PROTEOMIC IDENTIFICATION OF SERUM PROTEINS TO INDUCE OSTEOCONDUCTIVITY OF HYDROXYPATITE

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We performed proteomic analysis of rat serum proteins adsorbed on hydroxyapatite (HAp) and α-alumina (α-Al2O3) in order to identify proteins that specifically adsorb onto HAp and control cellular responses. Proteins with either or both molecular weight of 22–32 kDa and computed isoelectric point of 5.0–5.5 were preferentially adsorbed on HAp. In total, 182 proteins were adsorbed on both HAp and α-Al2O3, of which 14 were highly enriched on HAp, whereas 68 were adsorbed only on HAp. Therefore, 82 (14+68) proteins were further evaluated by bioinformatics and literature-based analyses. We predicted that hepatocyte growth factor and angiopoietin-like protein 3 (ANGPTL3) are candidate proteins responsible for the osteoconductivity of HAp. Although ANGPTL3 promoted the attachment and spreading of MC3T3-E1 cells, it did not promote their proliferation and differentiation. Our results suggest that specific adsorption of ANGPTL3 on HAp induced osteoconductivity by enhancing the attachment and spreading of osteoblasts.

Keywords: Proteomic analysis, Hydroxyapatite, α-Alumina, Osteoconductivity, Angiopoietin-like protein 3

INTRODUCTION

Artificial scaffold materials used in implants to treat bone injuries are typically encapsulated in fibrous tissues. However, few ceramic materials such as Bioglass41, hydroxyapatite (HAp)35, and glass-ceramic A-W9 exhibit osteoconductivity, by which bone tissues are formed along the surfaces of these materials and hence these materials bond to living bones without forming fibrous tissues. These ceramics are known as bioactive ceramics. HAp is the most widely used bioceramic material, primarily as a bone substitute or coating material on metallic orthopedic or dental implants. In 1977, Aoki et al.8 and Jarcho et al.5 demonstrated the clinical application of chemically synthesized HAp. In the 1980s, although de Groot6 coated HAp onto metallic implants by plasma spray, Oonishi et al.7 developed the interface bioactive bone cement technique, in which HAp granules are interposed at the interface of polymethyl methacrylate-based bone cement and living bone at cementation during surgery. This technique improves the osteoconductivity of polymethyl methacrylate-based bone cement. Furthermore, in recent years, HAp-collagen composites8 and carbonate-containing HAp9 have been used for clinical applications in Japan. These HAp-based materials show excellent osteoconductivity in vivo. Although cellular responses to osteoconductive materials have been investigated10,11, the molecular level mechanism underlying the osteoconductivity of HAp remains unclear.

Typically, when artificial materials are implanted into the body, many serum proteins immediately adsorb onto the material surface. Then, aided by these proteins, some cells attach to the surface of the materials10,13. We have been investigating the adsorption behavior of certain proteins on osteoconductive HAp and non-osteoconductive alpha-alumina (α-Al2O3) and their effects on cellular responses12–16. These investigations revealed that bovine serum albumin adsorbed onto HAp and α-Al2O3 to inhibit the attachment and spreading of osteoblast-like MC3T3-E1 cells but did not affect their proliferation. However, adsorption of bovine serum albumin led to inhibition of the attachment of RAW264.7 macrophages on α-Al2O3 but not on the HAp surface13. Further, we revealed that fibronectin or laminin adsorbed on HAp enhanced the attachment and spreading of MC3T3-E1 cells; however, on α-Al2O3, they showed no such effect14–16. These studies suggest that serum proteins display remarkably different functions depending on whether they are adsorbed on a bioresorbable material. Thus, we hypothesized that specific adsorption of serum proteins play an important role in and induce the osteoconductivity of HAp.

Recently, proteomic analysis has been used to study serum proteins adsorbed onto biomaterials17. Kaneko et al. conducted proteomic analysis of rat serum proteins adsorbed on the artificial bone materials octacalcium phosphate and HAp18. They found that
although complement 3 (C3) adsorption on octacalcium phosphate was greater than that on HAp, adsorption of apolipoprotein E, a protein known to promote osteoblast differentiation, was greater than on HAp than on octacalcium phosphate. Moreover, Romero-Gavilán et al. conducted proteomic analysis of human plasma proteins that were adsorbed on titanium and found that apolipoprotein E, antithrombin, and protein C were mainly adsorbed on blasted and acid-treated Ti, whereas C3 was mainly adsorbed on the smooth Ti surface. Therefore, in this study, we employed proteomic analysis to identify rat serum proteins that adsorbed onto HAp and α-Al2O3 and induced osteoconductivity. Furthermore, we studied the influence of the identified proteins on the attachment, spreading, proliferation, and differentiation of pre-osteoblastic MC3T3-E1 cells.

MATERIALS AND METHODS

Extraction of rat serum proteins adsorbed on HAp and α-Al2O3 samples

Commercially available rat serum was obtained from Japan SLC (Shizuoka, Japan), which was collected by cardiac puncture sampling from 8 Wistar rats (12 weeks, male). HAp powder (HAP-200, Taipei Chemical Industrial, Osaka, Japan) and α-Al2O3 powder (ALO14PB, Kojundo Chemical, Saitama, Japan) were used as materials for protein adsorption. We confirmed the composition and purity of HAP-200 and ALO14PB by X-ray diffraction analysis, which established that they contained only HAp and α-Al2O3, respectively. Based on the measured surface area, 867 mg of HAp powders and 386 mg of α-Al2O3 powders, which gave the same surface area of 3.2 m², were used for adsorption of serum proteins. The HAp and α-Al2O3 powders were separately incubated with 3 mL of rat serum for 24 h at 37°C under rotation (5 rpm). After incubation, the mixtures were centrifuged at 1,000 × g for 30 min to precipitate the powder as protein-adsorbed samples.

Proteins adsorbed on the HAp and α-Al2O3 were extracted by the procedure used to extract the bone matrices as described previously. Briefly, the protein-adsorbed powder was dispersed in 10 mL of extraction buffer [RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1.0% NP-40)], which was obtained from FUJIFILM Wako Pure Chemical (Osaka, Japan), and was supplemented with 1 mM phenylmethylsulfonyl fluoride. The mixtures were stirred for 3 days, transferred to 15 mL centrifuge tubes, vortexed, and centrifuged at 18,700 × g for 5 min at 4°C. After centrifugation, the supernatant was transferred to 15 mL centrifuge tubes, and the residue, 450 μL of ice-cold methanol was added, vortexed, and centrifuged at 13,800 × g for 3 min at 4°C. The upper layer just above the layer of white denatured protein was removed. To the residue, 450 μL of ice-cold methanol was added, vortexed, and centrifuged at 18,700 × g for 5 min at 4°C. The resulting precipitates were dried by vacuum centrifugation. The dried precipitates were dissolved in 10 μL of 6 M urea, diluted by addition of 35 μL of 0.1 M Tris-HCl, pH 8.8, and then 5 μL of 25 ng•μL –1 trypsin (Promega, Madison, WI, USA) was added to the solution. The mixtures were then incubated at 37°C overnight. Trypsin digestion was terminated by addition of formic acid at a final concentration of 0.1%.

The trypsin digest of each sample was analyzed with a nano flow-LC (Eksigent nanoLC with expekt cHiPLC, Sciex, Framingham, MA, USA) coupled with a tandem mass spectrometer (TripleTOF 5600+ Sciex). Each sample was analyzed in duplicates under the trap and elute mode using a ChromeXP C18 Chip column (200 μm×0.5 mm) as a trap column and ChromeXP C18 column (75 μm×150 mm) as an analytical column. Mobile phases A and B were 0.1% formic acid aqueous solution and 0.1% formic acid in acetonitrile, respectively. The peptides were eluted using a gradient from 2% to 32% B over 40 min at a flow rate of 300 nL·min–1. The MS spectrum (250 ms) and 10 MS/MS spectra (100 ms each) were acquired under data-dependent mode. The dynamic exclusion time was set to 12 s. Auto-calibration was performed for every 4 samples using 50 fmol of bovine serum albumin trypsin digest (KYA Technology, Japan).

The raw data files generated by Analyst TF1.6 (Sciex) were converted to mascot generic files by a MS Converter (Sciex). The two mascot generic files generated by duplicate runs for each of 3 samples from proteins adsorbed on either HAp or α-Al2O3 were merged and searched by the Mascot search engine (ver. 2.5) against an in-house built Rattus norvegicus proteome reference database (UniProt, downloaded May 29, 2015). Peptide tolerance and MS/MS tolerance were set to ±20 ppm and ±0.1 Da, respectively.

Data analysis

The identified proteins were quantitatively analyzed by using the normalized spectral abundance factor (NSAF), a spectral counting algorithm developed by Paoletti et al. In the spectral counting method, it is thought that a larger protein generates more peptides upon trypsin digestion, and consequently generates more spectral counts than smaller proteins. The number of spectral
counts for each identified protein was divided by protein mass or length to obtain the spectral abundance factor (SAF). To account for between-run variation, individual SAF values were normalized by dividing them by the sum of SAFs of all identified proteins to obtain the NSAF. The isoelectric point (pI) of the identified proteins was computed from the amino acid sequence using the Compute pI/MW tool in Expasy, the bioinformatics resource portal of the Swiss Institute of Bioinformatics. Statistical analysis to determine the mean molecular weight (Mw) and pI of proteins specifically adsorbed on HAp and α-Al₂O₃ was performed using Welch’s t-test, and p values<0.05 were considered to indicate significant results.

**MC3T3-E1 cell attachment, spreading, and proliferation assays**

Pre-osteoblastic MC3T3-E1 cells (approximately 1×10⁴), which were derived from the calvaria of a newborn C57BL/6 mouse, were seeded into a 24-well culture plate (TPP Techno Plastic Product, Trasadingen, Switzerland). To each well, 300 μL of Dulbecco’s Modified Eagle’s medium (DMEM) supplemented with 20% fetal bovine serum (FBS), penicillin/streptomycin, and 0, 0.3, or 1.5 μg•mL⁻¹ recombinant human angiopoietin-like proteins 3 (ANGPTL3; PTI 130-18, FUJIFILM Wako Pure Chemical) were added. Notably, the concentration of ANGPTL3 in human blood ranges from 0.2 to 0.3 μg•mL⁻¹. The isoelectric point (pI) of the identified proteins was almost the same (for identified proteins, refer to Supplementary material 3). MC3T3-E1 cells were seeded into a 24-well culture plate (TPP Techno Plastic Product, Trasadingen, Switzerland), and 300 μL of DMEM supplemented with 20% FBS and penicillin/streptomycin, and 0, 0.3, or 1.5 μg•mL⁻¹ ANGPTL3. As a positive control (BMP group), the cells were cultured in 300 μL of DMEM supplemented with 10% FBS, penicillin/streptomycin, and 0.1 μg•mL⁻¹ bone morphogenetic protein 2 (BMP-2) in place of ANGPTL3. After cell culture, the medium was replaced with 500 μL of phosphate-buffered saline (PBS; 05913 Nissui Pharmaceutical, Tokyo, Japan). PBS was then replaced with 200 μL of ALP yellow liquid substrate solution (P7998-100ML, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 2 μL of 10% Triton-X/PBS, and incubated at 37°C for 15 min. Finally, 75 μL of 2 M NaOH solution was added to terminate the phosphatase reaction. ALP activity was determined by measuring the absorbance of the solution at 405 nm with a microplate reader (GloMax®-Multi Microplate Multimode Reader, Promega). Statistical analysis was performed by two-way ANOVA followed by Tukey-Kramer multiple comparison test, and p values<0.05 were considered as significant.

**RESULTS**

Table 1 shows the total number of proteins found to be adsorbed on HAp and α-Al₂O₃. The total number of proteins identified on HAp or α-Al₂O₃ was almost the same (for identified proteins, refer to Supplementary materials 1 and 2). Among these, 182 proteins were found on both materials (Supplementary material 3). Sixty-nine proteins were uniquely identified on α-Al₂O₃, whereas 68 proteins were uniquely identified on HAp (Table 2).

**MC3T3-E1 cell differentiation assay**

Alkaline phosphatase (ALP) activity of adherent MC3T3-E1 cells was measured to determine their degree of differentiation. MC3T3-E1 cells (approximately 2×10⁴) were seeded into a 24-well culture plate, and 300 μL of DMEM supplemented with 20% FBS and penicillin/streptomycin was added to the wells. Cells were cultured for 3 days at 37°C and in a 5% CO₂ atmosphere, and further cultured for 14 and 28 days in 300 μL of DMEM supplemented with 10% FBS, penicillin/streptomycin, and 0, 0.3, or 1.5 μg•mL⁻¹ ANGPTL3. As a positive control (BMP group), the cells were cultured in 300 μL of DMEM supplemented with 10% FBS, penicillin/streptomycin, and 0.1 μg•mL⁻¹ bone morphogenetic protein 2 (BMP-2) in place of ANGPTL3. After cell culture, the medium was replaced with 500 μL of phosphate-buffered saline (PBS; 05913 Nissui Pharmaceutical, Tokyo, Japan). PBS was then replaced with 200 μL of ALP yellow liquid substrate solution (P7998-100ML, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 2 μL of 10% Triton-X/PBS, and incubated at 37°C for 15 min. Finally, 75 μL of 2 M NaOH solution was added to terminate the phosphatase reaction. ALP activity was determined by measuring the absorbance of the solution at 405 nm with a microplate reader (GloMax®-Multi Microplate Multimode Reader, Promega). Statistical analysis was performed by two-way ANOVA followed by Tukey-Kramer multiple comparison test, and p values<0.05 were considered as significant.

**Table 1 Numbers of proteins adsorbed onto HAp and α-Al₂O₃**

| Sample    | Total number | In common | Specifically |
|-----------|--------------|-----------|--------------|
| HAp       | 250          |           | 68           |
| α-Al₂O₃   | 251          | 182       | 69           |
| Gene symbol | Protein name | Accession number |
|-------------|--------------|-----------------|
| Adipoq      | Protein Adipoq | A0A0G2K845     |
| Al314180    | Protein Al314180 | F1M446       |
| Angptl3     | Angiopoietin-like 3, isoform CRA_b | F7HF01 |
| Apoe4       | Apolipoprotein A-IY | M0R5A9 |
| Brca2       | Breast cancer susceptibility protein 2 | Q66MH4 |
| Brca2       | Breast cancer type 2 susceptibility protein homolog | Q55923 |
| C1qa        | Complement C1q subcomponent subunit A | P31720 |
| C1qb        | Complement C1q subcomponent subunit B | G3V7N9 |
| C1qc        | Complement C1q subcomponent subunit C | P31722 |
| Cli         | Protein Cli | D4A1T6 |
| C4bpa       | C4-binding protein alpha chain | Q5S891 |
| C4bpb       | C4-binding protein beta chain | A0A5C5 |
| Cep350      | Protein Cep350 | F1LPD3 |
| Cfb         | Da1-24 | Q7TP05 |
| Clec11a     | C-type lectin domain family 11 member A | O88201 |
| Clu         | Clusterin | P05371 |
| Dmtf1       | Cyclin-D-binding Myb-like transcription factor 1 | Q66H1G |
| F9          | Coagulation factor IX | P6296 |
| Fgl2        | Fibrinogen-like 2 | G3V7P2 |
| Gc          | Group specific component | Q6S8Y4 |
| Gpx6        | Glutathione peroxidase | D3Z8H2 |
| Hbz         | Protein Hbz | G3V8R3 |
| Hgf         | Hepatocyte growth factor | P17945 |
| Itknap      | Elongator complex protein 1 | F1LP76 |
| Itih4       | Inter alpha-trypsin inhibitor, heavy chain 4 | Q5EB0 |
| Jade2       | PHD finger protein 15 (Predicted) | G3V9F5 |
| Kb15        | Protein Kb15 | G3V908 |
| Kcnq5       | Protein Kcnq5 | F1LS6D |
| Kif15       | Kinesin family member 15, isoform CRA_a | A0A0G2K0M1 |
| Kng111      | Kininogen | Q6LE85 |
| Kpp         | Keratinocytokeratin-like protein | G3V9A5 |
| Krt10       | Keratin, type I cytoskeletal 10 | A0A0G2K2V6 |
| Krt31       | Protein Krt31 | F1MAF7 |
| Krt40       | Keratin, type I cytoskeletal 40 | Q6IF2W |
| Krt73       | Keratin, type II cytoskeletal 73 | A0A0G2JXH6 |
| Krt79       | Protein Krt79 | F1M1D0 |
| Krt84       | Protein Krt84 | D3Z5Y5 |
| LOC100359993| Protein LOC100359993 | A0A0G2K3A6 |
| LOC100910255| Protein LOC100910255 | A0A0G2K9H4 |
| Lmap2       | Mannan-binding lectin serine protease 2 | Q9JJS8 |
| Olr1243     | Protein Olr1243 | A0A0G2K370 |
| Phf11       | PHD finger protein 11 | Q510E2 |
| Phf11       | Protein Setdb2 | F1LQ59 |
| Pla2g2a     | Phospholipase A2, membrane associated | P14423 |
| Prss1       | Protein S (Alpha), isoform CRA_b | M0R5R0 |
| Proz        | Protein Proz | D3ZKM4 |
| Prss1       | Anionic trypsin-1 | P00762 |
| Psap        | Sulfated glycoprotein 1 | F7EPE0 |
| Psma2       | Proteasome subunit alpha type-2 | P17220 |
| Psma4       | Proteasome subunit alpha type-4 | P21670 |
| Psma5       | Proteasome subunit alpha type | Q6F9V6 |
| Psma6       | Proteasome subunit beta type-6 | A0A0G2K0D7 |
| Psmb1       | Proteasome subunit beta type | Q6PDW4 |
| Psmb2       | Proteasome subunit beta type-2 | P40307 |
| Psmd1       | 26S proteasome non-ATPase regulatory subunit 11 | A0A0G2JWX1 |
| rCG_63409   | Protein rCG63409 | A0A0G2JSL0 |
| RGD1310257  | Protein RGD1310257 | D3ZHE3 |
| Rsu1        | Protein Rsu1 | D4A8F2 |
| Tec         | Protein Tec | A0A0G2KA94 |
| Tpfi        | Tpfi protein | Q5PQU9 |
| Timeless    | Protein timeless homolog | E9PTS7 |
| Try10       | Protein Try10 | W4V5R7 |
| Uttn        | Protein Uttn | G3V7L1 |
| Vtn         | Protein Vtn | Q3KR94 |
| Zscan30     | Protein Zscan30 | A0A0G2K290 |
|             | Uncharacterized protein | A0A0G2JUY3 |
|             | Uncharacterized protein | D3Z651 |
was 6.62±1.47, with values ranging from 4.31 to 9.39, whereas that of the 68 proteins specifically adsorbed on α-Al₂O₃ was 6.50±1.43, with values ranging from 4.17 to 11.57. The p values determined using Welch’s t-test for the Mₘ and computed pI of proteins specifically adsorbed on HAp and α-Al₂O₃ were 0.95 and 0.64, respectively. For the 182 proteins commonly identified on HAp and α-Al₂O₃, we compared their relative abundance by calculating the ratios of the NSAF (see the MATERIALS AND METHODS section for details) in the context of HAp versus α-Al₂O₃. The NSAF ratios were represented using box-and-whisker plots and analyzed by Tukey’s multiple comparisons using GraphPad Prism version 8 software (GraphPad, La Jolla, CA, USA). Proteins with ratios higher than 1.5-fold difference between the 25th and 75th percentiles were defined as significantly enriched in the context of HAp; 14 proteins in Table 3 showed this pattern. Notably, statistical analysis of the NSAF ratios gave a value of 0.991±1.311 (mean±SD) for the 25th percentile of 0.6206, 75th percentile of 1.018, and median of 0.7723. Thus, 82 proteins including the 68 proteins that were uniquely identified on HAp and 14 proteins that were found enriched on HAp were considered as protein candidates with potential roles in the osteoconductivity of HAp, as they displayed specific and preferential adsorption on HAp.

The subcellular distribution of proteins adsorbed on HAp (Supplementary material 1) and on α-Al₂O₃ (Supplementary material 2) were analyzed and categorized using Gene Ontology (GO) cellular component vocabulary (Fig. 2(a) and (c)). The subcellular distribution was similar for both HAp and α-Al₂O₃ except that the number of proteins categorized in the cell (27%) was slightly larger than that in the extracellular region (25%) and within the organelles (25%). Moreover, proteins categorized in the synapse were only detected in proteins adsorbed on α-Al₂O₃.

The results of pathway analysis of proteins adsorbed on HAp and α-Al₂O₃ are shown in Fig. 2(b) and (d), respectively. Proteins adsorbed on HAp were categorized into 22 pathways, and those on α-Al₂O₃ were grouped into 23 pathways. Among the pathways, 19 were common to both groups. Three pathways (Parkinson’s disease, ubiquitin proteasome, and cell cycle) were unique to proteins absorbed on HAp, whereas four pathways (synaptic vesicle trafficking, adrenaline and hypoxanthine salvage, purine metabolism, and 1-arachidonoylglycerol biosynthesis) were unique to those absorbed on α-Al₂O₃.

Based on literature analysis (discussed later), we selected two proteins from those adsorbed specifically on

### Table 3 Proteins adsorbed more onto HAp than α-Al₂O₃

| Gene symbol | Protein name                                | Accession number |
|-------------|---------------------------------------------|------------------|
| Apcs        | Serum amyloid P-component                   | P23680           |
| Apoa1       | Apolipoprotein A-I                          | P04639           |
| Apoc3       | Apolipoprotein C-III                        | A0A0G2K8Q1       |
| F2          | Prothrombin                                 | Q3V843           |
| Hps5        | Serum amyloid A protein                     | Q5M878           |
| Ighm        | Protein Ighm                                | A0A0G2JVP4       |
| Krt12       | Keratin, type I cytoskeletal 12             | A0A0G2K4H9       |
| Masp1       | Mannose-binding lectin serine protease 1    | Q8CHN8           |
| Mb1l        | Mannose-binding protein A                   | P19999           |
| Nes         | Nestin                                      | P21263           |
| Pon3        | Serum paraoxonase/lactonase 3              | Q68FP2           |
| Proc        | Protein C, isoform CRA_b                    | F7FMY6           |
| Serpina10   | Protein Z-dependent protease inhibitor       | Q62975           |
| Serpind1    | Heparin cofactor 2                          | A0A0G2K8K3       |
Fig. 2  Pie charts of the cellular component distributions of the proteins identified on HAp and α-Al₂O₃ ((a) and (c)) based on GO terms and functional classification of the proteins by pathway ((b) and (d)). Cellular component analysis and functional classification were carried out using the PANTHER Classification System.

HAp (Tables 2 and 3), hepatocyte growth factor (HGF) and angiopoietin-like protein 3 (ANGPTL3), for further analysis. While the effect of HGF on osteoblasts or osteoclasts has been reported in previous studies²⁴-²⁶, the effect of ANGPTL3 on these cells has not been understood. Therefore, we investigated the effect of ANGPTL3 on pre-osteoblastic MC3T3-E1 cell responses in this study. Figure 3 shows the number of adherent MC3T3-E1 cells cultured for 1, 6, or 24 h in medium containing different ANGPTL3 concentrations. Irrespective of the ANGPTL3 concentration, the number of adherent MC3T3-E1 cells increased with increasing culture period. Moreover, the number of adherent MC3T3-E1 cells cultured for 1, or 6 h in ANGPTL3-containing medium was significantly larger than that in ANGPTL3-free medium. For the culture period of 24 h, the number of adherent MC3T3-E1 cells cultured in 1.5 μg·mL⁻¹ ANGPTL3-containing medium was still significantly larger than that in ANGPTL3-free or 0.3 μg·mL⁻¹ ANGPTL3-containing medium. However, there was no significant difference in the number of adherent MC3T3-E1 cells cultured in medium containing 0.3 μg·mL⁻¹ and in that with no ANGPTL3.

Figure 4 shows maximum cell length of adherent MC3T3-E1 cells cultured for 1, 6, or 24 h in the medium with different ANGPTL3 concentrations. Irrespective of the ANGPTL3 concentration, the maximum cell length of adherent MC3T3-E1 cells cultured for 6 h was larger than that cultured for 1 or 24 h. At the given culture period, the ANGPTL3 concentration of 0.3 μg·mL⁻¹ showed the maximum cell length of adherent MC3T3-E1 cells.

Figure 5 shows proliferation of MC3T3-E1 cells cultured for 1, 3, or 7 days in medium with different ANGPTL3 concentrations. MC3T3-E1 cell proliferation increased with increasing culture period, irrespective of the ANGPTL3 concentrations. However, the cell proliferation rates were not significantly different at different ANGPTL3 concentrations.

Few markers such as Cbfa1, Sp7, and Spp1 have been used to assess the differentiation status of osteoblasts by in vitro calcification and are thought to be associated with increased ALP activity²⁷,²⁸. In addition, it is known that the level of markers including type I collagen, ALP, and Opn increases at the early stage of osteoblast differentiation while that of other makers such as Ocn increases at the later stages²⁹. Therefore, here, we decided to investigate the ALP activity to evaluate the early differentiation status of MC3T3-E1 cells in this study. Figure 6 shows ALP activity of MC3T3-E1 cells cultured for 14 or 28 days in medium with different ANGPTL3 concentrations, in comparison with those in medium containing BMP-2 without ANGPTL3. The ALP activity of MC3T3-E1 cells cultured in the medium containing 0, 0.3, or 1.5 μg·mL⁻¹ ANGPTL3 did
Fig. 4 Maximum length of adherent MC3T3-E1 cells cultured in medium with different ANGPTL3 concentrations for 1, 6, or 24 h (mean±SD, two-way ANOVA, **p<0.01, *p<0.05).

Fig. 5 Proliferation of MC3T3-E1 cells cultured in medium with different ANGPTL3 concentrations for 1, 6, or 24 h (mean±SD, two-way ANOVA, **p<0.01, *p<0.05).

Fig. 6 ALP activity of MC3T3-E1 cells cultured in the medium with different ANGPTL3 concentrations for 14 or 28 days compared with that in medium containing BMP-2 (mean±SD, two-way ANOVA, **p<0.01, *p<0.05).

not increase, but that of cells cultured in the medium containing BMP-2 without ANGPTL3 was significantly increased. Further, no significant difference was observed between the ALP activity of MC3T3-E1 cells cultured in ANGPTL3-containing medium and that in ANGPTL3-free medium, irrespective of the culture period; however, the ALP activity of MC3T3-E1 cells cultured in medium containing BMP-2 without ANGPTL3 was significantly higher than that in ANGPTL3-containing medium.

**DISCUSSION**

Physicochemical properties, such as the $M_w$ and pI, of proteins specifically adsorbed on HAp, may differ from those of proteins specifically adsorbed on $\alpha$-Al$_2$O$_3$. We found that the proteins with either or both $M_w$ values ranging from 22 to 32 kDa and computed pI values ranging from 5.0 to 5.5 tended to be adsorbed on HAp (Fig. 1), although there was no significant difference in the mean $M_w$ and computed pI of proteins specifically adsorbed on HAp and $\alpha$-Al$_2$O$_3$. It is difficult to explain why proteins with specific $M_w$ and calculated pI were preferentially adsorbed on HAp; however, this result may be related to the osteoconductivity of HAp. Bioinformatic analysis of subcellular distribution of proteins that were found to be adsorbed on HAp and $\alpha$-Al$_2$O$_3$ suggested a lack of synapse-related proteins in those adsorbed on HAp (Fig. 2(a) and (c)). This finding coincided with the result of pathway analysis in which proteins that were categorized into synaptic vesicle trafficking were identified only on $\alpha$-Al$_2$O$_3$ (Fig. 2(b) and (d)). Since $\alpha$-Al$_2$O$_3$ is a ceramic material that does not exhibit osteoconductivity, this finding implies that the proteins involved in synaptic vesicle trafficking adsorb on $\alpha$-Al$_2$O$_3$ and may inhibit the osteoconductivity of $\alpha$-Al$_2$O$_3$. Bioinformatic analysis also indicated that proteins categorized into three pathways are unique to HAp, while proteins categorized into 4 pathways including synaptic vesicle trafficking pathway are unique to $\alpha$-Al$_2$O$_3$. However, a survey of the literature indicated that these proteins are unlikely to be related to the osteoconductivity.

In the literature, two proteins among those specifically adsorbed on HAp (Tables 2 and 3) were recognized as being associated with bone formation and therefore they were selected as possible candidates for inducing osteoconductivity in HAp. These proteins were HGF and ANGPTL3.

In our bioinformatic analysis, HGF was categorized as a protein in the extracellular region but not categorized into any pathways. HGF is a growth factor that promotes tissue angiogenesis, cell motility, and cell differentiation. Interestingly, osteoblasts and...
osteoclasts express HGF receptor/c-Met and HGF24,25. HGF activates osteoblasts to promote proliferation and induces chemotactic migration of osteoclasts25,26. HGF in combination with vitamin D promotes the differentiation of mesenchymal stem cells into osteoblasts and chondroblasts25,26,27. HGF readily adsorbs on HAp; it does not detach even after 2 days and thereby promotes osteoblast differentiation28. It has also been reported that osteoblasts are activated on HGF-coated HAp29. Therefore, HGF likely contributes to the osteoconductivity of HAp.

Angiopoietin-like proteins (ANGPTLs) are secretory proteins with a molecular structure similar to that of angiopoietin. ANGPTL is an angiogenic factor with seven known subtypes30. We found that ANGPTL3, a member of the ANGPTL family, specifically adsorbs on HAp (Table 2). Our bioinformatic analysis suggested that this protein is localized in the extracellular region. Similar to HGF, it was not categorized into any pathway. A recent study revealed that ANGPTL3 contributes to maintaining the activity of hematopoietic stem cells in the bone marrow niche31. Maintaining the activity of hematopoietic stem cells in the bone marrow niche ensures the differentiation of monocyte-macrophage progenitor cells into osteoclast precursor cells. By the action of osteoclast differentiation factor present on the osteoblast cell membrane, osteoclast precursor cells differentiate into osteoclasts, which are essential for bone remodeling and bone metabolism in living organisms. Furthermore, some studies suggested that a close relationship exists between bone formation and hematopoiesis, and osteoblasts play an important role in regulating hematopoiesis and skeletal development32,33. Therefore, ANGPTL3 may be related to the osteoconductivity of HAp.

As the effect of ANGPTL3 on osteoblasts or osteoclasts is unknown, we investigated the effect of ANGPTL3 on pre-osteoblastic MC3T3-E1 cell responses. As shown in Fig.3, MC3T3-E1 cell attachment was enhanced by ANGPTL3; however, when the ANGPTL3 concentration was 0.3 μg·mL−1, which is the average level of ANGPTL3, cell attachment was not significantly promoted for a culture period of 24 h. This suggests that cell attachment for shorter periods such as 1 or 6 h was enhanced by ANGPTL3 with average levels in the blood, and the enhancement was prolonged when the ANGPTL3 concentration was increased above its average blood level.

Figure 4 shows that the spreading of MC3T3-E1 cells was significantly enhanced for 1 or 6 h in 0.3 μg·mL−1 ANGPTL3-containing medium, indicating that ANGPTL3 even at normal levels in the blood can promote spreading of MC3T3-E1 cells at an early stage. In contrast, enhancement of MC3T3-E1 cell spreading was not confirmed in 1.5 μg·mL−1 ANGPTL3-containing medium. This can be interpreted as follows. As shown in Fig. 3, the number of adherent MC3T3-E1 cells cultured in 1.5 μg·mL−1 ANGPTL3-containing medium was significantly higher than those in ANGPTL3-free or 0.3 μg·mL−1 ANGPTL3-containing medium. One mother cell divides into two daughter cells during proliferation, and the cytoplasm is evenly divided into both daughter cells; thus, each daughter cell cultured in 1.5 μg·mL−1 ANGPTL3-containing medium may have less cytoplasm than that cultured in ANGPTL3-free or 0.3 μg·mL−1 ANGPTL3-containing medium. The reduction in the amount of cytoplasm in each daughter cell may affect the maximum cell length.

As shown in Figs. 5 and 6, although ANGPTL3 did not enhance the proliferation and differentiation of MC3T3-E1 cells, there was no negative impact on proliferation and differentiation. Figures 3–6 show that ANGPTL3 enhances the attachment and spreading of MC3T3-E1 cells without negatively impacting the proliferation and differentiation of MC3T3-E1 cells, suggesting that ANGPTL3 was specifically adsorbed on HAp to enhance osteoblast attachment and spreading, and then induce osteoconductivity.

CONCLUSION

We conducted proteomic analysis of rat serum proteins to identify those that adsorb on osteoconductive HAp and non-osteconductive α-Al₂O₃ to identify proteins that can induce the osteoconductivity of HAp. Our results showed that: (1) 250 and 251 proteins adsorbed on HAp and α-Al₂O₃, respectively; (2) 182 proteins adsorbed on both ceramic powders; (3) 68 proteins were specifically adsorbed on HAp, and 69 specifically adsorbed on α-Al₂O₃; (4) proteins with either or both Ms values ranging from 22 to 32 kDa and computed pI values ranging from 5.0 to 5.5 were preferentially adsorbed on HAp; (5) 14 proteins adsorbed more on HAp than on α-Al₂O₃; and (6) in total, 82 proteins showed high affinity for HAp; (7) among the proteins specifically adsorbed on HAp, ANGPTL3 and HGF were the most probable candidate proteins. We also found that ANGPTL3 enhances the attachment and spreading of MC3T3-E1 cells without negatively impacting their proliferation and differentiation, suggesting that ANGPTL3 was specifically adsorbed on HAp to enhance osteoblast attachment spreading leading to osteoconductivity of HAp. Further in vitro and in vivo studies are needed to confirm the role of ANGPTL3. However, our findings provide a foundation for determining the molecular mechanisms underlying the osteoconductivity of HAp.

ACKNOWLEDGEMENTS

This work was partially supported by the Naito Foundation Natural Science Scholarship, and the Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, Project “Design & Engineering by Joint Inverse Innovation for Materials Architecture” from the Ministry of Education, Culture, Sports, Science, and Technology, Japan. The authors thank Masanobu KAMITAKAHARA (Graduate School of Environmental Studies, Tohoku University) for the surface area measurements.
CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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