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The influence of surface mineral and osteopontin on the formation and function of murine bone marrow derived osteoclasts.

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Abstract

The phosphorylated glycoprotein osteopontin (OPN) is involved in the regulation of biomineralization under normal and pathological conditions. Its actions include inhibiting apatite crystal growth and promoting the formation and function of mineral resorbing cells, including osteoclasts (OCL). The purpose of this study was to develop stable apatitic mineral surfaces and determine their influence on OCL formation and mineral resorption from bone marrow macrophages derived from OPN wild-type (OPN +/+) and OPN deficient (OPN -/-) mice. We demonstrated that these mineral coatings were stable and supported bone marrow-derived macrophage differentiation to OCL under our culture conditions. Macrophages harvested from OPN -/- mice had a greater capacity to form OCL than macrophages from OPN+/+ mice when allowed to differentiate on tissue culture plastic. In contrast, when allowed to differentiate on a mineral surface, no difference in OCL formation was observed. Interestingly, OPN+/+ OCL were more efficient at mineral dissolution than OPN -/- OCL, and this difference was observed regardless of differentiating surface. Our results suggest that mineralized substrates as well as ability to synthesize OPN both control OCL function in our model system. The exact nature of these effects may be dependent on variables related to mineral substrate presentation.

Keywords

Osteopontin; Osteoclasts; Biomineralization

1. Introduction

Biomineralization is a highly controlled process [1-4]. It occurs in a wide variety of species and tissues, with prominent examples being bone and tooth enamel [5-6].. In almost all cases of biomineralization, macromolecules play a vital role in the mechanisms that regulate crystal formation, growth, and dissolution [7-12]. An increasing number of studies indicate that loss of these regulatory macromolecules is a major mechanism underlying pathological mineralization [13].

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One such macromolecule is osteopontin (OPN), a phosphorylated glycoprotein involved in the regulation of bone development and pathological calcification, and secreted by many cell types including osteoblasts, osteoclasts (OCL), and macrophages [9]. Important structural elements of OPN include an arginine-glycine-aspartic acid (RGD) cell binding domain, putative calcium binding motifs, and numerous serine and threonine phosphorylation sites, which enhance calcium mineral binding once phosphorylated. OPN can physically inhibit mineral growth by binding to calcium phosphate minerals [14]. OPN can also play an active role in cell-mediated mineral dissolution by recruiting macrophages, promoting OCL formation, mediating OCL binding to mineral, and inducing carbonic anhydrase II (CAII) expression and subsequent local acidification of mineralized tissues [14, 15-18].

Previous *in vitro* studies have shown that OPN enhances OCL binding to mineral surfaces, facilitating osteoclast motility and bone resorption [19]. In addition, OPN appears to regulate OCL differentiation and function, since OPN-deficient (OPN^{-/-}) macrophages had a greater capacity to form OCL than wildtype macrophages (OPN^{+/+}) [20], and OPN^{-/-} derived OCL formed smaller and shallower resorption pits than OPN^{+/+} derived OCL, a phenotype that could be rescued partially by exogenous OPN [18]. Subsequent studies showed that the effect of OPN on OCL formation *in vitro* was time-dependent: down regulation of OPN gene expression early or prior to stimulation with macrophage-colony stimulating factor (M-CSF) and soluble receptor activator of nuclear factor κ B ligand (sRank-L) reduced OCL formation. In contrast, down-regulation of OPN gene expression immediately after macrophage stimulation with M-CSF and Rank-L showed no significant impact on formation [21]. However, in these studies, the down-regulation of OPN had no impact on OCL capacity for mineral dissolution.

More recently others have implicated physical properties such as surface mineral composition as an important affecter of cellular response to OPN [22]. Specifically, changes in mineral surface composition were shown to affect adhesion strength of OPN on a mineral substrate and consequently resulted in an increased OCL resorption response. Thus, it appears that physical conditions such as mineral surface characteristics in concert with OPN expression may contribute to the overall regulation of mineral resorptive cell formation and function. These findings are of particular importance with regard to understanding the mechanisms guiding mineral resorption of ectopic calcifications associated with various pathological conditions.

Furthermore, studies of implant biomaterials have also suggested that attributes such as wear particle composition in prosthetic materials can modulate cellular activity by altering bone-related gene profiles *in vivo* [23]. Recently, poly-methylmethacrylate (PMMA) particle-associated macrophages were shown to cause significantly more osteoclast formation and bone resorption than HA-associated macrophages, suggesting that particles derived from uncemented (HA-coated) implants are likely to induce less osteoclast formation and osteolysis than cemented implants [24]. These findings support the idea that a material composition induced effect on osteoclastic activity at the interface of prostheses may be a significant factor in the failure of orthopaedic implants.

In the current study, we developed a stable calcium phosphate coating on polystyrene to determine the impact of early exposure to a mineralized surface on the formation and function of OCL from macrophages, and the potential involvement of OPN. Our findings support the hypothesis that the presence of a mineral substrate affects the nature of OPN's influence on macrophage differentiation and function as mineral resorbing cells.

2. Materials and Methods

2.1. Plate mineralization using aqueous mineral growth solutions

Polystyrene (PS) plates were incubated with mineralizing solutions of varied ion concentration for 6, 12, 16, and 24 hours at 37°C. Optimal mineral coating was achieved when plates were incubated in an aqueous mineralizing solution (1.25mM CaCl₂·2H₂O, 2.4mM NaHCO₃, 73mM NaCl, and 0.05mM K₂HPO₄; adjusted pH = 7.4) at 37°C for 16 hours, followed by the addition of fresh mineralizing solution and an incubation period of 24 hours. Mineralized polystyrene (MPS) plates were allowed to air-dry after mineralization.

2.2. Surface elemental composition using X-ray photoelectron spectroscopy (XPS)

X-ray photoelectron spectroscopy was used to evaluate the surface composition of mineralized plates in comparison to a commercial mineral substrate (Osteologic™ discs, BD Biosciences). XPS was performed at NESAC/BIO, University of Washington. All XPS spectra were taken on a Surface Science Instruments S-probe spectrometer. The instrument has a monochromatized Al K_α x-ray source and a low energy electron flood gun for charge neutralization. X-ray spot size for these acquisitions was on the order of 800mm. Pressure in the analytical chamber during spectral acquisition was less than 5e10⁻⁹ Torr. Pass energy for survey spectra (composition) was 150 eV. The take-off angle was 55° (55 degree take-off angle at 50 Å sampling depth). The Service Physics ESCAVB Graphics Viewer program was used to determine peak areas, to calculate elemental compositions from peak areas and to peak fit high resolution spectra.

2.3. Surface topography using Scanning electron microscopy (SEM)

Scanning electron microscopy was used to determine the surface topography of substrate materials. SEM images were taken using an FEI (Hillsboro, OR) Sirion field-emission microscope at the Center for Nanotechnology, University of Washington. Samples were Au/Pd sputter coated for 30 seconds (3 angstroms/sec) (SPI Supplies, West Chester, PA) and scanned under high vacuum conditions at an accelerating voltage of 5kV and a fixed working distance of 5mm.

2.4. Quantitation of mineral surface stability using a modified colorimetric method

The stability of MPS was evaluated by incubating prepared plates in PBS at 37°C for a period of 3, 6, and 9 days. At each time point, the PBS was removed and stored at 4°C. Subsequently, each surface was washed with fresh PBS to collect residual dissolved mineral that may have remained on the surface. The plates were then air-dried and incubated in 0.6 N HCl for 24 hours at 37°C to completely dissolve remaining surface mineral. A modified colorimetric method (Teco Diagnostics, Anaheim, CA) was used to evaluate the calcium content of the recovered PBS, residual PBS wash, and 0.6 N HCl solutions. All calcium

content measures were normalized using appropriate volume and diluent controls. The absolute calcium dissolution was calculated using the following equation:

$$Mass_{Ca^{++}} = \text{Concentration (mg/mL)} \times \text{Volume (mL)}$$

2.5. Murine bone marrow-derived macrophage isolation

OPN mutant mice were generated in a 129/SvJ X Black Swiss background as previously described [25]. Hybrid mutant mice were backcrossed onto the C57Bl/6 background for greater than 10 generations. OPN homozygous wild-type (OPN+/+) and homozygous null (OPN-/-) mice on a C57Bl/6 background were used in these studies. Femora from 4-6 week old OPN+/+ and OPN-/- mice were aseptically harvested and adherent soft tissue dissected away. All macrophage preparations were carried out under sterile tissue culture conditions and following appropriate University of Washington animal use protocols. Briefly, the ends of femora were cut and the marrow cavities flushed with RPMI 1640 (Gibco BRL, Carlsbad, CA) culture medium. Dispersed marrow cells were expanded in supplemented media (50% RPMI 1640 (RPMI), 30% fibroblastic cell (L929) conditioned medium (L929-CM), and 20% fetal bovine serum (FBS)) for three days. The cultures were subsequently maintained in a minimal media formulation (70% RPMI, 20% L929-CM, and 10% FBS) that was changed every three days until confluence. Mature macrophages were collected by repeated washing with cold PBS after 10 days for osteoclast preparations [26].

2.6. Osteoclast differentiation conditions

Bone marrow-derived macrophages were plated on either PS or MPS plates at a concentration of 5×10^5 cells/well and incubated at 37°C for 6 days. All cultures were maintained in D-MEM (with 10%FBS) supplemented with macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor κ B ligand (sRankL) at 25 ng/mL and 40 ng/mL respectively, to induce osteoclast differentiation. The media was changed every three days and assays were terminated at day 10.

2.7. Osteoclast characterization

Samples were fixed and stained to detect tartrate-resistant acid phosphatase (TRAP) for OCL characterization. Briefly, cultured cells were fixed with 10% formalin for 5 minutes followed by ethanol:acetone (50:50 v/v) for 1 minute. After fixation samples were stained for TRAP by incubating in acetate buffer (pH 4.8) containing naphthol AS-MX phosphate (Sigma Chemical Co., St. Louis, MO), fast red violet LB salt (Sigma), and 50mM sodium tartrate at room temperature for 30 minutes. The samples were then counter-stained with Methyl Green for 2 minutes, rinsed with tap water and allowed to dry.

2.8. Osteoclast functional assay

After 6 days of differentiation as described above, OCL preparations from PS and MPS plates were trypsinized and replated on Osteologic™ discs (BD Biosciences, Chicago, IL) at of 1×10^5 cells/well and supplemented with M-CSF and RankL at 25 ng/mL and 40 ng/mL respectively. The supplemented media was changed every 3 days for 10 days. At termination of the assay, culture media was removed and wells were rinsed with deionized water. A

bleach solution (~6% NaOCl, ~5.2% NaCl) was added to each well and flushed to dislodge all cells. Bleach solution was aspirated after five minutes at room temperature. Each well was subsequently washed with distilled water and allowed to air dry. Resorption pits were visualized under light microscopy and quantified by image analysis (ImageJ, version 1.32; National Institutes of Health, Bethesda, Md.) as a percentage of total area [27].

2.9. Statistical Analysis

Data is presented as means $\pm$ SEM of triplicate measures. Using SPSS 14 for MS Windows (SPSS, Inc.), analysis of variance tests were used to evaluate differences between sample groups. Differences with p-values of $p < 0.05$ were considered significant.

3. Results

3.1. Plate mineralization, composition, and stability

In order to carry out culture experiments, it was important to create a mineral surface on our culture plates of appropriate composition and stability in solution. Using these metrics, we evaluated several mineralizing solutions. A mineralizing solution of relatively high ion concentration (e.g. 2.5 mM CaCl $\cdot$ 2H $_2$ O, 4.8 mM NaHCO $_3$, 146 mM NaCl, and 1.0 mM K $_2$ HPO $_4$) resulted in spontaneously precipitated mineral in solution with little or no mineral surface deposition. At low ion concentration (1.25 mM CaCl $\cdot$ 2H $_2$ O, 2.4 M NaHCO $_3$, 73 mM NaCl, and 0.5 mM K $_2$ HPO $_4$), no deposition was observed until 16 hours of incubation. A visible thin film of mineral developed after incubation times of 16 and 24 hours (data not shown). SEM showed the mineral coating on the MPS surface to be similar in appearance to an OsteologicTM substrate, a known commercial mineral substrate (Fig. 1).

Surface composition was evaluated using XPS. The ratio of phosphate to oxygen and calcium to phosphate was determined and is presented in Table 1. XPS survey scans of the MPS plate and the commercial OsteologicTM disc are shown in Figures 2A and 2B. Sodium and chloride peaks were observed because NaCl was used in the mineralization process of the PS plates (arrows, Fig. 2A). The silicon peak present in the scan of the OsteologicTM disc is expected because the mineral is coated on a glass coverslip (arrows, Fig. 2B). The MPS surface also had a very strong carbon signal (from the underlying PS substrate). It is very likely that this is a result of the mineral film being very thin or patchy [28].

To determine the stability of the mineralized surfaces under culture conditions, plates were incubated for various times in PBS at 37°C. No significant differences in the percent mineral dissolved (normalized to total mineral deposition) between 3, 6, and 9 days were observed. The highest percentage of mineral dissolved occurred at 3 days with an average of 4.1 percent of total mineral deposited (data not shown).

3.2. Osteoclast differentiation and resorption

Bone marrow-derived macrophages from OPN $^{+/+}$ and OPN $^{-/-}$ mice were induced to differentiate into osteoclasts on either PS or the MPS described above. To determine the effect of surface composition on OCL differentiation, TRAP $^{+}$ /multinucleated cell formation was determined for OPN $^{+/+}$ and OPN $^{-/-}$ -derived macrophages (n=3 for each treatment),

and expressed as a percentage of total cells (Fig. 3). The data show that OPN^{-/-} bone marrow-derived macrophages had a greater capacity to form OCL than OPN^{+/+} macrophages when allowed to differentiate on a PS surface. However, this difference was attenuated when differentiation occurred on a MPS surface (Fig. 3).

To determine whether resorptive behavior was altered as a result of the differentiating surface, OCL were allowed to differentiate on PS and MPS, and then replated onto OsteologicTM discs. Mineral resorption was determined for both OPN^{+/+} and OPN^{-/-} derived OCL (n=3 for each treatment). For each treatment, the percent of total area of osteologic disc resorbed (Fig. 4A) and percent area osteologic disc resorbed per osteoclast (Fig. 4B) were determined. The data suggest that while there was no statistically significant difference in the percent area resorbed by OPN^{+/+} and OPN^{-/-} OCL on OsteologicTM discs regardless of differentiating surface, a trend for decreased resorbed area in OCL differentiated on MPS was noted (Fig. 4A). However, when normalized to cell number, OPN^{+/+} OCL were more efficient at mineral dissolution than OPN^{-/-} OCL (Fig. 4B). No statistically significant differences were observed in resorption/cell between OCL that were allowed to differentiate on a PS or MPS surface, regardless of genotype.

4. Discussion

Osteoclasts have the unique capacity to resorb bone and are derived from monocyte/macrophage precursors found in bone marrow [29]. OPN has high binding affinity for calcium-containing mineral and an RGD motif that can interact with the $\alpha_v\beta_3$ integrin on OCL. Indeed, OPN has been shown to play an essential role in bone resorption, in part by facilitating the attachment of OCL to mineralized tissues [30-32]. In addition, OPN has been implicated in the regulation of mineral resorption of ectopically calcified tissues by regulating the differentiation of circulating macrophages to OCL-like cells that upregulate carbonic anhydrase expression and acid production [14, 17]. Recently, surface mineral composition has been reported to regulate cellular response to OPN [22], suggesting that OCL formation and function may be substrate dependent.

In the current study, we developed a stable calcium phosphate coating on PS to determine the impact of early exposure to a mineralized surface on OCL differentiation from OPN^{+/+} and OPN^{-/-} macrophages. Mineral coatings were stable and had a surface topography similar in appearance to OsteologicTM mineral coatings as determined by SEM. The composition of MPS closely resembled that of hydroxyapatite. XPS results showed a calcium-phosphate ratio of 1.7 and a phosphate-oxygen ratio of 0.2 – matching the theoretical ratio seen in pure hydroxyapatite (Table 1). Interestingly, the mineral surface we prepared had a closer resemblance to pure hydroxyapatite than the commercial material (calcium-phosphate ratio (C/P) of 1.5). While a mineral with calcium-phosphate ratio of 1.5 could be more favorable in studying resorption (due to a higher solubility product and similarity in Ca/P ratio to more immature bone minerals such as octa-calcium phosphate and β -tricalcium phosphate), a more stoichiometric mineral may be preferable for culturing purposes since it may be more stable in solution.

We exposed primary cultures of bone marrow-derived macrophages to PS or MPS plates and stimulated OCL formation to determine the effect of a mineralized substrate on OCL formation and functional capacity. Our data show that bone marrow-derived macrophages from OPN^{-/-} mice had a greater capacity to form OCL on PS than macrophages from OPN^{+/+} mice, consistent with previous studies [20], and suggesting that OPN was an inhibitor of OCL differentiation. However, in the current study, the enhanced OCL differentiation in OPN^{-/-} cells was lost when macrophages were allowed to differentiate on a mineralized surface. These data suggest that OPN does not inhibit macrophage differentiation to OCL under conditions that more closely resemble conditions found at sites of bone and ectopic mineralization.

On the other hand, OCL from OPN^{+/+} macrophages resorbed mineral more efficiently than OPN^{-/-} OCL, independent of differentiating surface. These results are in line with previous studies that have shown that OPN is required for efficient osteoclast motility and resorption [32-34]. In addition, OPN was required for CAII expression and acidification of calcified implants by circulating macrophages [14,17]. Thus, effects on increased motility and gene expression, rather than on OCL differentiation, may underlie the ability of OPN to promote mineral resorption by macrophage-derived cells.

Ultimately, our findings may be important in understanding the mechanisms of action for OPN in mineral resorption relevant to both bone and ectopic calcifications. OPN, in these cases, is a known regulator of mineralization and bone turnover. As stated previously, there is significant evidence implicating OPN in the dissolution of calcified tissues *in vivo* [14-18]. Recent studies have shown that mineralization under pathologic conditions is regulated in much the same way as physiologic mineralization, including the formation of distinct mineral species and site specific architectures [13, 35]. Coupled with evidence suggesting that physical properties such as mineral composition are important effectors of cellular response to OPN [22] and that cells treated with only synthetic apatite or calcium phosphate materials are induced to express significant amounts of OPN [36], it is possible that local differences in mineral composition and structure of ectopic calcification deposits may play an important role in dictating cell-mediated responses. Thus it is also possible that our findings may have further reaching implications towards understanding the mechanism through which material composition influences periprosthetic bone loss [37].

5. Conclusion

In this study, we have demonstrated that bone marrow-derived macrophages have a reduced capacity to differentiate into OCL on a mineralized substrate compared to a PS surface independent of OPN genotype. Furthermore OCL from OPN^{+/+} macrophages resorb mineral more efficiently than OPN^{-/-} osteoclasts regardless of the differentiating surface. These results suggest that mineralized substrates as well as ability to synthesize OPN both control OCL function in our model system. The exact nature of these effects may be dependent on variables related to mineral substrate presentation, and will be the topic of future studies.

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Fig. 1.

SEM image of (A) MPS and (B) commercial Osteologic™ disc material.

Fig. 2.

XPS survey scan of (A) MPS and (B) the commercial Osteologic™ disc. Note the presence of a sodium and chloride peak impurities in scan of MPS.

Fig. 3.

Assessment of osteoclast formation by quantitation of TRAP⁺, multinucleated (MNC; 3+ nuclei) cells from PS or MPS differentiated OPN ^{+/+} and OPN ^{-/-}-macrophages. Percent TRAP⁺ cells/treatment $\pm$ standard error (n=3). Significant differences are indicated with brackets.

A

B

Fig. 4.

Assessment of osteoclast function (resorption). (A) Percent of total surface area resorbed PS and MPS differentiated OPN^{+/+} and OPN^{-/-} OCL +/- standard error (n=3). Significant differences are indicated with brackets, (B) Average dissolution capacity for PS- and MPS differentiated OPN^{+/+} and OPN^{-/-} OCL +/- standard error (n=3). Significant differences are indicated with brackets.

Table 1

	Component Elements							Element Ratio	
	C	O	P	Si	Ca	Cl	Na	P/O [*]	Ca/P [*]
Commercial HA (Osteologic™ Disc)	22.9	46.6	9.2	7.6	13.7	ND	ND	0.2	1.5
MPS	57.7	26.8	4.9	ND	8.4	0.9	1.3	0.2	1.7
Theoretical ratio for HA								0.2	1.7

Notes:

(A) C=carbon, O=oxygen, P=phosphorus, Si=silicon, Ca=calcium, Cl=chloride, Na=sodium

(B) ND = not detected

(C) HA = hydroxyapatite, $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$

(D) MPS = mineralized polystyrene

* ratios of indicated elements