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Recombinant osteopontin fragment coating on hydroxyapatite for enhanced osteoblast-like cell responses

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Surface engineering approaches using biomimetic peptides provide promising strategies to improve the bioactivity of materials. In this study, to improve the bioactivity of hydroxyapatite (HA), a recombinant osteopontin fragment (rhOPN) was devised so as to retain its specific binding domains to HA, as well as to cells, in order that it function as a bridge between the two. A protein adsorption assay was performed to determine the optimal coating conditions, and the bioactivity of rhOPN coated HA was evaluated by conducting various *in vitro* cellular assays, such as the evaluation of cellular attachment, proliferation and differentiation. The rhOPN was adsorbed to the HA by an electrostatic attraction which was facilitated by the former's HA binding domain. The osteoblast-like cell attachment was significantly simulated through the RGD-integrin interaction. Moreover, HOS cells attached to the adsorbed rhOPN ligands on the HA surfaces showed significantly enhanced proliferation and differentiation behaviors ($P < 0.05$). These enhanced cellular responses can be attributed to changes in gene expression via the signal transduction cascade initiated by the RGD-integrin interaction.

1. Introduction

Hydroxyapatite [HA, $Ca_{10}(PO_4)_6(OH)_2$] is the main inorganic component of human hard tissues, thus its possible utilization as a biomaterial has attracted a great deal of interest. Actually, HA has been widely used in dentistry and orthopedic applications in the form of powders, granules and composites with resins [1]. Most *in vivo* studies have proven that HA bonds directly with host tissues and achieves complete osteointegration [2, 3]. However, chemically synthesized HA has apparent limitations in terms of cellular responses when compared to the biological apatite.

To improve the cellular responses and biocompatibility of HA, considerable effort has been made to modify its physico-chemical properties. The cell responses on HA, including the initial attachment, proliferation and differentiation, are known to be significantly affected by its surface characteristics, such as the chemical composition (Ca/P ratio and impurity) and physical properties (grain size, roughness and crystallinity) [4–8]. Since anchorage dependent cells require the mediation of adhesion molecules in response to substrates, this variation in the cellular responses depending on the surface characteristics is mostly associated with the mechanism of protein adsorption and further alteration. As such, in order to enhance the cellular responses, significant attempts have been made to modify the surface of materials using proteins [9–12].

The proteins in the extracellular matrix (ECM) of hard tissues are known to play a significant role in cell attachment, and to further regulate the cell behaviors via an internal signaling mechanism. The ECM proteins are known to regulate the mineralization process of calcium phosphate crystals. The vitronectin-modified surface was reported to promote the osteoblast phenotype and the formation of mineralized tissues [9]. Recombinant fragments of proteins, such as vitronectin, fibronectin and osteopontin, have been observed to improve biological activity [10–12].

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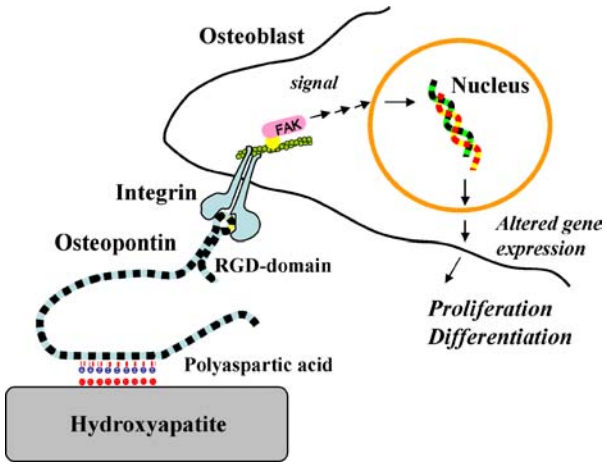

Figure 1 Schematic diagram of the proposed function of the rhOPN coating, namely to act as a bridge between hydroxyapatite and osteoblasts, in order to enhance the cellular responses.

In this study, the authors modified the surface of HA by coating it with osteopontin (OPN), which has an HA specific binding domain (immobilizing bioactive sequence to HA). A recombinant fragment of OPN (rhOPN), which includes both the HA and cell binding domains, was designed and produced. This rhOPN coating is expected to play a significant role in the improvement of osteoblast cell responses as a bridge between HA and cells, because of its biological importance in the signaling pathways of osteoblast cells through the binding of its RGD domains to the integrins of the cells. The basis and purpose of the present work, which involves the use of an rhOPN specific coating on HA to improve the cellular responses, is schematically illustrated in Fig. 1. The specific adsorption of rhOPN to HA was measured, in order to determine the optimal coating condition, and the effect of the rhOPN coating on the various cellular responses, such as cell adhesion, proliferation and differentiation, was assessed using osteoblast-like HOS cells.

2. Materials and methods

2.1. Production and characterization of rhOPN

OPN cDNAs were amplified from an adult human cDNA library. PCR primers were designed to recognize the NH_2-terminal fragment (18–154 a. a.) encompassing a cell adhesion recognition motif RGD amino acid: forward "rOPNF" primers, 5′-GAAGATCTGCCCAGTTAAACAGGCTGATTC-3′; reverse "rOPNR" primers, 5′-GGGGTACCTACCTC-AGTCCATAAACCAC-3′. PCR was performed in a 50 μl reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 100 μg ml^{-1} gelatin, 0.2 mM dNTPs, 1.25 units of Taq polymerase (Perkin-Elmer Corp.), and 50 pmol each of the upstream and downstream primers. The thermocycling parameters used in PCR were as follows: annealing at 55°C for 1 min; extension at 72°C for 2 min; denaturation at 94°C for 1 min. After 30 cycles, the amplified cDNA products were digested with NdeI and BamHI and separated using a PCR Purification kit (Qiagen, Chatsworth, CA). The nucleotide sequence inserts were determined by using dideoxy terminator cycle sequencing (Applied Biosystems), and compared with those in the GenBank database by means of the BLAST program of the NCBI (National Center for Biotechnology Information, Bethesda, MD). The rhOPN fusion proteins containing the poly-His tag were expressed and purified using an Ni^{2+} affinity column under denaturing conditions according to the manufacturer's protocol (Invitrogen, Carsbad, CA).

2.2. Adsorption assay of rhOPN to HA

The HA powder was molded in a metal die and then cold isostatically pressed (CIP) at 300 MPa. The discs so obtained were pressurelessly sintered at 1250°C for 2 h in air. The heating and cooling rates were 5°C /min and furnace cooling, respectively. The specimens were sterilized in 100% ethanol and dried for subsequent tests.

The HA discs were coated with the rhOPN at various concentrations in 1 ml of fetal bovine serum (FBS)-free Rosewell Park Memorial Institute (RPMI) Media at 4°C for 24 h. After rinsing the unbound rhOPN, the coated surfaces were incubated at room temperature for 90 min with 1:1000 diluted HRP-conjugated His antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) corresponding to the polyhistidine domain of expression vectors containing the polyhistidine sequence. After rinsing the unbound His-antibodies, 1-step^{TM} TMB-blotting(Pierce, Rockford, IL) was added to measure the amount of bound His-antibodies, by developing the HRP for 30 min in the dark. After adding stop solution (2M-H_2SO_4), the absorbance was measured at 450 nm using a microplate reader (PowerWave340, BioTek).

2.3. *In vitro* cellular assay

Based on the rhOPN adsorption data, the HA coated with rhOPN at a concentration of 1 μM was used for further cellular assessments.

Human osteosarcoma (HOS) cells were cultured at a density of 1×10^4 cells/ml for the cell adhesion assay, 7×10^5 cells/ml for the proliferation assay, and 5×10^4 cells/ml for the differentiation assay. The cell morphology was observed with SEM after fixing with glutaraldehyde (2.5%), dehydrating with graded ethanols (70, 90 and 100%), critical point drying and gold coating.

For the cell adhesion test, the cells were cultured for 35 min. After rinsing the unbound cells and washing with PBS, the adhered cells were fixed with 3.7% formaldehyde, and stained with 0.25% (w/v) Crystal Violet in 2% (w/v) ethanol/water for 24 h. After vigorous washing with distilled water, 2% SDS was added and the cells were vortexed for 5 min and the absorbance was measured at 570 nm using a microplate reader.

To assess the cell proliferation, the cells were cultured for 1 and 3 days, and then washed with PBS. The viable cell number was measured by means of the MTS assay. An MTS solution kit (Celltiter 96 AQ Non-radioactive Cell proliferation Assay, Promega,

Madison, WI), was added into the specimen-containing well, and the cells were incubated at 37°C for 2 h. The soluble formazan product was measured at an absorbance of 490 nm using a microplate reader.

For the assessment of the alkaline phosphatase (ALP) activity, the cells were cultured for 7 days. After harvesting the cells, the cell pellets were resuspended by vortexing them in 150 μl of 0.1% Triton X-100 and further disrupted by means of successive freezing/thawing cycles. The cell lysates were quantified using a protein assay kit (BioRad, Hercules, CA) and assayed colorimetrically for their ALP activity using *p*-nitrophenyl phosphate as a substrate (ALP yellow liquid substrate for ELISA, Sigma, St. Louis, MO) and measured at 405 nm using a microplate reader.

Statistical analysis was performed using the Student's *t*-test and significance was considered at $p < 0.05$.

3. Results and discussion

The optimal conditions for the coating of rhOPN on HA were determined by varying the OPN concentration. The amount of rhOPN adsorbed to the HA surface with respect to the OPN concentration is represented in Fig. 2A. The degree of rhOPN adsorption to HA increased proportionally to the rhOPN concentration up to 0.1 μM, and then became saturated at higher concentrations, with only a slight increase being observed.

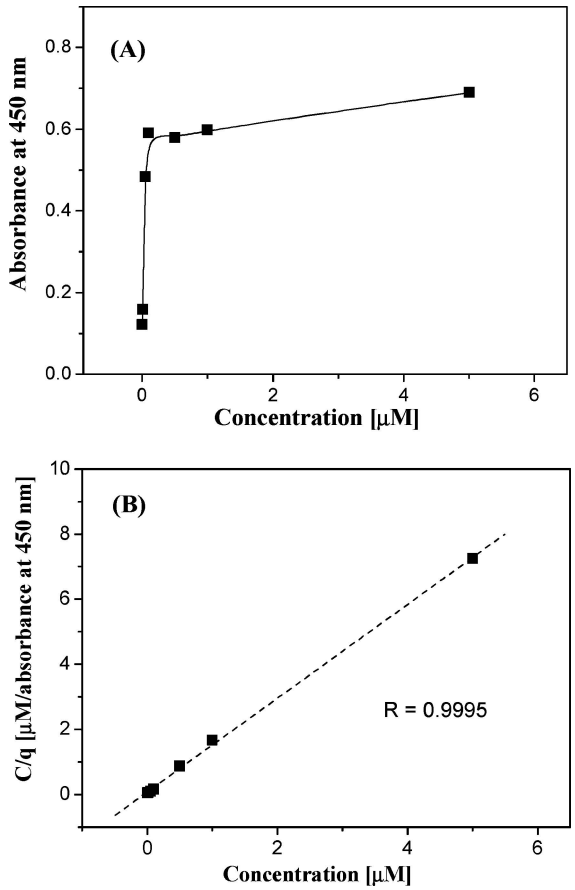

Figure 2 Protein fragment (rhOPN) adsorption result: (A) Adsorption of rhOPN as a function of rhOPN concentration in solution and (B) the adsorption data plotted as C/Q versus C and its linear regression result ($R = 0.9995$, C: equilibrium concentration of the adsorbate, Q: the amount adsorbed).

The equilibrium isotherms for the adsorption of proteins are often described by means of the Langmuir equation, as follows [13–15]:

$$q = \frac{Kq_0C}{1 + KC}$$

where C is the equilibrium concentration of the adsorbate, q is the amount of adsorbate adsorbed, K is the equilibrium constant and q_0 is the saturation capacity. This equation was transformed into the following equation:

$$C = -\frac{1}{K} + q_0\left(\frac{C}{q}\right)$$

The adsorption data were plotted in the form of C/q versus C, as shown in Fig. 2B. The observed high correlation coefficient (0.999) indicated that these results fit the Langmuir model very well. The rhOPN is deemed to be adsorbed on the HA by the following Langmuir-type reaction, via the electrostatic attraction between the positively charged group of HA surfaces and the negatively charged domain of rhOPN, as follows:

$$R + \text{rhOPN} \overset{K}{\longleftrightarrow} R \cdot \text{rhOPN}$$

The Langmuir model is the fundamental adsorption model of chemisorption [16]. In this model, the chemical specificity is very high and the energy of adsorption is so large that chemical bonding occurs eventually. This adsorbate forms a homogeneous and strong monolayer on the solid surface, such that the chemisorbed adsorbate may be difficult to remove, and desorption may be accompanied by chemical change.

The adsorption of rhOPN was facilitated by the positively charged Ca^{2+} ions of HA and the negatively charged rhOPN, as shown in Fig. 1. The region consisting of nine consecutive aspartic acid residues has previously been proposed as a potential domain for mineral binding in OPN [17]. Furthermore, it was proven experimentally in a recent study that OPN binds to HA via its polyaspartic acid sequences [18].

Based on these adsorption data, the HA was coated with rhOPN at a concentration of 1 μM rhOPN. Fig. 3 shows the cell adhesion morphologies on the rhOPN coated HA after culturing for 35 min at 37°C. Pure HA was tested for the purpose of comparison. It was observed at low magnification (Figs 3A–B) that a large number of cells spread and adhered well on both the coated and uncoated surfaces. At higher magnifications (Figs 3C–D), the cell membranes were seen to be well flattened and in intimate contact with the materials. In particular, the cells adhered on the rhOPN coated HA were more flattened. Specifically, the average area covered by an individual cell on the rhOPN coated HA (992 μm^2) was ~4 times higher than that on pure HA (258 μm^2), as measured from the SEM image of at least 20 different cells.

The cell attachment was quantified after staining the cells with Crystal Violet, as shown in Fig. 4. More cells were attached on the rhOPN coated HA than on pure

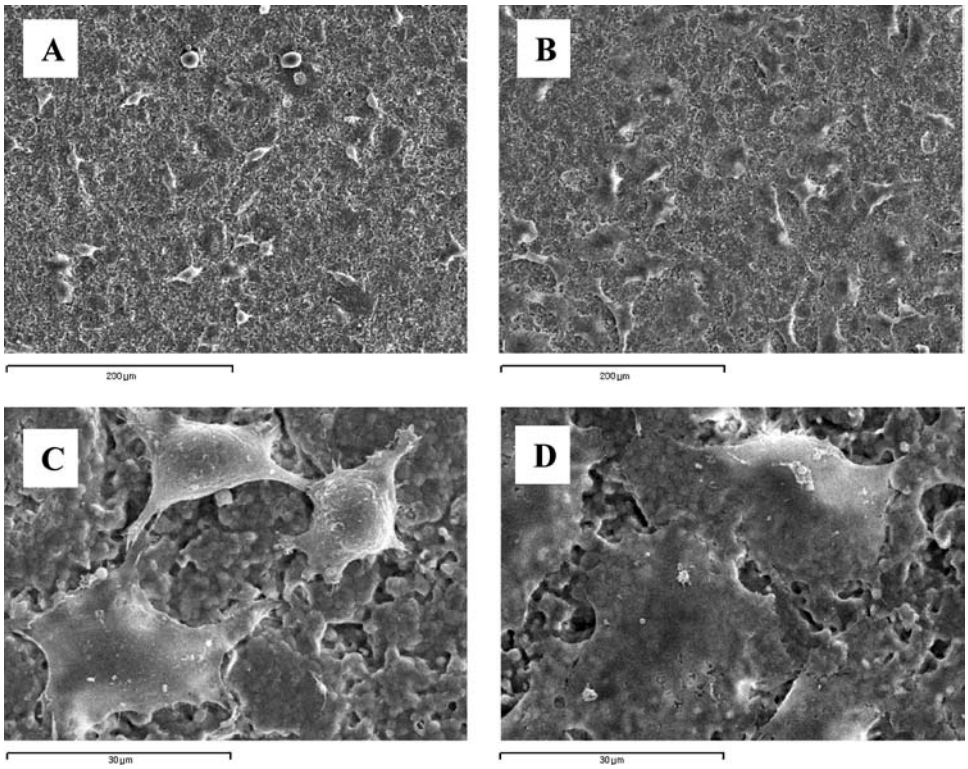

Figure 3 SEM morphology of HOS cells attached on samples (A, B) at low magnification and (C, D) high magnification; (A, C) pure HA and (B, D) rhOPN coated HA.

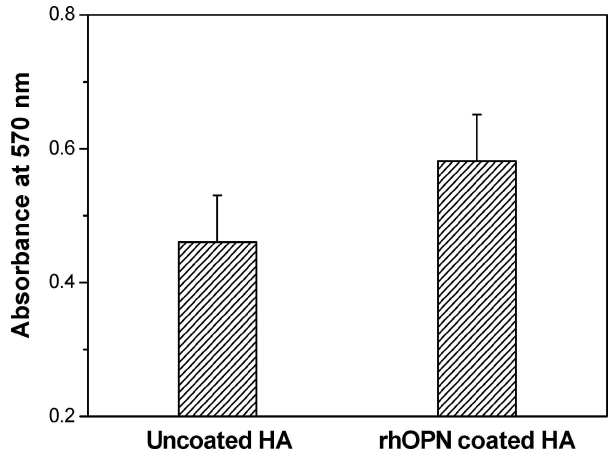

Figure 4 HOS cell attachment levels on pure HA and rhOPN coated HA after 35 min from seeding ($P < 0.05$).

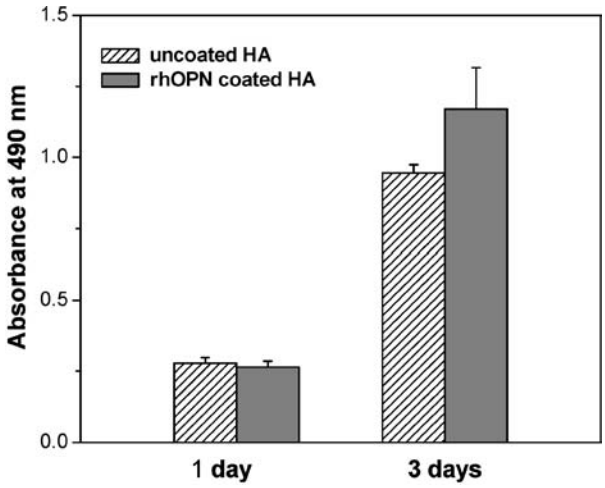

Figure 5 Proliferation levels of HOS cells on pure HA and rhOPN coated HA after culturing for 1 and 3 days ($P < 0.05$ at day 3).

HA ($p < 0.05$). The improvement in the adhesion behavior of the cells on HA afforded by the rhOPN coating was believed to be attributed to the specific attachment mechanism of OPN, namely the integrin/RGD-mediation, because the RGD sequence in OPN is well known to interact with integrin receptors at the cell surface, and serve to anchor the bone cells tightly to the extracellular matrix [17, 19–21].

The cell proliferation level on the rhOPN coated HA was assessed by an MTS method after culturing for 1 and 3 days, as shown in Fig. 5. The cell proliferation at 1 day was similar, but the cell number at day 3 was significantly higher ($p < 0.05$) on the rhOPN coated HA than on the uncoated HA.

The differentiation characteristics of the cells were evaluated by measuring the ALP activity after culturing for 7 days, as shown in Fig. 6. The HOS cells on the rhOPN coated HA expressed a significantly higher ($p < 0.05$) ALP level compared to those on the uncoated HA.

All of the data on the cellular responses, namely, the cell attachment, proliferation and ALP activity were significantly improved by the rhOPN coating. It is known that OPN promotes changes in gene expression via a signal transduction cascade, which is initiated by the interaction with cell surface receptors such as $\alpha_v\beta_3$ integrin or CD44 [22–24]. These integrins translate the attachment of external ligands to internal information, thus regulating the cell behaviors, such as cell growth and differentiation [25–27]. Therefore, the improved proliferation and ALP activity of the cells on the rhOPN

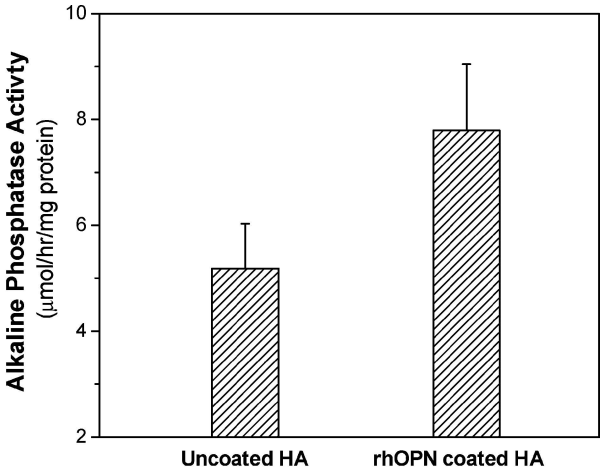

Figure 6 Alkaline phosphatase activity of HOS cells on pure HA and rhOPN coated HA after culturing for 7 days ($P < 0.05$).

coated HA can be attributed to the stimulated intracellular signaling pathway and a further up-regulation of the cell behaviors. Together with the specific binding of rhOPN to the HA surface, the improved cellular responses afforded by the rhOPN coating suggest that rhOPN is an efficient protein to use as a coating on HA, and that rhOPN coated HA has the potential to be used as a biomedical material.

4. Conclusion

To improve the biocompatibility of HA, a recombinant fragment of osteopontin (rhOPN) was specifically coated on the HA surface. When the HA was coated with rhOPN at a concentration of 1 μM, the osteoblast-like HOS cells exhibited significantly higher attachment, proliferation and differentiation levels. These enhanced cellular responses were attributed to the interaction between the cell's integrins and the RGD domains of the rhOPN coated on HA.

Acknowledgements

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