Proteomic analysis of bone proteins adsorbed onto the surface of titanium dioxide

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ABSTRACT

Osseointegration is the structural and functional connection between bone tissues and implants such as titanium dioxide (TiO2). The bone-TiO2 interface is thought to contain proteoglycans. However, exhaustive analysis of the proteins in this layer has not been performed. In this study, we evaluated the bone protein adhered on the surface of TiO2 comprehensively. Pig bone protein was extracted by sequential elutions with guanidine, 0.1 M EDTA, and again with guanidine. The proteins obtained from these extractions were allowed to adhere to an HPLC column packed with TiO2 and were eluted with 0.2 M NaOH. The eluted proteins were identified by LC/MS/MS and included not only proteoglycans but also other proteins such as extracellular matrix proteins, enzymes, and growth factors. Calcium depositions were observed on TiO2 particles incubated with bone proteins, guanidine-extracted proteins adhered to TiO2 displayed significantly high amounts of calcium depositions.

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1. Introduction

Osseointegration refers to a direct bone-to-metal surface connection without interposition of fibrous tissue [1]. Based on this concept, implant treatment has become one of the prosthetic treatments for replacing missing teeth. Almost all dental implants are made of titanium dioxide (TiO2), because of its superior biocompatibility and mechanical resistance to force [2]. Osseointegration is mainly evaluated by observing the bone interface using light microscopy or by comparing the bone-to-implant surface contact rate in vivo [1]. However, a layer at the bone-TiO2 interface has also been reported based on transmission electron microscopy [3,4]. This layer was suggested to be essential for osseointegration.

Previous reports speculated that this zone was filled with glycosaminoglycans (GAGs) as observed by immunohistochemical analysis and ruthenium red staining [4,5].

A previous biochemical study attempted to identify the biomolecules adsorbed on TiO2 [5]. GAG chondroitin-4-sulfate (C4S) was adsorbed onto the surface of TiO2 powder via calcium ions [6]. However, the proteoglycans adsorbed on TiO2 were not released upon EDTA-mediated calcium ion chelation, invalidating the electrostatic bridge hypothesis. These histological and biochemical data are several decades old and have previously been summarized elsewhere [7].

Recently, TiO2 has been used in proteomics for chromatography-based protein purification because of its high affinity for phosphopeptides [8]. TiO2 has the capacity to bind not only to phosphorylated peptides but also to non-phosphorylated or acidic peptides, excluding hydroxycarboxylic acids, 2,5 dihydroxybenzoic acid, and glycolic acid [9]. The mechanism that binds TiO2 and the adherent proteins has not been fully elucidated. Gertler and colleagues investigated the binding mechanism and relative binding strength between peptides and a TiO2 surface using chromatography [10]. This biochemical analysis was performed with peptides or trypsin-digested proteins that were no longer in their three-dimensional conformation, and therefore did not represent the in vivo behavior of proteins. Recently, Tsuchiya and colleagues reported that secreted proteins derived from cultured bone marrow stromal cells, including extracellular matrix (ECM) proteins, cytokines, growth factors, and cellular components, were capable of adhering to TiO2 particles [11]. Another study revealed that the proteins attached to TiO2 were related to cell adhesion, transport, coagulation, immune response, and cytokines [12]. Taken together, these previous reports demonstrate that various proteins can be immobilized on TiO2 surfaces, and the TiO2 chromatography system is a useful proteomics tool. Research is needed to identify the specific bone-related proteins that are immobilized on the TiO2 surface.

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There were only a few report of biochemical analysis with in vivo proteins on the surface of TiO2. To clarify the phenomenon of osseointegration, this study was a new trial to use in vivo proteins for proteome analysis of proteins adhered on TiO2.

To achieve this, chromatographic analysis was performed. Commercially available TiO2 particles were used as a carrier. Immobilized proteins were analyzed via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and liquid chromatography-tandem mass spectrometry (LC/MS/MS). The mineralization capacity of these proteins was also evaluated by field emission scanning electron microscopy with X-ray spectroscopy (SEM).

2. Materials and Methods

2.1. Preparation of Bone Powder

Pig calvaria were surgically extracted with a hammer and chisel from the skulls of 24 pigs (6-month-old) with in minutes after sacrifice at the Nambu Market (Nagoya City Central Wholesale Market, Nagoya, Japan). The calvaria were cut into small pieces (approximately 20 × 10 mm) and the tissue was powdered using a grinder (ASONE, Osaka, Japan).

2.2. Dissociative Extraction Procedure

The procedure for sequential extraction of bone proteins was performed at 4 °C as described previously [13]. A 50 g bone powder sample was taken from each density fraction. Two 48-h extractions, each at a volume:weight ratio of 50:1, were performed at 4 °C with a solution of 4 M guanidine hydrochloride (GuHCl), 50 mM Tris-HCl (pH 7.4) containing a protease inhibitor cocktail (1 mM AEBSF, 0.8 μM aprotinin, 50 μM bestatin, 15 μM E-64, 20 μM leupeptin, and 10 μM pepstatin (Calibiochem EMD Chemicals Inc., Gibbstown, NJ, USA)), and 1 mM 1,10-phenanthroline (Sigma-Aldrich, Tokyo, Japan). Insoluble material was pelleted by centrifugation and the supernatant was designated the first guanidine extract (G1S). The remaining insoluble bone powder was washed twice for 30 min in phosphate-buffered saline (PBS) at a volume: weight ratio of 200:1. The samples were then extracted twice with 0.5 M EDTA/50 mM Tris-HCl (pH 7.4) containing the same protease inhibitor cocktail as previously described, each at 100X volume, for 48 h. Insoluble material was pelleted by centrifugation and the supernatant was designated the EDTA extract (E-Sup). The PBS wash procedure was repeated and the tissue residues were again extracted twice at 4 °C with the guanidine extraction solution for 48 h at a volume: weight ratio of 50:1. Residual insoluble material was pelleted by centrifugation. Following centrifugation, the supernatant was designated the second guanidine extract (G2S). All extractions were then dialyzed exhaustively in distilled water at 4 °C.

2.3. Protein adherence on TiO2 and Elution using Chromatography

Before chromatographic analysis, it was confirmed that G1S, E-Sup, and G2S proteins were adhered on the TiO2 powder (particle size, 45 μm) (WAKO, Osaka, Japan) and were subsequently eluted with 0.2 M NaOH (Supplementary Fig. 1). A Bio-Scale MT10 Column (12 × 88 mm) (Bio-Rad, Tokyo, Japan) packed with TiO2 powder was used for chromatography. The packed columns were washed consecutively with water and 0.2 M NaOH to desorb surface contaminants. The columns were then pre-equilibrated with 0.01 M PBS. Chromatography was performed on an Agilent 1220 Infinity HPLC system (Agilent Technologies, Tokyo, Japan). Lyophilized protein samples were dissolved in running buffer (10 mg/mL), centrifuged at 5000 × g for 5 min, and filtered through a 0.45-μm syringe filter (Thermo Fisher Scientific K.K., Nagoya, Japan) to remove aggregates prior to injection into the column. Five milliliters of supernatant was injected into the TiO2 column at a flow rate of 0.02 mL/min to attain a 40 min retention time for adsorption and flow-through of non-adhesive proteins [14]. After 40 min, the flow rate was increased to 3.0 mL/min. PBS (10 mM) lacking Mg2+ and Ca2+ (pH 7.4), was used as the adsorption (running) medium. Elution was performed using gradients of 0.2 M NaOH and monitored at 254 nm.

2.4. SDS-PAGE

A 100 ng sample of each of the G1S, E-Sup, and G2S extracts was resuspended in 50 μL SDS sample buffer (0.125 mM Tris-HCl, 4% SDS, 10% sucrose, 0.01% bromophenol blue, and 10% 2-mercaptoethanol) and heated to 95 °C for 1 min. Proteins were separated in a Nihon Eidol system (Nihon Eido, Tokyo, Japan) using 12% Tris-Tricine gels, alongside a protein ladder (Spectra Multi-color Broad Range Protein Ladder, Thermo Fisher Scientific K.K.) to estimate molecular weights. Proteins were stained with Coomassie Brilliant Blue (CBB R-250) and 0.01% Stains-All (Sigma Chemical Co., St Louis, MO).

2.5. Protein Identification with Liquid Chromatography-tandem Mass Spectrometry (LC/MS/MS)

Proteins in the elution fractions were digested with trypsin (Promega KK, Tokyo, Japan) for 16 h at 37 °C after reduction, alkylation, demineralization, and concentration. Nanoelectrospray tandem mass analysis was performed using an LTQ Orbitrap XL mass spectrometry system (Thermo Fisher Scientific Inc., Wallingford, MA) combined with a Paradigm MS4 HPLC system (Michrom BioResources Inc., Auburn, CA). Samples were injected onto the Paradigm MS4 HPLC system equipped with an L-column2 ODS 0.1 mm in diameter and 150 mm in length (Chemical Evaluation and Research Institute, Japan). Reversed-phase chromatography was performed with a linear gradient (5% B at 0 min; 50% B after 100 min) of solvent A (2% acetonitrile with 0.1% formic acid) and solvent B (90% acetonitrile with 0.1% formic acid) at an estimated flow rate of 500 nL/min. Ionization was performed by an ADVANCE Spray Source (Michrom BioResources Inc., Auburn, CA) at 150 °C with a capillary voltage of 1.7 kV. A precursor ion scan was carried out using a mass to charge ratio (m/z) of 400–2000 prior to MS/MS analysis. Multiple MS/MS spectra were submitted to the Mascot program, version 2.4.1 (Matrix Science Inc., Boston, MA) for the MS/MS ion search.

2.6. Mineralization Capacity of Proteins Adhered on TiO2

About 100 μg each of G1S, E-Sup, and G2S proteins were dissolved in separate 100-μL aliquots of PBS, added to TiO2 particles, then incubated overnight at 37 °C. As a negative control, a sample of TiO2 particles was immersed in PBS alone. After incubation, TiO2 particles were rinsed three times with PBS, and 100 μL of 1 mM CaCl2 was added for another overnight incubation at 37 °C. Field emission scanning electron microscopy with X-ray spectroscopy (JEOL-JSM7610F SEM) was performed to confirm the presence of calcium deposits on the TiO2 particle surfaces for the G1S, E-Sup, G2S, and control groups. The amount of calcium deposition was measured with an Alizarin Red S (ARS) Staining Quantification assay (ScienCell Research Laboratories, Carlsbad, USA). Briefly, 40 nM ARS was added to the TiO2 particles for 30 min at 37 °C. After incubation, semiquantification of ARS in TiO2 particles was measured by acetic acid extraction and neutralization with ammonium hydroxide, followed by colorimetric detection at 405 nm.

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3. Results

3.1. TiO₂ chromatography

The results of TiO₂ chromatography of the G1S, E-Sup, and G2S proteins are shown in Fig. 1A–F. Most of the proteins injected into the column eluted in the flow-through fraction (Fig. 1A, C and E). However, several smaller peaks were detected after elution with 0.2 M NaOH (Fig. 1B, D and F). For the G1S proteins, only one peak was detected in the flow-through fraction (Fig. 1A), while 6 peaks were detected in the eluted fraction (Fig. 1B). For E-Sup proteins, 2 peaks were detected in the flow-through fraction (Fig. 1C) and 5 peaks were detected in the eluted fraction (Fig. 1D). For the G2S proteins, one peak was detected in the flow-through fraction (Fig. 1E) and 4 peaks were detected in the fraction (Fig. 1F).

3.2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Fig. 2A–F shows the 12% gel electrophoretic profiles of peaks from the TiO₂ chromatography performed on the G1S, E-Sup, and G2S proteins. Several bands were detected with CBB and Stains-All staining. Proteins with various molecular weights were detected in each experimental group. The expression pattern of each sample derived from the peaks was not significantly different between the three experimental groups.

3.3. Protein identification with liquid chromatography-tandem mass spectrometry (LC/MS/MS)

Using LC/MS/MS, the proteins from each fraction that adhered to TiO₂ were identified. From the 0.2 M NaOH elution peaks, 151, 116, and 43 proteins were detected from the G1S sample, E-Sup sample, and G2S sample, respectively. Table 1 shows representative proteins detected in the three experimental groups based on their Exponentially Modified Protein Abundance Index (emPAI) value.
Table 1
List of Proteins Identified on TiO2 Particles.

| # | Accession no | Protein | emPAI |
|---|-------------|---------|-------|
| 1 | P02007      | Hemoglobin subunit beta | 30.17 |
| 2 | P00835      | Serum albumin | 21.95 |
| 3 | P085571     | Serotransferrin | 21.67 |
| 4 | P01846      | Ig lambda chain | 14.46 |
| 5 | Q97788      | Fatty acid-binding protein, adipocyte | 5.55 |
| 6 | P50828      | Hemopexin | 4.26 |
| 7 | Q83575      | Haptoglobin | 4.21 |
| 8 | P01965      | Hemoglobin subunit alpha | 4.0 |
| 9 | Q29116      | Tenascin | 3.9 |
| 10| P28178      | Thrombospondin-1 | 3.59 |
| 11| P02465      | Collagen alpha-2(I) chain | 3.57 |
| 12| Q05443      | Lumican | 1.1 |
| 13| P14287      | Osteopontin | 1.33 |
| 14| Q28944      | Cathepsin L1 | 0.46 |

Table 1 (continued)

| # | Accession no | Protein | emPAI |
|---|-------------|---------|-------|
| 15| Q20835      | Protein disulfide-isomerase | 0.21 |
| 16| P01965      | Hemoglobin subunit alpha | 0.19 |
| 17| Q09959      | Prolargin | 0.18 |
| 18| P00039      | Interferon alpha-2 | 0.09 |
| 19| Q29156      | Platelet-derived growth factor-receptor-like protein | 0.07 |
| 20| Q64222      | Spermatogenesis-associated protein 1 | 0.06 |
| 21| Q83575      | Platelet-derived growth factor-receptor-like protein A4 | 0.05 |
| 22| Q60753      | Triosephosphate isomerase | 0.05 |
| 23| Q05116      | Thrombospondin-2 | 0.03 |

3.4. Mineralization assay

Fig. 3 shows the mineralization capacity of the proteins that adhered on TiO2 particles. TiO2 particles incubated with proteins from the G1S and G2S fractions displayed increased calcium deposition compared to TiO2 particles incubated with PBS or the E-Sup fraction (Fig. 3A). As measured by the Alizarin Red S Staining Quantification Assay, more calcium depositions were observed on TiO2 particles treated with G1S and G2S proteins in comparison to the PBS control group. Few calcium depositions were seen in the TiO2 particles treated with E-Sup protein (Fig. 3B).

4. Discussion

The specific bone-related proteins adhered on TiO2 are not completely understood. Previous reports on the bone-TiO2 interface rely on histological observations, but this method is not able to comprehensively identify the adherent biomolecules. In this report, native proteins were extracted from pig bone and subjected to TiO2 particle binding using chromatography. Adhered proteins were analyzed using SDS-PAGE and LC/MS/MS. This analysis revealed that the interface between bone and TiO2 existed as a sort of proteins, such as extracellular matrix, enzyme, and growth factor and some of the adhered proteins showed mineralization capability.

Evaluation of the proteins that adhered to TiO2 particles was performed with chromatography. In contrast to the current findings, a previous report analyzing TiO2 adhesive proteins using chromatography detected only one peak [15]. However, in that study, bovine metatarsal proteins were dissolved in 2 M urea, which denatures proteins and disrupts their conformation [16]. In the current study, extracted proteins were dissolved in PBS before analysis so that their conformation would be maintained closer to their actual in vivo state. Thus, it is possible that the buffer caused the differences in results between this previous report and the current study. Moreover, the presence of one peak in their study indicates that this protein fraction was adsorbed on TiO2 with a certain affinity at a neutral pH, and was released at an alkaline pH. The presence of multiple peaks during a linear gradient elution with 0.2 M NaOH buffer indicates that the eluted proteins have different retention times. This suggests that the analyzed bone proteins display differences in affinity for TiO2. At about 15% of 0.2 M NaOH (30 mM NaOH), the column was completely eluted. This is in accordance with results from a previous study that reported complete protein elution using 25 mM NaOH [14]. This suggests that the adherence strength between TiO2 and the bone proteins did not depend on protein conformation.

Furthermore, there was substantial overlap between the peaks found for each experimental group. At the same time, there were significant differences between the chromatograms of all three
experimental groups. This suggests that the type of chemical bond responsible for the adhesion of proteins on TiO$_2$ is not dependent on the protein. The gradient conditions of the 0.2 M NaOH elution buffer were changed in an attempt to improve peak resolution. However, complete separation of the peaks could not be obtained (data not shown). Further optimization of the TiO$_2$ carrier column is required to improve the separation of the protein peaks upon elution. In this study, the method of chromatography method was available to evaluate soluble proteins adhered to TiO$_2$. Evaluation of the insoluble proteins derived from bone could not be performed with chromatography, because samples to analysis with this chromatography system were limited to soluble molecules. Further research is required to address the effect of insoluble proteins adsorption.

SDS-PAGE and LC/MS/MS revealed the presence of a wide variety of proteins in each of the three experimental fractions. In the G1S fraction, the most abundant TiO$_2$ adhesive proteins were albumin and hemoglobin subunit beta, according to the current emPAI results. These serum proteins are known to adhere to TiO$_2$ [17] and are found in bone tissue upon extraction. However, previous studies reported that fibronectin was the most abundant TiO$_2$-adhesive protein in serum [12,18]. This discrepancy can be explained by the fact that small proteins such as albumin may be replaced by larger proteins such as fibronectin and fibrinogen during chromatography, due to an exchange process known as the Vroman effect [19]. Additionally, the protein samples analyzed in the two studies are completely different. While bone tissue and serum both contain fibronectin, the relative protein content is different, possibly affecting the degree of fibronectin adsorption on TiO$_2$.

Previous studies used TiO$_2$ disks to study protein adherence [20,21]. It is known that, besides structural selectivity, affinity of large biomolecules can also arise from physical surface properties including hydrophobicity, topology [21], and charge [20]. Our results indicate that these factors were different between TiO$_2$ particles and TiO$_2$ disks.

The ECM components detected, especially biglycan, glycosylated decorin, and chondroitin/dermatan sulfate, which contain sulfate groups, were negatively charged [22]. The keratin sulfate proteoglycans fibromodulin, lumican, and mimecan also contain sulfate groups [23]. A previous report showed that these small leucine-rich proteoglycans (SLRPs) can adhere to TiO$_2$ [24]. However, this report was based on in vitro gene expression analysis, which does not provide direct evidence for the adherence of SLRPs onto TiO$_2$. This is the first report that unequivocally demonstrates SLRPs adhering to TiO$_2$.

Thrombospondin-1 [25], SPARC [26], osteopontin [27], and tenascin [28] are glycoproteins with O- and N-linked glycosylation, but they have not yet been reported to have sulfate groups. Osteopontin was detected in the interface between TiO$_2$ and bone in
a previous histological analysis [29]. This indicates that sulfate groups are not necessary for TiO₂ adhesion. Furthermore, osteocalcin, periostin, and matrix gla protein were also detected. These proteins play a role in mineralization and do not have a carbohydrate chain. Osteocalcin was also previously detected in the bone-TiO₂ interface [29]. Our results are in agreement with these previous reports and suggest that these proteins, with the exception of proteoglycan, can be found in the interface between TiO₂ and bone.

It was previously suggested that GAGs bind TiO₂ via Ca²⁺ [6]. However, the proteins in this study had the capacity to bind TiO₂ in the absence of Ca²⁺ and Mg²⁺, as these ions were not present in the buffer. This inconsistency was previously reported by Ozawa and Kasugai (1996) who suggested that proteins from bone marrow stromal cells and osteoblasts could adhere on TiO₂ in the absence of these divalent cations [30]. Further research is required to address the effect of Ca²⁺ and Mg²⁺ on TiO₂ protein adsorption.

Proteins can have several binding sites, depending on their conformation and amino acid sequence. Some growth factors that were detected in this study have the ability to bind proteoglycans [22], and SLRPs are known to interact with collagen during collagen fibrillogenesis [31]. The protein samples used in this study were dissolved in 0.01 M PBS (pH 7.4) to mimic an in vivo environment, and it is therefore possible that the proteins detected in this study were bound to each other in a complex before adhering to TiO₂. Therefore, future investigations should analyze purified proteins of interest to evaluate whether they adhere to TiO₂ directly.

The mineralization capacity of the proteins experimentally found to adhere on TiO₂ was evaluated by SEM. In the G1S fraction, the most abundant protein was albumin. However, several studies have reported that the presence of albumin decreases the amount of calcium phosphate precipitate on a Ti surface [32]. The G1S fraction included not only albumin but also other proteins, and the findings suggest that one or several of these other proteins are involved in the formation of calcium deposits. However, the specific protein(s) in G1S that are associated with mineralization were not identified and further study is required to determine the players contributing to increased mineralization. The E-Sup fraction contained calcium-binding proteins such as osteocalcin, periostin, and matrix gla protein, however, we only found limited calcium deposition in this fraction. It is possible that the calcium-binding proteins directly adhere onto TiO₂, inhibiting the binding of calcium. The role of the interaction between calcium-binding proteins and TiO₂ in the mineralization process is unclear and requires further research. The most abundant protein in the G2S fraction was the SLRP decorin. SLRPs control the formation and orientation of collagen fibrils and contribute to mineralization [33]. A previous study showed that the GAG chain of decorin can bind calcium [33]; it is possible that decorin contributed to an increased formation of calcium deposits in comparison to the control.

Besides using histological analysis, osseointegration has also been studied by comparing the removal torque or the force necessary for pushing out implants placed in animals [13]. For obvious ethical concerns, these experimental methods are not appropriate to study osseointegration in clinical applications. In contrast to these invasive methods, which require the removal of the operated implants from patients, biochemical methods that detect a given protein profile related to osseointegration cause no harm to the bone tissue or implant. The current study represents an important step forward in the development of a biomarker of osseointegration for clinical application.

To conclude, native proteins were extracted from pig bone in this study. These proteins were subjected to TiO₂ chromatography and were identified with LC/MS/MS. We identified that the interface between bone and TiO₂ existed a sort of proteins, such as extracellular matrix, enzyme, and growth factor. Interestingly, except for previously detected proteins, the detected ECM proteins were not glycosylated. TiO₂ incubated with proteins from guanidine-extracted proteins displayed increased calcium depositions. Proteome analysis using TiO₂ chromatography is a useful tool for investigating which bone proteins adhere to TiO₂.

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Appendix A. Transparency document

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.07.007.

Appendix B. Supporting information

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