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Interaction between titanium and phosphoproteins revealed by chromatography column packed with titanium beads

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Abstract

The biochemical mechanism behind the strong binding between titanium and living bone has not been fully elucidated, in spite of worldwide clinical application of this phenomenon. We hypothesized that one of the core mechanisms may reside in the interaction between certain proteins in the host tissues and the implanted titanium. To verify the interaction between titanium and proteins, we chose the technique of chromatography in that titanium spherical beads (45 μm) were packed into a column to obtain a bed volume of 16 x 50 mm,

which was eluted with physiological saline (PBS) and a straight gradient system made by using PBS and 25 mM NaOH. Fetal calf serum, albumin, lysozyme, casein, phosvitin and dentin phosphoprotein (phosphophoryn) were applied to the column. Most part of albumin and lysozyme eluted with the breakthrough peak, indicating practically no affinity to titanium. Fetal bovine serum also eluted mostly as the breakthrough peak, but distinct retained peak was observed. On the other hand, α-casein, phosvitin and phosphophoryn exhibited a distinct retained peak separated from the breakthrough peak. We proposed that phosphate groups (phosphoserines) in the major phosphoproteins, α-casein, phosvitin and phosphophoryn may be involved in the binding of these proteins with titanium.

Introduction

Titanium is widely applied as a biomaterial in various orthopedic and dental fields, including dental implants and artificial joints, mainly due to its remarkable property to bind strongly to living bone. This unusual phenomenon was discovered more than 50 years ago and has been studied extensively in relation to many aspects of medical and dental therapies [1-4]. We propose that the biochemical interaction between titanium and protein is a primary event that occurs immediately on implantation of titanium. However the biochemical mechanism of the binding has still not been clarified and only a few systematic studies in this field have been reported. We considered that the first group of molecules which titanium encounters when it is implanted into bone tissues will be serum proteins and ECM (extracellular matrix) components. Therefore we thought it reasonable to clarify the interaction of titanium with serum proteins as the first step in our investigations. We decided to use technique of chromatography, which we have previously used successfully to study the interaction between carbon nanotubes and serum proteins [5].

2. Materials and Methods

2.1 Chromatography

Pure titanium beads with an average diameter 45 μm were obtained from a commercial company (Osaka Titanium Technologies, Co., Japan). Finer particles were carefully removed by repeated decantation from the suspension in distilled water. The beads were

packed into a commercial glass chromatography column (XK16/20, GE Health Care, Tokyo, Japan,) to obtain a bed volume of 16 x 50 mm. The column was eluted with Dulbecco's physiological saline (PBS) at a flow rate of 180 ml/hr using a ceramic pump (VSP-3200W, Eyela, Japan). One ml of fetal calf serum (Cansera International, Toronto, Canada) and various proteins (2.5 - 10 mg/1 ml of PBS) were applied to the column, which were first eluted with 60 ml of PBS, then with a straight gradient system made by using PBS and 25 mM NaOH, (100 ml of each). Elution was monitored by an automatic UV monitor system at 254 nm (AC 5100, Atto Co., Japan). The samples were selected from three groups of proteins, typical acidic and basic proteins, and phosphoproteins for analysis. We applied 10 mg of albumin (bovine serum albumin, Sigma-Aldrich, Tokyo, Japan), 5 mg of lysozyme (chicken egg white lysozyme, Sigma-Aldrich, Tokyo, Japan), 10 mg of phosvitin (chicken egg yolk, Sigma-Aldrich, Tokyo, Japan) and 10 mg of α-casein (bovine milk, Sigma-Aldrich, Tokyo, Japan), which were commercially obtained. Bovine phosphophoryn (dentin phosphoprotein) (2.5 mg), was prepared by the method previously reported [6]. Polyphosphate (30 mg) of average tri-phosphate size was obtained from Wako, Co., Japan.

2.2 Polyacrylamide Gel electrophoresis

SDS-PAGE gel electrophoretic profiles were obtained from bovine fetal serum, chromatography breakthrough fraction and the second peak, which were desalted by dialysis against distilled water at 5° C and lyophilized. Five μg of each sample was dissolved in SDS sample buffer and applied to a 7.5% polyacrylamide gel containing 0.1% SDS. A stained standard marker kit was purchased from APRO Co. (Naruto, Japan). The gel was stained with 0.25% Coomassie Brilliant Blue G250.

3. Results

3.1 Adsorption of proteins on titanium column

Result of titanium chromatography of serum protein is shown in Fig. 1A, in that most of the serum proteins applied to the column eluted at the breakthrough fraction, indicating that they were not adsorbed to the titanium. However, a small second peak was observed after the gradient was elevated to about 4 mM NaOH, which indicates that this protein fraction adsorbed to titanium with a certain affinity.

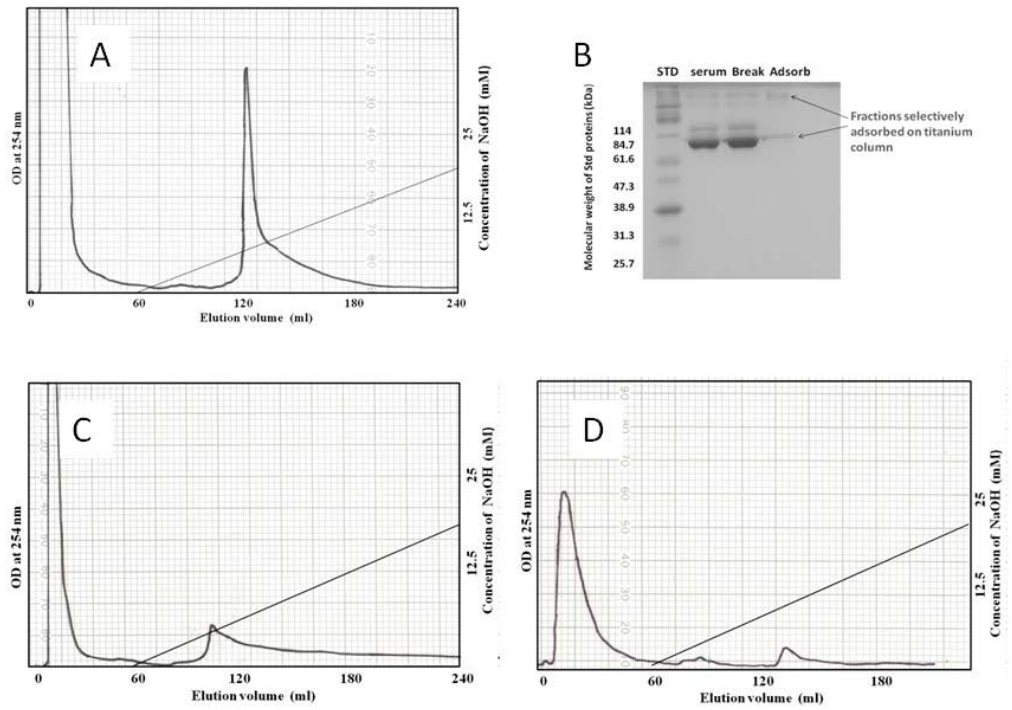


Figure 1

Comparative titanium chromatograms of fetal calf serum (A), and authentic proteins of albumin (C) and lysozyme (D) which are not regarded to be phosphoproteins, together with electrophoretic profile of eluted fractions of chromatography of the serum (B). These three proteins eluted mostly with the breakthrough fraction and showed minimum retained peaks. SDS gel electrophoretic pattern of fatal calf serum, the breakthrough fraction (break) and the second chromatography peak (adsorb). STD was the commercial mixture of standard molecular weight marker proteins.

Bovine serum albumin (Fig. 1C) and lysozyme (Fig. 1D) eluted mostly with the breakthrough fraction and showed minimum retained peaks. In contrast, phosphoproteins retained in the column and eluted later at approximately 5-10 mM NaOH. Alfa-casein (Fig. 2A), phosvitin (Fig. 2B) and phosphophoryn (Fig. 2C) showed large retained peaks, although there were certain breakthrough fractions. Poly-phosphate with average size of

tri-phosphate was eluted as a control (Fig. 2D), which also retained in the column.

3.2 Characterization of the titanium binding proteins in serum

As shown in Fig. 1B, the electrophoretic analysis of the second peak from the chromatography of the serum (Fig 1A) showed 2-3 bands in the profile, which were not detected in the pattern of total serum protein or the breakthrough fraction. A major band had an apparent molecular weight of less than 60 kDa in the electrophoretic gel. Further characterization of this protein is being done by mass spectrum and other tools.

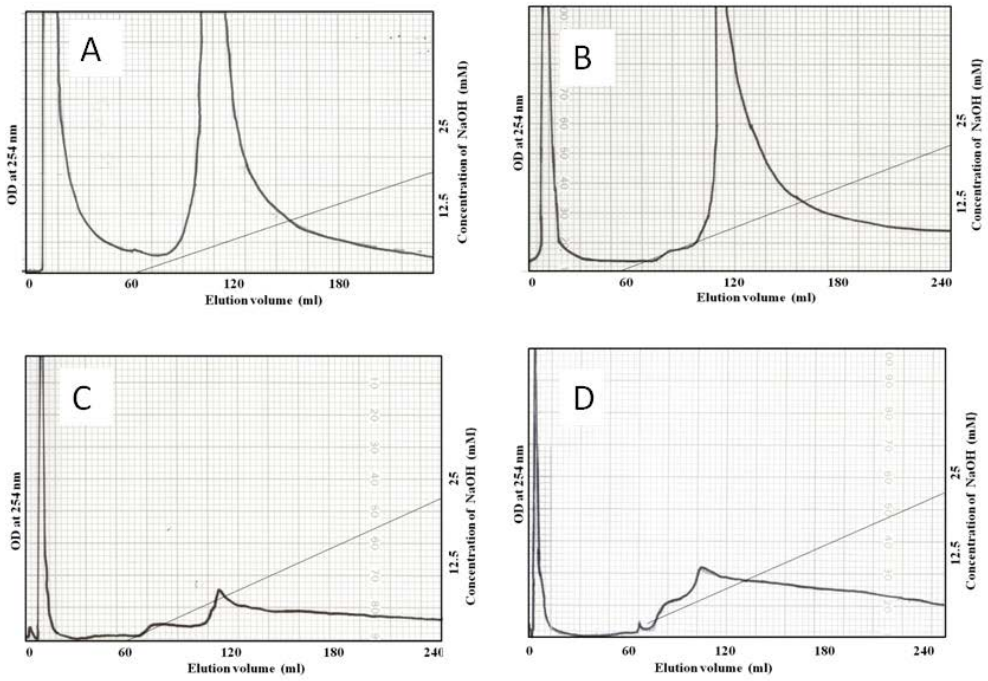


Figure 2

Comparative titanium chromatograms of various phosphoproteins

α-casein (A), phosvitin (B), phosphophoryn (C), and poly-phosphate (D) as a control eluted at breakthrough fraction to a certain extent, but larger retained peaks appeared at the beginning of the gradient, at approximately 5-10 mM NaOH.

4. Discussion

Since a typical acidic protein, albumin and also a typical basic protein lysozyme were not retained on this column (Fig. 1C and D), it is difficult to attribute the interaction of proteins with titanium to positive or negative charges. Rather we would like to suggest that phosphorylated groups (phosphoserine) in the α-casein, phosvitin and phosphophoryn are responsible for the interactions with titanium. Bovine α-casein (essentially α-S1) which is a typical phosphoprotein has 8 phosphoserines out of 214 amino acids [7-8]. Phosvitin [9] and phosphophoryn [10], both are highly phosphorylated proteins, showed much larger retained peaks, compared with those of breakthrough fractions. It was assumed that the breakthrough fractions of the phosphoproteins may be de-phosphorylated forms of the same proteins, possibility of which needs further verification.

Recently it was reported that mesoporous silica modified by addition of titanium phosphonate groups could selectively captured phosphoproteins in peptides [11]. The affinity of phosphate groups in peptides for titanium compounds has attracted an increasing attention in the fields of detection and analysis of protein phosphorylation [12, 13]. These studies did not mention in details on the affinity of phosphoproteins as whole molecules, but their studies strongly support our hypothesis that the retention of proteins in the titanium column of this study was caused by phosphorylated amino acids in the proteins. To our knowledge, there has been little systematic study of various proteins, not peptides by using titanium beads chromatography. One potential of the titanium beads chromatography is the initial separation of phosphorylated proteins from non- or less phosphorylated proteins, which will be useful for further analysis or diagnostic purpose. For instance, a small retained peak in the chromatogram of Fig 1C has possibility of being phosphorylated bovine serum albumin, which was recently identified its presence by a CdSe/ZnS quantum dots based electrochemical immunoassay [14].

It should be noted that casein is a phosphoprotein which forms a calcium-phosphate complex in milk due to calcium binding to its phosphorylated amino acids. In bone there are many phosphorylated proteins. In addition to phosphophoryn, if a group of bone phosphoproteins are shown to bind with titanium surface, they may be related to early mineralization of the bone-titanium interface when this metal is implanted into bone. Further verification of this hypothesis is currently being carried out in our laboratory.

5. Conclusion

This paper clearly showed that α–casein, phosvitin and phosphophoryn, which are typical phosphoproteins have binding abilities with titanium under physiological conditions, while the other proteins examined have not. By this chromatography with titanium beads, it is possible that many other matrix proteins in bone are examined for their titanium binding ability. Such finding may open a new area of interest in titanium applications for bone reconstruction therapy.

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