) bone matrix constituents are dissolved. In non-resorbing osteoclasts actin is arranged at the cell periphery as podosomes, whereas in the resorptive phase active osteoclasts can easily be detected by staining actin; functional osteoclasts form a so-called sealing zone consisting of a ringlike actin structure.[2−4]

Lectins compose a molecular family of sugar-binding proteins or glycoproteins, some of which, such as wheat germ agglutinin (WGA) and *Arachis hypogaea* peanut agglutinin (PNA), are reported to have target cells also in bone tissue.[5,6] Interestingly, WGA recognizes and binds to glycomolecules revealed in resorbed bone matrix area. Osteoclastic resorption pits on bone substrate in vitro can be detected by WGA−lectin staining indicating their intimate reaction with bone matrix sugar epitopes.[7]

Osteoblasts differentiate from mesenchymal stem cells. Mature osteoblasts are capable of producing collagen-rich osteoid matrix and mineralizing it. Osteoblasts are adherent and anchorage-dependent cells, which have to attach to certain extracellular matrix (ECM) proteins, such as fibronectin, to function and differentiate properly.[8] This attachment is mediated by specific structures called focal adhesions (FAs), which function as anchoring protein complexes between the actin cytoskeleton of the cells and ECM.[9] Focal adhesions contain many proteins, of which paxillin and vinculin can easily be stained for FA visualization. The main interconnecting protein in FAs is integrin.[9] Integrins bind to a specific amino acid motif (Arg-Gly-Asp) in ECM proteins, which then stimulates the attachment and spreading of osteoblasts as well as mineralization processes.[10−12] Sugar epitopes of ECM proteins also play a key role in bone formation. For example, Nagahata and colleagues postulate that a sulfated ECM polysaccharide hyaluronan induces the expression of cell attachment proteins in osteoblasts.[13] In addition, the importance of polysaccharides in bone biology is emphasized via growth factors; bone matrix proteoglycans biglycan and decorin control the effects of growth factors and cytokines on the cells of osteoblastic lineage,[14] and heparin sulfate affects the bone tissue morphogenesis via, for example, fibroblast growth factors.[15]

Problems in biocompatibility may cause severe clinical complications and implant failure; successful implant attachment requires stable fixation of biomaterial with bone cells during the healing period.[16,17] Thus, in orthopedic as well as dental implants osteointegration is a critical factor. Reactions following implantation occur at the implant material/bone tissue interface, which in successful operation can be seen as intensified bone formation and bone tissue bonding at the interface.[18] According to these demands it would be important to develop appropriate molecular coatings for bone implants.

Biocompatibility of medical devices is characterized mainly by interactions with cells and adsorbed proteins at the implant/tissue interface, which is the reason for the growing interest for modifying implant surface chemistry and topography.[17,19]

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Various chemicophysically appropriate carbohydrates and polyelectrolytes, such as alginic acid,[20,21] hyaluronan,[22] heparin,[23] and dextran hydrogels,[24] have been used as biomaterial coatings. Carbohydrate molecules seem to have many roles in biological processes, which are still poorly understood. Recently, this research area has been increasingly focusing on the applications of bioactive polysaccharides, such as chitosan.[25] Pectins are potential candidates for coating applications because of the possibility of controlling of their structure and their anti-inflammatory properties.[26] One aspect of using pectins is that these plant-derived molecules are not degraded by the human body.

Pectins are large polysaccharide molecules found in the cell wall structure of higher plants, in which these molecules provide strength to the wall and control the porosity and water-holding capacity of the wall. Pectins are also involved in cell expansion and separation and are important during fruit ripening.[27−29] Pectins can be isolated on an industrial scale to yield hydrocolloids for the food industry but can also be found as modified hairy regions (MHRs) in, for example, juices after enzymatic modification during the isolation process. The composition, charge, and wettability of these MHRs can be controlled. Pectins are composed of various structural elements. Different combinations of these elements organized in so-called smooth and hairy regions produce a wide spectrum of these large molecules. The smooth region consists of α-1,4-linked and partly methanol-esterified D-galacturonic acid residues (α-D-Gal) and is called homogalacturonan. In contrast, the hairy regions (also called ramified regions) consist mainly of alternating α-1,2-linked L-rhamnosyl (α-L-Rha) and α-1,4-linked D-galacturonosyl (α-D-Gal) residues, a backbone called rhamnogalacturonan I (RG-I). The hairy region may also contain arabinan and arabinogalactan side chains linked to the rhamnosyl residues and may be substituted with acetyl groups at O-2 and/or O-3 of the galacturonic acid moieties. Other structural elements of a hairy region, such as xylogalacturonan, contain in turn xylose residues and may be esterified with methanol. In addition, pectins may also contain a conserved and complex rhamnogalacturonan II element.[30−32]

Pectin molecules differ in their side chain composition, branching, total charge, and molecular weight, which is seen as varying physicochemical properties of these polysaccharides. In addition, pectin compositions depend on the plant species and tissue types. With specific enzymes, such as endopolygalacturonases and pectin methylesterases, it is possible to release the hairy regions from the smooth region for subsequent modifications with, for example, rhamnogalacturon hydrolase and lyase, rhamnogalacturonan, and α-D-galactopyranosylurunohydrolase.[33−36]

The aim of this study is to obtain a view of how bone cells attach, proliferate, and differentiate on modified pectin coatings. By investigating the molecular interactions of bone cells and modified pectin molecules we can further tailor the coatings for better biocompatibility and functionality.

Experimental Section

Osteoblast-like Cells. MC3T3-E1 (subclone IV) are murine preosteoblastic cells, which are not classified as cancer cells despite their ability to divide immortally. MC3T3 cells (LGC Promochem) were cultured in α-MEM (Sigma) containing 10% heat-inactivated (56 °C, 30 min) fetal bovine serum, FBS (Gibco), 1% L-glutamine (Gibco), and 100 U/mL penicillin + 100 μg/mL streptomycin (Sigma). Cells are cultivated in T25 cell culture bottles (Greiner) in a 37 °C incubator (5% CO_2, 95% air) and divided 1:6 every second day before culturing on test materials (described below) for 48 h.

Table 1. Percent Molar Amounts of Different Sugar Components of MHR-A and MHR-B after Enzymatic Modifications[a]

sugar	MHR-A (mol %)	MHR-B (mol %)
arabinose	50	11
galactose	10	20
galacturonic acid	26	37
glucose	1	3
mannose	0	0
rhamnose	5	11
xylose	8	18
methyl groups/100 mol of galacturonic acid	40	34
acetyl groups/100 mol of galacturonic acid	55	11
rhamnose/galacturonic acid	0.19	0.30
% (w/w) sugar	66	78

[a] Partly modified from ref 32.

Primary Bone Cells. A mixed primary bone cell population was isolated from long bones of 1−2-day-old Sprague−Dawley rat pups. Pups were decapitated, and each femur, tibia, and humerus was aseptically removed. In a laminar hood, bones were sterilized in 70% ethanol for 30 s, after which they were rinsed in 1× PBS. Muscle and cartilage tissues surrounding the bones were removed, and the bones were transferred into 10 mL of culturing medium (Dulbecco's modified Eagle's medium, alpha modification (α-MEM, Sigma)) containing 10% heat-inactivated (56 °C, 30 min) FBS (Gibco), 1% L-glutamine (Gibco), 100 U/mL penicillin + 100 μg/mL streptomycin (Sigma), and 20 mM HEPES buffer (Sigma). The pH of the medium was adjusted to 7.0. Bones were vertically cleaved into halves, and bone marrow cells were mechanically harvested into the medium. The suspension was centrifuged (1000 rpm, 8 min), after which the cell pellet was resuspended in α-MEM and spread (50 μL/cm^2) onto different test materials (described below). Cells were allowed to attach in a 37 °C incubator (5% CO_2, 95% air) for 30−60 min, after which detached cells were rinsed away. Cells were cultured for 48 h in a 37 °C incubator.

Test Materials. Bone cells were cultured on tissue culture polystyrene (TCPS) dishes (Ø 6 cm) or on objective glasses coated by the covalent linking of different MHRs of apple pectins. In short, test materials had been surface-functionalized by an aminating plasma deposition process and coated with MHRs using carbodiimide-mediated condensation between deposited amino groups and carboxyl groups of MHRs.[32] Apple MHR-A had been produced using a commercial enzyme preparation Rapidase C600 (DSM Food Specialities), and Apple MHR-B using a commercial enzyme preparation Rapidase liq+ (DSM Food Specialities). Coatings will be later denoted as MHR-A (RC600) and MHR-B (Rliq+). Enzyme treatments had released the homogalacturonan part of the pectins thus producing hairy regions. These two apple pectin derivatives contain different amounts of different kinds of neutral side chains, even though the basic structural moieties remain mainly the same.[32] Molecular compositions (mol %) of MHR-A and MHR-B are presented in Table 1. Substrate samples were sterilized with 10× penicillin−streptomycin solution (Sigma) at 4 °C for 2−24 h and rinsed with 1× PBS before culturing. Uncoated TCPS or glass, aminated surfaces, or bovine bone slices were used as control substrata depending on the cell type cultured.

Fixation, Staining, and Microscopy. After 48 h of culturing, cells were fixed with a 3% paraformaldehyde (PFA) solution containing 4% saccharose for 10 min at room temperature. The fixative was rinsed off with 1× PBS.

After fixation, cells were fluorescently labeled with FITC−phalloidin, Hoechst, Texas red conjugated paxillin antibody, or monoclonal vinculin antibody (Sigma). For actin staining with FITC−phalloidin (Sigma Chemical Co.), a 1:200 concentration of stain was used. Incubation conditions were 20 min at 37 °C. Hoechst 33258 stain (Sigma Chemical Co.) was used as a 1:800 dilution for 10 min at room temperature. For

paxillin and vinculin stainings, 0.1% (v/v) Triton-X-100 in PBS was used for permeabilization of the cells (10 min on ice), after which cells were treated with 0.2% BSA (30 min at room temperature). Staining reactions were performed using 1:100 mouse monoclonal paxillin (ZYMED Laboratories) or vinculin (Sigma) antibody for 45 min and secondary antibodies (ALEXA Fluor 568 or ALEXA Fluor 488 goat anti-mouse IgG, Molecular Probes) for 30 min on ice.

Osteoclasts were stained for TRACP using Sigma's Leukocyte Acid Phosphatase Kit according to the manufacturer's instructions.

Stainings were visualized with a Nikon Optiphot II fluorescence microscope coupled with a Sony DXC 930P video camera or with a confocal microscope (LSM 510, Zeiss) using appropriate filtersets.

Cell Counting. Focal adhesions of MC3T3-E1 cells were quantitatively analyzed based on paxillin and vinculin stainings. The proportions of FA-containing cells of the total cell number were calculated from 10 microscopic fields (63× objective) per sample type (MHR-A, MHR-B, aminated, TCPS). Vinculin staining was performed for MHR-B and TCPS samples, from which the size of the FAs was analyzed with a digital image analysis system (MCID-M5+, Imaging Research, Inc., Canada) from 10 microscopic fields (100× objective).

The amounts of TRACP+ cells of pit assay primary cell cultures were counted to obtain data indicating differentiation and activation of osteoclastic cells. From each sample (MHR-A-TCPS, MHR-B-TCPS, uncoated TCPS, and bone slice) 50 fields (40× objective) were visualized for manual cell counting. From MHR dishes, both total cell numbers as well as TRACP+ cell proportions were counted. From control samples the absolute TRACP+ cell numbers were counted. In addition, TRACP+ cells containing 1 or 2 nuclei and TRACP+ cells containing 3 or more nuclei were counted separately in each field observed.

From the pit assay primary cell cultures, also the proportions of osteoclastic cells containing actin rings were evaluated from a few microscopic fields (40× objective).

Statistical Analyses. Data sets were analyzed with a nonparametric Kruskal−Wallis test, and different sample groups were compared with a Mann−Whitney or *t*-test for two independent sample populations using SPSS 14.0 or Origin 6.0 software. The level of significance was set at $p < 0.05$. The graphs (Figure 4) were produced with Origin 6.0 software.

Results

Osteoblastic Cell Line. Murine preosteoblastic MC3T3-E1 (subclone IV) cells were cultured on TCPS dishes coated with MHR-A and MHR-B as well as on aminated and uncoated TCPS. These osteoblastic cells were cultured to verify the cell attachment and to study the behavior of immortal but noncancerous cells on polysaccharide coatings. MC3T3-E1 cells grew more numerously on MHR-B than on MHR-A. Clear and numerous focal adhesions were detected on MHR-B and uncoated TCPS, only a few on the aminated surface, and none on MHR-A (Figure 1). The average percent amounts of FA-containing cells on different sample types were as follows: MHR-A 0.0%, aminated 4.1%, TCPS 72.2%, and MHR-B 81.5%. The difference of FA cell proportions between MHR-B and TCPS was not significant. However, the size and morphology of FAs differed between MHR-B and TCPS, which was verified by measuring vinculin-stained FA spots (Figure 2). The average area of FAs on MHR-B was 0.57 μm^2 whereas the area of FAs on TCPS was approximately 1.45 μm^2. The average FA length was 1.28 μm on MHR-B and 2.11 μm on TCPS. Both of these differences were statistically significant ($p < 0.001$).

Primary Bone Cells. Cells scraped from the endosteal surfaces of newborn rat bones were cultured on MHR-A- and MHR-B-coated TCPS dishes, on pure TCPS, and on bovine bone slices. Bone substrate was generally preferred by cells, as expected. Between polysaccharides, cells preferred MHR-B to MHR-A. Both the density and the spreading of the cells were clearly diminished on MHR-A compared with MHR-B, TCPS, or bone. Stress fibers indicate the spreading and proper attachment of the cells. Numbers of osteoclasts were counted based on TRACP+ staining. The total number of TRACP+ cells on MHR-B exceeded that of TCPS, whereas it did not significantly differ between MHR-B and bone. The amount of

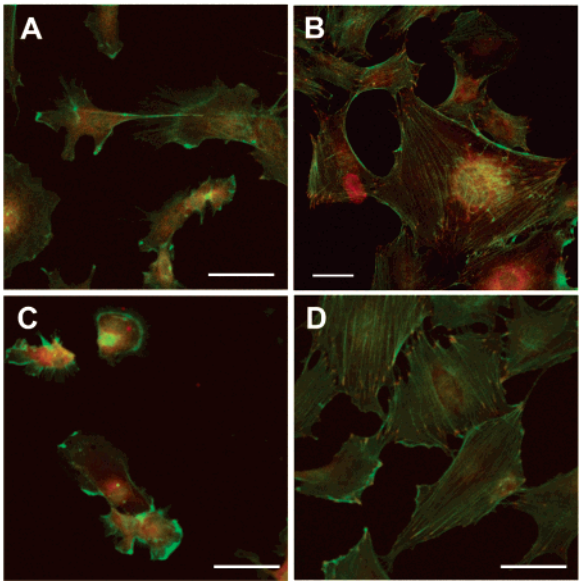

Figure 1. Confocal images of MC3T3-E1 cells growing on (A) aminated surface, (B) uncoated TCPS, (C) MHR-A, and (D) MHR-B. Focal adhesions stained with paxillin antibody can be seen as red spots (40× objective, 50 μm scale bar).

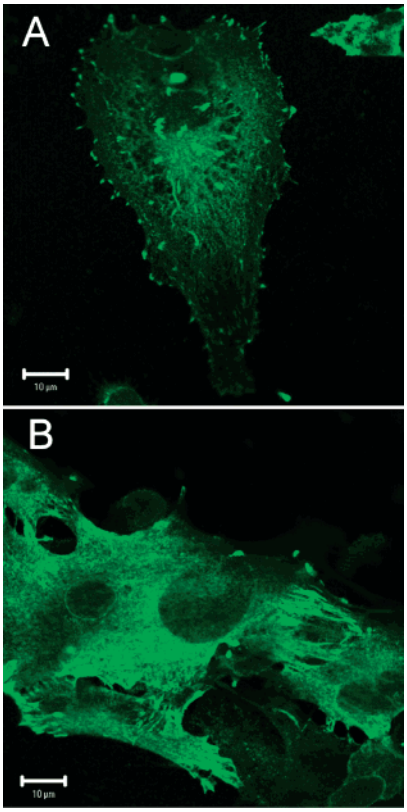

Figure 2. Confocal images of MC3T3-E1 cells growing on (A) MHR-B and (B) TCPS. Focal adhesions stained with vinculin antibody can be seen as green spots (100× objective, 10 μm scale bar).

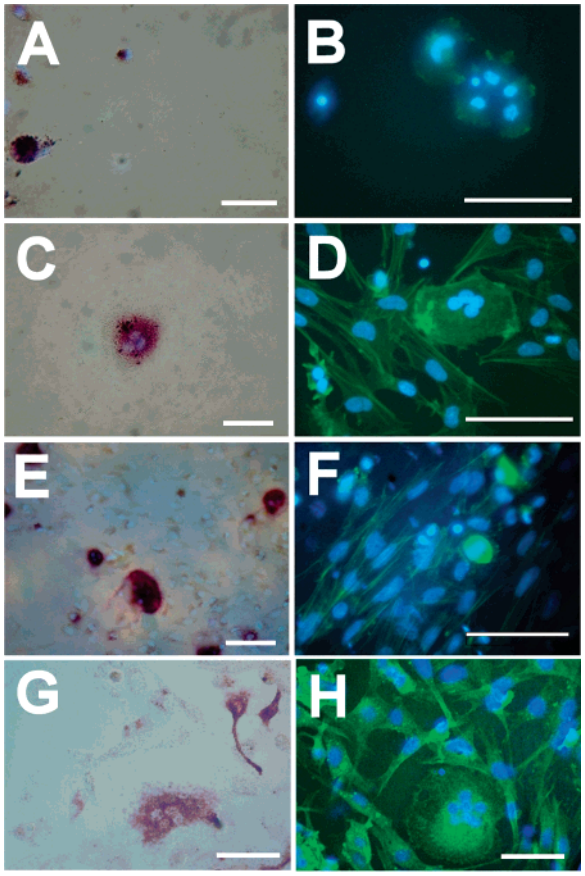

Figure 3. General views of primary rat bone cells growing on (A and B) MHR-A, (C and D) MHR-B, (E and F) bone, and (G and H) TCPS. Nuclei are stained with Hoechst (blue fluorescence) and actin with FITC−phalloidin (green fluorescence). TRACP is seen as a purple stain (40× objective, 50 μm scale bar).

TRACP+ cells was clearly diminished on MHR-A compared with MHR-B, TCPS, and bone (Figures 3 and 4). Between control surfaces, the number of TRACP+ cells was greater on bone than that on pure TCPS.

In addition, TRACP+ osteoclasts with 1−2 nuclei and with 3 or more nuclei (multinuclear) were counted separately. On MHR-A, the amount of 1−2 nuclear cells was clearly lower than those on MHR-B, TCPS, and bone. The amount of 1−2 nuclear cells did not differ significantly between control substrata. Interestingly, MHR-B contained more TRACP+ cells with 1 or 2 nuclei than TCPS or bone. Instead, the amount of multinuclear osteoclasts was significantly greater on bone than on TCPS or either of the polysaccharides, between which the number of multinuclear osteoclasts did not differ (Figure 4). We also calculated the ratios of multinuclear TRACP+ cells of all TRACP+ cells. This ratio between both MHRs and bone was significantly different; on bone the proportion of multinuclear TRACP+ cells of all the TRACP+ cells proved to be superior compared with that on polysaccharides. In contrast, between the MHRs such a difference was not detected.

The activity of the osteoclasts on MHR-B and bone was also verified by detecting the actin rings. On MHR-B, approximately one-third of the osteoclasts exhibited an actin ring, whereas on bone all of the osteoclasts contained an actin ring. In contrast, on MHR-A actin rings were not detected (Figure 5).

Total cell numbers representing the whole mixed cell populations were counted from the MHR samples. On MHR-B the total cell number proved to be superior compared with that of MHR-A. We also calculated the proportional amounts of

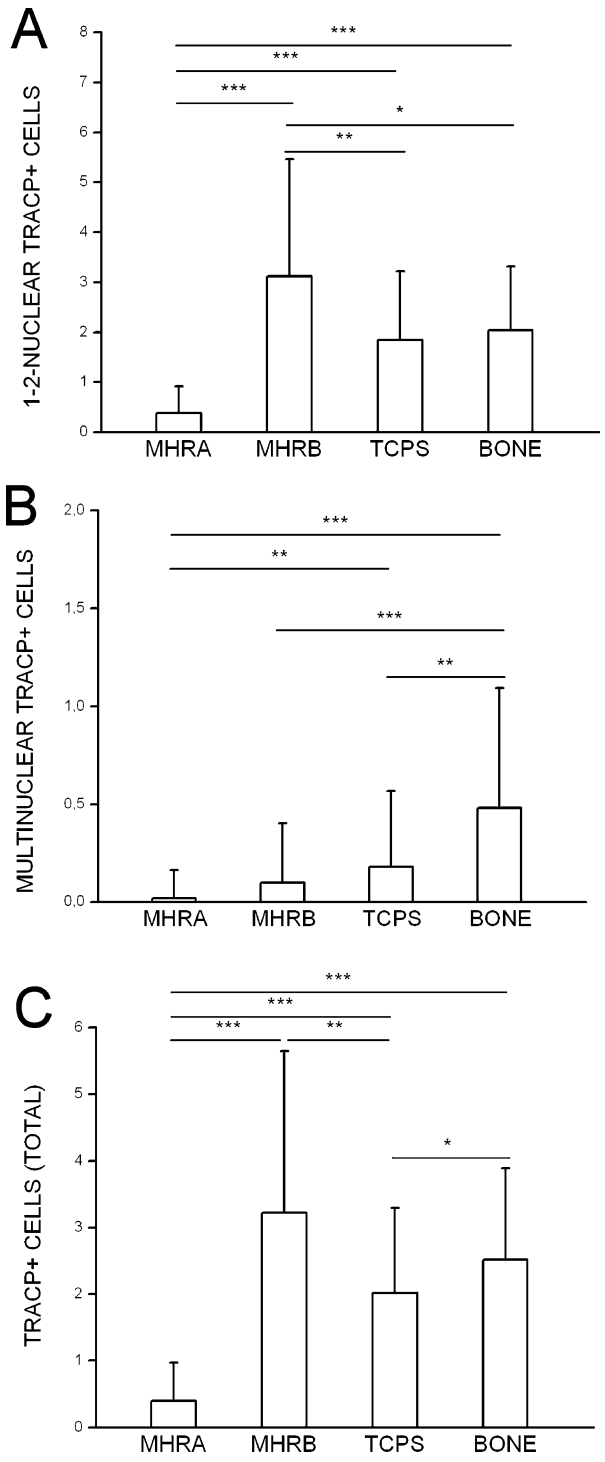

Figure 4. Results of a Kruskal−Wallis test illustrating the number of (A) 1−2 nuclear TRACP+ cells, (B) multinuclear TRACP+ cells, and (C) total number of TRACP+ cells on MHR-A, MHR-B, TCPS, and bone. Values of the *y*-axis represent the numbers of cells per a microscope field (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

TRACP+ cells of all of the cells on polysaccharide coatings. All of the ratios as well as the total amount of TRACP+ cells were significantly greater on MHR-B than on those on MHR-A.

In general, cell attachment, indicated by paxillin-stained focal adhesions, was more abundant on MHR-B and bone than on MHR-A. Osteoblastic cells of the primary bone cell pool showed clear paxillin spots on MHR-B and bone, on which focal adhesions were well-formed and numerous (Figure 5). This observation can be considered consistent with MC3T3-E1 data.

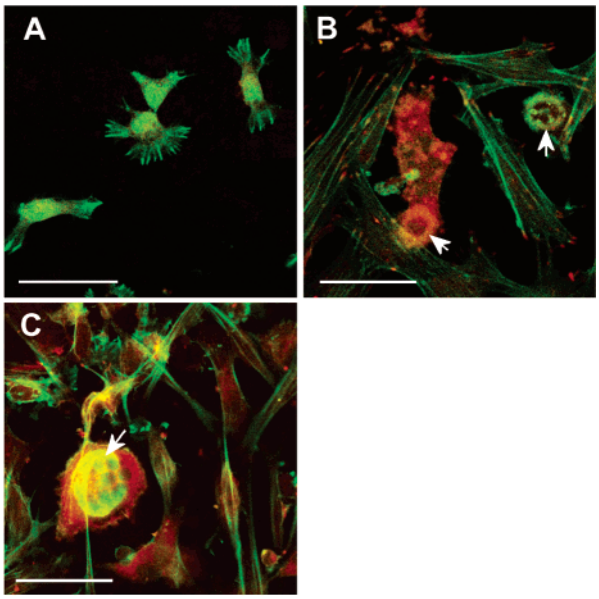

Figure 5. Endosteal cells from newborn rat bones (osteoclasts and osteoblasts) on (A) MHR-A, (B) MHR-B, and (C) bovine bone slice. Osteoclastic actin rings seen as green fluorescence are indicated with arrows (40× objective, 50 μm scale bar).

Discussion and Conclusions

We have assessed the effects of MHRs of apple pectin polysaccharides on the growth and differentiation of mammalian bone cells. According to our results, bone cells are sensitive to modifications of pectic coatings. The cells preferred rhamnogalacturonans with shorter side chains in all parameters studied. This preference can be seen both as an increased number as well as a more spread morphology of the cells on MHR-B coating in comparison to that on MHR-A coating. Similar results have been reported by Morra and colleagues with different cell types: Aortic smooth muscle cells and murine L-929 fibroblasts were cultured on MHR-A- and MHR-B-coated Petri dishes, and both of these cell types proved to grow better on MHR-B than on MHR-A.[32] Our results prove that bone cells can tolerate pectin-coated surfaces. Moreover, the behavior of bone cells depends at least on the length of the hairy regions of the rhamnogalacturonan molecules. This opens up possibilities to either stimulate or inhibit local bone growth.

Osteoclasts are appropriate cells for testing implant material biocompatibility because of their fine-tuned appearance and sensitivity, which can be exploited as a responsive marker of growth substrate suitability.[4] Mature osteoclasts are terminally differentiated and therefore remarkably demanding cells concerning their growing surroundings. Thus, these cells provide an advantageous cell model compared with immortal cell lines. It is very promising for the use of pectin-coated biomaterials in orthopedic implants that osteoclasts can organize the sealing zone. We doubt that bone-resorbing osteoclasts could also resorb pectin, but this remains to be confirmed. Even though Chambers and colleagues state that osteoclasts require a mineralized substratum for resorption to occur,[37] contradictory observations by Jones and colleagues showed that osteoclasts are able to resorb unmineralized dental tissue and also other substrata without specificity.[38] According to Redey and coworkers, the resorptive activity may be limited to the substratum solubility.[39]

When bone-marrow-derived cells were cultured on bone slices most of the observed factors, cell number, morphology, and osteoclast number, seemed to be superior compared to both MHR-A and MHR-B or pure TCPS. Interestingly, however, the amount of 1−2 nuclear osteoclasts was slightly greater on MHR-B than that on bone or TCPS. In addition, the total number of TRACP+ cells on MHR-B exceeded that of TCPS. According to these observations it seems that MHR-B is well tolerated by osteoclast precursors, whose differentiation process is still disturbed to some extent compared to that on bone. The superlative differentiation properties of bone substratum is further indicated by observing that the amount of multinuclear osteoclasts on pure TCPS was significantly lower compared to that on bone.

The differences between MHR sample types are supposed to be due to the differences in pectin nanocoatings. In general, only a few multinuclear osteoclasts were detected on both pectin samples, which probably explains the nonsignificant difference in multinuclear (3 or more nuclei) TRACP+ cell number between MHR-A and MHR-B, even though MHR-B otherwise proved to be clearly favored by bone cells. However, TCPS did not contain significantly more multinuclear osteoclasts than MHR-B, which further might indicate the cellular competence of this nanocoating. In addition to osteoclastic cells, other cell types in the primary cell pool also verify the biocompatible characteristics of MHR-B. Paxillin staining showing numerous well-formed focal adhesions revealed tight attachment of the primary osteoblastic cells on MHR-B nanocoating.

In addition to primary bone cells, we studied the interactions of MHRs and well-differentiated murine preosteoblasts (MC3T3-E1). These immortal cells showed clear FAs on MHR-B similarly to the osteoblastic cells diverging from the primary cell pool. Clear paxillin spots at the tips of the spread actin stress fibers of the MC3T3-E1 cells were detected on MHR-B-coated TCPS and on uncoated TCPS control but not on MHR-A. We also tested MC3T3-E1 cell attachment on aminated but not on MHR-coated TCPS: Only a few FAs were detected (Figure 1). The proportional amounts of FA-containing cells did not significantly differ between MHR-B and TCPS, whereas the appearance of FAs did. This was confirmed by staining vinculin: The size of the FAs was significantly greater on TCPS than on MHR-B as indicated by both the area and the length of the FAs (Figure 2). Thus it can be concluded that MHR-B allows cells to attach well but for some yet unknown reason the FAs are not as mature as on TCPS.

Differences in cellular behavior are detected both as nonconformist densities and morphologies of the cells. Our results imply that the more cell-resistant MHR-A produces a weaker interaction between the coated material surface and the bone cells, whereas MHR-B supports cellular adhesion and spreading. On MHR-A, the morphology of all the cell types tested was rounded, and the cell density was clearly lower than that on MHR-B, on which the cells displayed a flattened and spread morphology (similarly as on uncoated control materials). It is generally thought that cell spreading can be considered as a characteristic of proliferating cells, whereas rounded cells indicate differentiation or apoptosis.[40,41] On the basis of these assumptions our results suggest that MHR-B supports cell spreading and proliferation, whereas MHR-A could promote cell apoptosis. Hoechst staining did not, however, reveal morphological signs of apoptosis. However, it is also possible that the rounded cell morphology on MHR-A at the time point 48 h indicates probably differentiating cells, which could not fully maturate because of the hindered attachment onto the surface.

Cell attachment and growth on biomaterial surfaces depend, among other factors, on the ability of the surface to adsorb

proteins from surrounding fluid (ECM proteins), which determines subsequent biological responses, such as cell attachment, differentiation, growth, and even apoptosis.[42,43] Protein adsorption is, in turn, influenced by many factors, such as carbohydrate moieties of plasma proteins[44] as well as by the wettability (hydrophobicity/hydrophilicity) of the surface coating. It is generally assumed that protein adsorption and cellular attachment prefer hydrophilic surfaces, but the degree of this interaction seems to vary between different cell types and proteins adsorbed. For example, a moderately wettable surface is preferred by endothelial cells[45] and fibroblasts,[46] whereas in some studies fibroblasts[47] and liver cells[48] grow better on extensively hydrophilic surfaces. Surprisingly, however, according to our results bone cells seem to prefer a MHR-B type coating providing a less wettable interface, which might be the outcome of many interacting factors.

In comparison with traditionally used polysaccharide coatings, such as hyaluronan, the modification of pectins concern side chains and methyl and acetyl moieties, thus influencing ionic interactions instead of weak (hydrogen bonds) interactions.[32] The contents of these chemical groups in a molecule might have notable biological effects. For example, according to Amaral and colleagues the degree of acetylation of a polysaccharide coating chitosan affects the cellular functionality and osteogenic activity of rat bone marrow stromal cells.[49] The amounts of esterified methyl and acetyl groups differ between MHRs. MHR-A contains more methyl and acetyl groups (Table 1), which chemically indicate greater hydrophobicity.[32,46] However, according to atomic force microscopy force separation studies made by Morra and colleagues, the wettability of MHR-A is actually greater than that of MHR−B.[32] The bonding mechanisms of the MHRs may affect the wettability properties. Usually carboxyl (−COOH) groups make surfaces moderately wettable favoring the attachment and growth of cells.[46] Instead, the effects of carboxyl groups on the wettability of MHR-coated surfaces might not be so straightforward because of the linking reactions of these groups to amino groups in carbodiimide condensation.[32] In addition, long and bulky arabinan-containing side chains of MHR-A produce a hydrated gel-like surface.[32] The side chain length correlates with protein adsorption so that longer side chains resist adsorption more effectively because of steric repulsion.[32,50] If the adsorption of proteins that promote cellular adhesion is this way hindered, then the longer side chains of MHR-A may partly explain the poor attachment of the bone cells.

Different adsorbed proteins may have variable effects on cellular functions. For example, fibronectin has proven to promote cell attachment, spreading, and proliferation,[47] whereas albumin may be considered as an adhesion-reducing protein.[51] According to some studies, albumin has the greatest affinity toward hydrophobic substrata.[17] In contrast, albumin has been reported as a main adsorbing protein onto hydrophilic, vitamin-E-coated surface, which MC3T3-E1 cells then repelled.[52] The different adsorption profiles of antiadhesive albumin and/or proteins favoring cellular attachment onto different MHRs probably explain our results to a large extent.

The effects of ECM proteins on the growth of bone cells are mediated mainly via integrin receptors. Integrins form a part of FAs, which are primarily responsible for cell attachment and spreading by physically linking integrins to the actin cytoskeleton.[53,54] Staining paxillin and vinculin proteins, crucial components of FAs, indicates that MHR-B supports FA formation in bone cells whereas MHR-A does not. This might be due to wettability differences of coatings; according to Lim and colleagues the expression of the genes coding integrin polypeptides depends on the wettability of the surface on which anchorage-dependent cells are growing. This effect was demonstrated with human fetal osteoblasts (hfOBs), which grew better on hydrophilic than on hydrophobic substrata.[55]

Integrins are associated with ECM protein binding in the formation of actin rings of active osteoclasts: Osteoclasts that perform actin organization thus act as a marker indicating tolerance toward the growth substratum.[3] A contact between osteoclasts and mineralized bone tissue in vivo ignites the resorptive action of osteoclasts.[37] In our study TRACP+ osteoclasts with an actin ring indicate merely active but not resorbing cells. This, nevertheless, provides valuable information of the biocompatibility of the underlying culturing material: According to Saltel and colleagues, active osteoclasts are able to grow and migrate also without resorptive phases in vitro.[3] Integrin-binding ECM proteins have to be properly folded. The possible effect of MHRs on polypeptide conformation at the biomaterial surface has to be considered, since the secondary structures of the adsorbed proteins may be altered due to the surface wettability.[56] Roach and colleagues have reported that albumin molecules display a more organized secondary structure on a hydrophilic surface than on a hydrophobic surface.[17] One explanatory factor of the cell-resistant nature of MHR-A might thus be related to protein folding chemistry.

Despite of the great significance of carbohydrate molecules in biological processes, the polysaccharide biology of bone tissue, "the sugar language of bone cells", still remains a largely obscure field in glycoscience. Information about the effects and interactions of plant polysaccharides and mammalian bone cells could have significant impacts on both basic research of sugar biology of bone as well as on bone implant applications. Pectins could serve as a relatively cheap and easily available device coating material. From an immunological point of view, pectins might, however, have some effects that should be considered to avoid excessive stimulation of the immune system. For example, Dourado and colleagues have shown that arabinan-rich almond pectins are able to stimulate murine lymphocytes.[57] Similar responses have been observed by Sakurai and colleagues: Rhamnogalacturonan regions of *Bupleurum* root pectin containing neutral sugar chains can stimulate lymphocytes.[58] In addition, pectins from different plant sources have been shown to activate the complement system.[59,60] Immunologically active structures of pectins should thus be studied concerning also bone implant coatings. Interestingly, in microencapsulation of Langerhans islets alginate poly-L-lysine rich in mannuronic acid has been shown to be more immunogenic causing strong antibody-producing responses than alginate rich in glucuronic acid.[61] MHR-A and MHR-B do not contain mannuronic acid (Table 1), but the potential immunological impacts of other monosaccharide components should be evaluated. According to Wang and colleagues, the immunological activity of pectins is at least to some extent dependent on acetyl and carboxyl groups,[62] which might indicate that the immunological effects of pectins could possibly be affected with enzymatic modifications.

The potential for diverse molecular modifications of pectins support their usefulness: By controlling the length of side chains of pectins their polarity can be affected.[32] In addition, the charge and wettability of hairy regions can be altered with the removal of acetyl groups as well as neutralizing methyl esters by tailoring the side chains.[32] We have verified the possibility to impact mammalian cells with tailored pectin molecules and

demonstrate that by modifying the sugar molecule side chains it is possible to trigger bone cell attachment on an artificial material.

The effectiveness of using pectic MHRs as bone implant nanocoatings should further be studied and verified from other aspects of bone biology as well. In this study we used primary bone cells as a source of osteoclastic cells. The next step with osteoblastic cells is to assess the effect of MHRs on the capability of mature osteoblasts to secrete osteoid matrix and to mineralize it. Resorption pits of bone can be stained with WGA−lectin, which binds to some yet largely unknown sugar epitopes unveiled in resorbed bone matrix. These epitopes are probably responsible for alluring osteoblasts to the resorption site. Identifying these sugar molecules could serve as a starting point and as a template for further tailoring of pectic nanocoatings to improve interactions and functionality of bone cells at the bone/implant interface.

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