Interaction of human mesenchymal stem cells with osteopontin coated hydroxyapatite surfaces

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**Abstract**

In vitro studies of the initial attachment, spreading and motility of human bone mesenchymal stem cells have been carried out on bovine osteopontin (OPN) coated hydroxyapatite (HA) and gold (Au) model surfaces. The adsorption of OPN extracted from bovine milk was monitored by the quartz crystal microbalance with dissipation (QCM-D) and the ellipsometry techniques, and the OPN coated surfaces were further investigated by antigen–antibody interaction. It is shown that the OPN surface mass density is significantly lower and that the number of antibodies binding to the resulting OPN layers is significantly higher on the HA as compared to the Au surfaces. The initial attachment, spreading and motility of human mesenchymal stem cells show a larger cell area, a faster arrangement of vinculin in the basal cell membrane and more motile cells on the OPN coated HA surfaces as compared to the OPN coated Au surfaces and to the uncoated Au and HA surfaces. These in vitro results indicate that there may be great potential for OPN coated biomaterials, for instance as functional protein coatings or drug delivery systems on orthopaedic implants or scaffolds for tissue-engineering.

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1. Introduction

When designing new artificial biomaterials for application in the fields of bone tissue-engineering and orthopaedic implantology, it is mandatory to ensure a high degree of osteoconductivity and/or osteoinductivity. Hydroxyapatite (HA) [Ca\textsubscript{10}(PO\textsubscript{4})\textsubscript{6}(OH)\textsubscript{2}], which is the most abundant inorganic mineral compound in skeletal tissue of vertebrates, is widely acknowledged in orthopaedic surgery, since the tissue response evoked by HA coated implants and HA containing grafts displays a low degree of inflammation, low level of intervening fibrous tissue and good ingrowth of bone [1,2]. Therefore HA coating of implants is generally considered “the golden standard” in orthopaedics.

The biological response of any biomaterial is related to how cells interact with the surface, which in turn is influenced by the adsorbed biomolecules at the surface [3]. A group of genetically linked bone proteins with similar structure and function, called small integrin binding ligand N-glycosilated proteins (SIBLINGs), are considered important in the generation and remodelling of skeletal tissue [4] and have in the past attracted considerable interest as biomolecular components in functional coatings that aim to improve the performance of orthopaedic materials for guided tissue engineering [5–8].

Osteopontin (OPN) is a SIBLING protein, which was recognized in bovine bone already in 1985 by Franzén and Heinegard [9] and later in human bone in 1988 [10]. The OPN protein consists of approximately 300 amino acids with small variations in the length of the amino acid polypeptide chain depending on the species in question. The native structure of OPN closely resembles a random coil with a flexible peptide backbone [11] and contains the following domains, all of which are considered highly conserved among different species [11,12]: (i) a HA-binding polyaspartic acid sequence, (ii) an integrin binding RGD sequence, (iii) a cryptic integrin binding sequence (human: SVVYGLR, bovine: SVAYGLK), (iv) a thrombin-cleavage site, (v) a CD44 binding domain, and (vi) several glycosylation and phosphorylation sites that are present in a random fashion throughout the entire protein play an important part in the functional state of OPN [12,13]. Bovine OPN consists of 266 amino acid residues and removal of the C-terminal fragment of thrombin-cleaved OPN has...
been reported to enhance the interaction between OPN and cells [14].

Numerous in vitro cell assays on different synthetic materials coated with OPN have been studied in the past with the purpose to explore whether OPN may influence the functionality of biomaterial surfaces [5,6,15–17]. For instance, it is found that OPN on hydrophilic polystyrene surfaces enhance the adhesion and spreading area of MG63 cells [15] and that the amino end-groups enhance the OPN mediated adhesion and spreading area of bovine endothelial cells as compared to carboxylic, hydroxyl, and methylic functionalized surfaces [8]. It is reported that OPN attached to a 2-D interfacial collagen layer enhances the cell adhesion of the mouse pre-osteoblastic MC3T3-E1 cell line [16], whereas on HA no such influence was seen [5]. It was shown that OPN covalently immobilized on poly(hydroxyethyl methacrylic acid) (poly(HEMA)) increase the adhesion of bovine endothelial cells as compared to pure poly(HEMA) [17]. Previous studies [5,6,15–17] thus indicate that OPN may act as a functional coating on orthopaedic implants, e.g., for joint prostheses to improve remodelling of bone tissue around biomedical implants.

Naturally occurring OPN is present at the surfaces of the non-organic phase of mineralized tissues [18], and several in vivo studies have examined the influence of OPN in the formation and remodelling of mineralized tissue. Changes in hardness and relative mass of mineralized tissue have been reported as well as the speed of bone remodelling and vascularisation in OPN-knockout mice [19–22]. OPN coated coralline porous HA granulae was used in a study of bone remodelling and vascularisation in OPN-knockout mice [19]. OPN coating was examined by investigating the initial motility, spreading area, morphology and vinculin distribution of human mesenchymal stem cell interacting with HA and Au preadsorbed with OPN or standard cell medium.

2. Materials and methods

2.1. Surfaces and characterization

Quartz crystal microbalance sensor surfaces coated with either a 10 nm thick HA layer (QSX-327) or with 100 nm thick Au thin film (QX3-301) were purchased from Q-Sense AB, Sweden. The sensor crystals (diameter 14 mm) were used as substrates in all experiments throughout the study. A Nanoscope IIIa Multimode SPM Atomic Force Microscope from Veeco instruments, Santa Barbara, CA, was used to characterize the surface topography at the nanometer resolution at ambient conditions. The applied silicon cantilevers (NSG01, NT-MDT, Russia) had a resonance frequency around 150 kHz, a spring constant of 5.5 N/m and a tip curvature radius below 10 nm. The root mean square (RMS) roughness was determined to be 2.2 ± 0.2 nm and 0.9 ± 0.2 nm for the HA and Au surfaces, respectively, as quantified by SPIP software (Image Metrology, Copenhagen, Denmark) in accordance with the model employed in a previous study [24]. The HA coatings were furthermore examined with grazing incidence X-ray diffraction, and several peaks which belong to the crystalline phase of HA were observed alongside with the prominent Au peaks from the supporting Au layer below the HA coating. This showed that the HA coating was partly crystalline (data not shown). Water contact angle measurements were carried out with the sessile drop method using a DSA100 (Krüss, Hamburg, Germany) after the respective samples were ozone cleaned for 25–30 min (Bioforce Nanosciences UVO cleaner). Both the HA and Au surfaces were found to be hydrophilic with water contact angles less than 10°. To test the stability of the HA coating in liquid a dissolution test of the HA coated crystals was conducted by the QCM-D technique by injecting a 10 mM Tris buffer with 1 mM CaCl$_2$ and 100 mM NaCl adjusted with HCl and NaOH to pH 7.4 at 37°C in the QCM-D chambers and examining the QCM-D response over a time span of 30 h. No QCM-D frequency shift was observed indicating that during this time span the HA substrates are stable/crystalline and suitable for protein adsorption and cell experiments. All surfaces were ozone cleaned for organic contaminants by means of the Bioforce Nanosciences UVO cleaner for 25–30 min immediately prior to use.

2.2. OPN and antibodies

Osteopontin (OPN) was purified [25] by Arla Foods amba, Denmark. The standard grade OPN used contains both full length OPN (35 kDa) and an N-terminal fragment cleaved immediately downstream of the cryptic integrin binding sequence (25 kDa). By weight the standard grade comprises 30% full length OPN and 70% cleaved OPN (unpublished results). No C-terminal fragments are present. Osteopontin extracted from bovine milk contains posttranslational modifications and is highly phosphorylated [26]. Polyclonal OPN antibodies from rabbits immunized with full length OPN purified from the standard grade OPN, were provided by Esben Skipper Sørensen, Department of Molecular Biology, Aarhus University, while goat albumin antibodies for unspecific binding were purchased at Sigma–Aldrich, Tastrup, Denmark. The proteins were dissolved in a 10 mM Tris buffer with 1 mM CaCl$_2$ and 100 mM NaCl adjusted with HCl and NaOH to pH 7.4 at 37°C. OPN was dissolved in the buffer at a concentration of 100 μg/ml and the antibodies at 300 μg/ml. OPN solutions were kept in a freezer and thawed at room temperature prior to use. The dissolved antibodies were kept at 4°C at maximum time three months.

2.3. The ellipsometry studies

Ellipsometry is a well established technique for quantification of surface mass densities of, e.g., thin organic films at a solid–liquid interphase with a typical thickness resolution of 0.5 nm [27]. The method relies on the change in the polarization of elliptically polarized light reflected at the interface, and the observed change is caused by the change in ratio of electric field amplitudes ($\Psi$) and the change in phase ($\Delta$) between the p- and the s-component. The recorded signal refers to the complex reflectance ratio given by:

$$\rho = \frac{R_p}{R_s} = \tan(\Psi) e^{i \Delta}$$  \hspace{1cm} (1)

where $R_p$ and $R_s$ are the reflection coefficients for the p- and s-components, respectively, as given by the Fresnel equations and $\rho$ is the complex reflectance ratio [28]. By using a layer-by-layer combi-
nation of the Fresnel equations it is possible to derive the thickness of films [28]. The surface mass density of non-hydrated protein films, \( \Gamma_{\text{Ellipsometry}} \), is subsequently calculated from the derived thickness by means of the de Feijter's formula [29]:

\[
\Gamma_{\text{Ellipsometry}} = d \left( \frac{n_{\text{protein}} - n_{\text{buffer}}}{dn/dc} \right) \frac{dn}{dc}
\]

where \( d \) is the thickness of the protein film, \( n_{\text{protein}} \) and \( n_{\text{buffer}} \) the refractive indices of protein layer and buffer, respectively, and \( dn/dc \) is the refractive index increment for a protein concentration change. The values used for the refractive indices are 1.465 and 1.335 for \( n_{\text{protein}} \) and \( n_{\text{buffer}} \), respectively [30], while for \( dn/dc \) a value of 0.18 was chosen, in accordance with what is commonly reported in the literature [31,32].

The surface mass densities were recorded with an ELX-02C (DRE GmbH, Ratzeburg, Germany) rotating analyzer ellipsometer setup with a single wavelength \( \lambda = 632.8 \text{ nm} \) He–Ne laser and a static liquid cell from DRE GmbH. A fixed angle-of-incidence of 70° was used to detect the dynamic change in surface mass densities of OPN on the HA and Au surfaces during adsorption process at room temperature.

2.4. QCM-D

Quartz crystal microbalance with dissipation (QCM-D) is an acoustic technique suitable for direct quantification of surface mass densities and viscoelastic properties of thin films [31,33–35]. The QCM-D technique relies on the use of a quartz crystal oscillating in shear mode. A change in mass on the crystal induce a measurable change in resonance frequency, and in case of a thin non-dissipative layer with no slip, the frequency shift, \( \Delta f_m \), and non-coated hydroxyapatite (HA−OPN), OPN coated hydroxyapatite (HA−OPN), and non-coated hydroxyapatite (HA−OPN). After immersion in Tris buffer \( \pm \text{OPN} \) all the surfaces were immersed in 5 ml MEM/FBS at 37°C for 15 min, after which the surfaces were used immediately for the cell studies. 6-well culture plates precoated with agarose gel (1% in MilliQ water, Sigma–Aldrich, Brøndby, DK) were filled with 5 ml MEM/FBS at 37°C and afterwards one surface was transferred directly to each culture well. 200 μl cell suspension (8000 cells) was seeded immediately beneath the air/liquid interphase by a pipette above the location of the surface. The culture plates were incubated in a humidified atmosphere of 37°C and 5% CO\textsubscript{2}. At times 1, 3, 6 and 24 h the surfaces were removed from culture plates, rinsed shortly in 37°C PBS and fixated with Lillies 10% neutral PBS buffered formalin (Gibco, Taastrup, Denmark).

An actin cytoskeleton and focal adhesion staining kit (Chemicon International, USA) was used to fluorescently stain the cells. Staining of actin was done with tetramethyl rhodamine iso-thiocyanate (TRITC) conjugated phalloidin to analyze cell morphology and cell nucleus with 4,6-diamidino-2-phenylindole (DAPI), while vinculin was stained with mouse monoclonal anti vinculin and fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG to analyze the distribution of vinculin in the basal membrane. Details can be found in ref. [39].

Throughout the staining procedure phosphate buffered saline (PBS) was used as buffer. The surfaces were washed twice, permeabilized with 0.1% Triton X-100 and washed twice. 1% bovine serum albumin (lyophilized powder >98%, Sigma–Aldrich, Brøndby, DK) was used for blocking in 30 min at room temperature followed by washing twice. 1.25 μg/ml mouse monoclonal anti vinculin was added for 60 min at 37°C followed by washing three times. 10 μg/ml FITC conjugated goat anti mouse IgG and 37.5 ng/ml TRITC conjugated phalloidin in the same solution were added for 60 min at room temperature. Surfaces were washed three times, 0.1 μg/ml

where \( N_{\text{OPN}} \) is the number of OPN molecules in the protein layer, \( N_{\text{antibody}} \) the number of antibodies bound to the protein layer, \( \Delta f_{\text{antibody}} \) and \( \Delta f_{\text{OPN}} \) the respective frequency changes, and \( m_{\text{OPN}} = 28 \text{ kDa} \) and \( m_{\text{antibody}} = 150 \text{ kDa} \) the respective molecular weights.

2.5. Cell culture

For the cell experiments, adult human mesenchymal stem cells (hMSC) transduced with the telomerase reverse transcriptase gene (hTERT) (often named hMSC–TERT) was employed [36]. These cells exhibited 4.3 population doublings per week and the cells were used at a population doubling level (PDL) 266 at which level recent studies using the same cells have reported osteogenic potential [37,38]. The cells were seeded at a density of 4000 cells/cm\textsuperscript{2} in 150 cm\textsuperscript{2} flasks in modified essential medium (MEM) that contained 10% fetal bovine serum (FBS) (all products from Gibco, Taastrup, Denmark) and cultivated in a humidified atmosphere of 37°C and 5% CO\textsubscript{2}. After 1 week, cells were washed in PBS (pH 7.4 at 37°C), trypsinized with 1.25% trypsin and 5 mM EDTA in PBS, seeded at 4000 cells/cm\textsuperscript{2} in 150 cm\textsuperscript{2} flasks and cultivated for another week. Cells were trypsinized and used (PDL 274) for the experiments at a concentration of 40,000 cells/ml. The substrate seeding density in the cell studies was fixed at approximately 5200 cells/cm\textsuperscript{2}. The effective cell density was most likely smaller since the cells were applied locally above the substrates, which were placed in a larger container (see Sections 2.6 and 2.7).

2.6. Fixation/fluorescence staining

OPN was preadsorbed on the HA and Au surfaces by immersing the samples in TRIS with 100 μg/ml OPN and in control experiments, pure TRIS for 150 min at room temperature. Four different surfaces were prepared: OPN coated gold (Au + OPN), non-coated gold (Au – OPN), OPN coated hydroxyapatite (HA + OPN), and non-coated hydroxyapatite (HA – OPN). After immersion in Tris buffer \( \pm \text{OPN} \) all the surfaces were immersed in 5 ml MEM/FBS at 37°C for 15 min, after which the surfaces were used immediately for the cell studies. 6-well culture plates precoated with agarose gel (1% in MilliQ water, Sigma–Aldrich, Brøndby, DK) were filled with 5 ml MEM/FBS at 37°C and afterwards one surface was transferred directly to each culture well. 200 μl cell suspension (8000 cells) was seeded immediately beneath the air/liquid interphase by a pipette above the location of the surface. The culture plates were incubated in a humidified atmosphere of 37°C and 5% CO\textsubscript{2}. At times 1, 3, 6 and 24 h the surfaces were removed from culture plates, rinsed shortly in 37°C PBS and fixated with Lillies 10% neutral PBS buffered formalin (Gibco, Taastrup, Denmark).

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DAPI added for 5 min at room temperature and finally rinsed three times. Stained cells were kept in PBS at 4 °C until the surfaces were investigated by optical microscopy.

Optical microscopy was performed with a Leica DM 6000B microscope, where 20–25 images at x400 magnification were acquired at random on each surface. Area and circularity of isolated cells were analyzed with the grain analysis tool provided with the ImageJ software [40]. Circularity is defined in the software as $4\pi (\text{area}/\text{perimeter})^2$, so a circularity of 1.0 indicates a perfect circle and as the value approaches 0 indicates an increasingly elongated shape. Distribution of vinculin assemblies near the basal cell membrane was estimated and categorized by eye. The experiment was performed once with single surfaces and once with triplets. Data from all surfaces was included.

2.7. DIC cell surveillance

Differential interference contrast (DIC) microscopy is a suitable technique for time-lapse observations of live and incubated cells. The technique was employed to analyze the cell motility during the first 8 h after cell seeding.

Surfaces were OPN precoated in the same way as described earlier in Section 2.6. Four surfaces: HA + OPN, HA − OPN, Au + OPN and Au − OPN were placed in one single Petri dish precoated with agarose gel (1% in double distilled sterile water). 200 μl cell suspensions (8000 cells) were slowly seeded by a pipette immediately beneath the air/liquid interphase above each surface. The petri dish was placed on an automated sample holder in an incubator at 37 °C and 5% CO2 mounted on a Leica DM 6000B microscope. The microscope was run in differential interference contrast (DIC) mode [41] at x200 magnification and programmed to obtain an image every 4th minute at four predefined sites on each of the four surfaces. The seeded surfaces were analyzed in the period 1–7 h after seeding and cells lying isolated on the surfaces were selected for motility analysis. 28 min intervals were used for calculation of motility. Cell position every 28th minute was determined as center of mass with the grain analysis tool provided with the basic ImageJ software [40] and the linear interval between positions was considered the distance migrated by the cell. Inaccuracies in the automated sample holder and surface movements were identified with a fixed surface spot and the position corrected accordingly before the migration distance was calculated. The motility experiment was performed once.

2.8. Data analysis

All data was analyzed parametrically and Students t-test was used to estimate significance of difference. A two-sided p-value less than 0.05 was considered significant. Unless otherwise stated data is presented as mean ± standard error of mean (SEM) along with sample size. Statistical analysis was performed with Stata v9.0 software, Statacorp, USA.

3. Results

3.1. Protein adsorption

In Fig. 1 the QCM-D results are shown for the n = 3, 5 and 7 overtones. After the injection of the OPN-containing buffer an immediate fast drop in frequency and increase in dissipation is observed. Only very small frequency shifts and dissipation changes were observed 1–2 h after the OPN injection and data was read after 2 h. The second frequency decrease observed after 300 min is due to the injection of polyclonal antibodies, which interact with the OPN layer.

![Fig. 1. Typical adsorption curves on Au and HA of OPN and subsequent anti-OPN binding obtained from QCM-D.](Image)

From Fig. 1 it is clearly seen that the OPN adsorption induced frequency shifts belonging to the different overtones nearly superimpose, when scaled by their respective overtone numbers: the relative difference in frequency shifts between third and seventh overtone on HA and Au was approximately 8% and 15%, respectively. Therefore the Sauerbrey equation is a valid approximation for estimating the OPN surface mass densities. In Table 1, the hydrated surface mass densities of OPN layers measured by QCM-D and calculated with Eq. (3), $f_{\Delta \text{DCM-D}}$, are listed, and it is shown that the hydrated OPN surface mass density for the HA surface is observed to be approximately 45% of that detected on Au. As seen in Table 1 the same trend is observed with ellipsometry: a significantly higher surface mass density of a non-hydrated OPN film adsorbed to the Au as compared to the HA surface. The water contents of the protein films formed on HA and Au obtained from the ratio of hydrated to the non-hydrated surface mass densities ($f_{\Delta \text{CM-D}}/f_{\Delta \text{Ellipsometry}}$) are depicted in Table 1. It is seen that the water factor is slightly higher on the HA (3.0 ± 0.5) coated substrates as compared to the Au (2.65 ± 0.10) surface.

3.2. Dissipation and antibody binding

The QCM-D technique also provides information on the dissipation in the resulting protein layer from the detected change in dissipation values per frequency shift ($\Delta D/\Delta f$) [31,34]. A small but significant difference in the $\Delta D/\Delta f$ value (10−8 Hz−1) was observed with $-0.078 \pm 0.003$ and $-0.0975 \pm 0.0010$ on the HA and Au surfaces, respectively (Table 1). This indicates that a stiffer protein layer is formed on the HA surface as compared to the Au surface. The binding of polyclonal OPN antibodies to the OPN saturated surfaces results in an additional frequency change (see Fig. 1) and the additional detected mass was analyzed as an estimate of "epitope availability" with Eq. (5). The results are listed in Table 1 and on average 935 and 471 ng/cm² of antibodies are attached to OPN layers on HA and Au, respectively. This is approximately four times as many antibodies bound per OPN molecule on the HA as com-
pared to the Au surface. Test for unspecific antibody binding to the surfaces showed an unspecific binding of less than 8% of the specific antibody binding values observed on both the HA and the Au surfaces (data not shown).

### 3.3. Cell area, morphology and vinculin distribution

Over time a steady increase in area is evident on all surfaces apart from HA + OPN where a small decrease is observed between 6 and 24 h (Fig. 2). At times 1, 3, and 6 h the cell area on HA + OPN is approximately 100% larger than the cell area on the remaining groups and at all times a p-value below 0.01 was observed between the area measured on the HA + OPN surfaces compared to the other reference surfaces (HA – OPN, Au + OPN and Au – OPN).

From the results depicted in Table 2 it is seen that the cells initially exhibit a circular morphology when attached to the surfaces, after which the circularity decrease as a function of time on all surfaces. This indicates that the cells elongate after attachment to the respective surfaces. At 1, 3 and 24 h cells on the respective surfaces follow each other closely. After 6 h HA + OPN clearly shows a lower circularity as compared to the remaining surfaces, which indicate a more rapid elongation of cells on this surface.

Vinculin assembly into detectable focal adhesion spots was observed on all surfaces at times from 3 h and forward, and the vinculin assemblies were either distributed throughout the cell or mainly localized in a corona shaped band around the nucleus (Fig. 3). The percentage of cells on the respective surfaces dominated by corona distribution was estimated by eye observation of each cell and listed in Table 2 with 20% interval designations.

### 3.4. Cell motility

Most cells moved in a single direction during the observation time and no change of direction in the remaining cells took place without at least an hour of standstill. Fig. 4 displays the average migration velocity of cells in 28 min intervals and Fig. 5 displays the cumulated movement of cells during the observation period. The migration velocity at every 28 min time interval, as seen in Fig. 4, was significantly higher on the cells attached to HA + OPN surfaces resulting in a significant difference in cumulated movement, Fig. 5. The velocity on HA + OPN was initially 6–8 μm/h and decreased to 4–5 μm/h towards the end of observation. No significant differences were observed among the other surfaces.

### 4. Discussion

#### 4.1. Protein adsorption characteristics

OPN adsorption on HA and Au was characterized with the ellipsometry and QCM-D techniques and as seen from the results in Table 1, a significantly lower OPN surface mass density is observed on the HA surfaces as compared to Au surfaces. This difference in OPN surface mass uptake can be ascribed to a larger average protein contact area on HA as compared to Au.

Additional information about the dissipative properties of the resulting OPN-layer was obtained from the recorded QCM-D ΔD/Δf results. From Table 1 it is seen that the ΔD/Δf value is slightly lower on the HA as compared to the Au surface, which indicates that the protein film formed on the HA surface is stiffer and more rigid with proteins that establish a large contact area with the interface [31,34].

The contact area of proteins typically increase as protein unfolding occur on a surface, which in turn might be reflected in a change of available epitopes on the protein due to both a different protein configuration/orientation on the surface and a changed degree of steric hindrance between the adsorbing antibodies and proteins attached to the surface. Several studies have shown that antibodies can be applied in combination with QCM-D studies as a binding probe to estimate the epitope availability [33,35,42]. By complimenting the QCM-D measurements with polyclonal antibody-antigen recognition, it was observed that the amount of antibodies binding to each OPN molecule on the Au surfaces was app. 25% as compared to the amount bound to the HA surfaces. Since the amount of unspecific binding was much smaller than the difference in the number of antibodies per protein found on Au and HA,
the issue of a different amount of unspecific binding to the protein films on Au and HA was not taken into consideration. The observation of more antibodies binding to OPN on HA is in agreement with the OPN molecules binding in a configuration/orientation with higher epitope availability on HA as compared to Au. The protein studies suggest that OPN binds to the HA surface in a more active state with more available cell active domains as compared to the Au surface.

4.2. Initial cell interactions

The cell area, cell morphology (circularity), vinculin distribution and cell motility of human mesenchymal stem cells was studied dynamically on the HA and Au surfaces with and without the OPN precoating. On the HA surfaces it was observed that the presence of an OPN adsorbed layer influenced both the cell area and changes in the cell morphology over time (drop in cell circularity), while the OPN coating on Au only had a minor impact on these cell surface interactions.

From the vinculin immunostaining images it was noticed that the vinculin distribution in the cells was affected by OPN which was in accordance with the cell area and morphology results. The distribution of vinculin observed on HA + OPN at 3, 6 and 24 h after the cell seeding depicted in Fig. 3 is in agreement with expected characteristics of cells undergoing active change in morphology and migration: a low density of vinculin spots near the protruding edge, fibrillar assemblies in the center and larger diffuse assemblies at the rear end of the cells [43–46]. Moreover, a rather interesting
Table 2
Circularity of cells and presence of corona band distribution of vinculin.

<table>
<thead>
<tr>
<th>Cell characteristics</th>
<th>1 h</th>
<th>3 h</th>
<th>6 h</th>
<th>24 h</th>
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<tbody>
<tr>
<td></td>
<td>Circularity</td>
<td>Circularity</td>
<td>Circularity</td>
<td>Circularity</td>
</tr>
<tr>
<td>Au − OPN</td>
<td>0.670 ± 0.010</td>
<td>0.511 ± 0.014</td>
<td>0.414 ± 0.009</td>
<td>0.336 ± 0.011</td>
</tr>
<tr>
<td>Corona distribution</td>
<td>N.A.</td>
<td>++</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Au + OPN</td>
<td>0.672 ± 0.012</td>
<td>0.583 ± 0.012</td>
<td>0.445 ± 0.010</td>
<td>0.362 ± 0.011</td>
</tr>
<tr>
<td>Corona distribution</td>
<td>N.A.</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Ha − OPN</td>
<td>0.710 ± 0.007</td>
<td>0.583 ± 0.013</td>
<td>0.512 ± 0.012</td>
<td>0.269 ± 0.009</td>
</tr>
<tr>
<td>Corona distribution</td>
<td>N.A.</td>
<td>++</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Ha + OPN</td>
<td>0.679 ± 0.010</td>
<td>0.513 ± 0.014</td>
<td>0.293 ± 0.012</td>
<td>0.301 ± 0.009</td>
</tr>
<tr>
<td>Corona distribution</td>
<td>N.A.</td>
<td>+</td>
<td>−</td>
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</tr>
</tbody>
</table>

Corona band distribution is listed with approximate 20% interval designations. (++++) = 80–100%, (+++) = 60–80%, (++) = 40–60%, (+) = 20–40%, (+) = 0–20% and (−) = none. Distinction between corona and non-corona was estimated by eye. Sample size ranges from 126 to 209.

Fig. 4. Average cell speeds in 28 min intervals. Cells on Ha + OPN moved at a significantly higher velocity at all intervals. Sample sizes ranging from 28 to 30 cells. Asterisk (*) denotes $p < 0.05$ for indicated samples.

Fig. 5. Cumulated cell migration in period 1–7 h following cell seeding. Sample sizes ranging from 28 to 30 cells. Asterisks (**) denote $p < 0.01$ for indicated samples.

corona distribution of vinculin assemblies dominates the remaining surfaces (HA − OPN, Au + OPN and Au − OPN) at 3 h. The corona distribution is compatible with cells at an early stage of cell/surface interaction. Corona distribution of surface contact associated proteins in newly attached cells has been reported previously, but was present only shortly after attachment [47,48]. Time-lapsed studies of the corona distribution presence in response to the substrate may have the potential to be a sensitive marker of early cell/substrate interactions. After 24 h a random distribution of mostly fibrillar like vinculin assemblies were observed on all surfaces, which is characteristic for more mature cell surface contacts [49].

Cell motility was analyzed as velocity in 28 min intervals of single cells to provide a time-lapsed motility overview that is unbiased by change of direction. As expected from the results of the vinculin immunostaining, the cell motility was found to be promoted significantly by the presence of OPN on HA. Several bone related cells type have been reported to be induced in migration by OPN: mesenchymal stem cells [50], osteoblasts [51] and osteoclasts [52].

The RGD-sequence is a well known integrin binding motif, which controls cell spreading [47,53–55], motility [56] and focal adhesion spot assembly on the surface [53]. Several studies have shown that the RGD mediated cellular responses on interfaces mainly depend on the amount of available RGD sequences on the surface [47,53–56]. For instance Maheshwari et al. found cell motility of murine fibroblast cells to be controllable with different densities and clustering of RGD-peptides—higher RGD density generally promoted motility [56]. The OPN induced motility may not solely be connected with the RGD-sequence, since the CD44 ligand, which is also displayed by the OPN molecule, has also been shown to induce motility [50,52,57].

The impact on cell area, shape, vinculin distribution and motility further supports the assumption that a specific configuration/orientation of OPN bound to HA exists and it favours the availability of the OPN cell binding domains to the surrounding environment. This is in line with the protein results discussed previously, which showed that the individual OPN molecules are in general more exposed on the HA surface as compared to the Au surface.

5. Conclusion

We have studied the adsorption of osteopontin (OPN) on hydroxyapatite and gold and in particular the influence of the resulting OPN protein layer on the adsorption of human mesenchymal stem cells. It is found that the individual OPN molecules adsorb to hydroxyapatite coated surfaces with a larger contact area leading to a more rigid state with more cell-guiding domains exposed.
as compared to the reference Au surface. Furthermore, the OPN-layer attached to the hydroxyapatite surface catalyses a larger and faster cell spreading as well as higher cell motility. From the present model studies it thus appear that osteopontin derived from bovine milk is indeed a new interesting bioactive molecule, for example for the functionalization of biomaterials, due to low cost and high availability. To further explore the full potential of OPN and the long-term influence on cell activity in connection with new and novel biomaterials the present in vitro studies must be extended by in vivo animal studies to assay the in vivo response of OPN coated surfaces.

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References