Journal of Hard Tissue Biology 29[1] (2020) 45-48

CODEN-JHTBFF, ISSN 1341-7649

Original

Discovery of Ti-Binding Abilities of Phosphorylated-Chitin and -Collagen

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(Accepted for publication, January 23, 2020)

Abstract: Previously we have discovered that titanium (Ti) binds with bone phosphoproteins SIBLING protein family, by using a Ti beads chromatography. Furthermore, we showed that the isolated bone phosphoproteins remarkably enhanced bone formation when we coated the Ti device with them and implanted into rat calvaria. Therefore, we have called the Ti-binding bone phosphoproteins as "the implant proteins." This discovery encouraged us to create a new biomolecule that can simulate the functions of the implant proteins. Since significant characteristics of the implant proteins are the presence of multiple phosphate groups and the occurrence of single cell-adhering RGD sequence, we decided for the first place to phosphorylate chitin and collagen to see whether they acquire or increase Ti-binding ability. Results showed that more than 70% of phosphorylated chitin bound with Ti, and phosphorylated collagen enhanced about 7% of its Ti-binding ability. These modified biomolecules, P-chitin and P-collagen will become highly useful for new development of Ti-related bone regenerative medicine.

Key words: Titanium (Ti), Binding with Ti, Phosphorylation, Chitin, Collagen

Introduction

Brånemark[1,2)] accidentally discovered the phenomenon of the strong affinity between Ti and living bone and the discovery is now applied worldwide in clinical applications in dental and orthopedic fields[3-5)]. However, the biochemical mechanism behind this unique phenomenon has been unsolved until we had discovered that phosphoproteins in bone bind with Ti and induced remarkable bone formation when we coated the Ti device with these proteins and implanted into rat bone. From these results, we concluded that the bone phosphoproteins (SIBLINGs) play core biochemical mechanisms of the strong bond between the living bone and Ti. Thus, we proposed to call the bone phosphoproteins (SIBLING) as "implant proteins".

The name of SIBLING is an acronym for small integrin-binding ligand, N-linked glycoprotein, a group of the extracellular non-collagenous matrix of bone and dentin, which plays key roles in the mineralization of these tissues[6,7)]. This category of the proteins includes: 1) osteopontin (OPN), 2) bone sialoprotein (BSP), 3) dentin matrix protein 1 (DMP1), 4) dentin sialophosphoprotein (DSPP) and 5) matrix extracellular phosphoglycoprotein (MEPE)[6,7)]. Among them, we consider BSP and DMP1 to be the two most important members in terms of bone enhancing functions and quantities in bone tissue[5)], which suggests future fruitful clinical applications. On the other hand, one difficulty is the purification of the implant protein on a large scale, sufficient for therapeutic purposes. Considering the above situations, we decided to create a synthetic molecule which simulate the function of implant proteins, namely SIBLINGs. There are at least two unique common properties in SIBLINGs. One is the presence of numerous serine- or threonine-phosphate residues through which SIBLINGs bind itself on the surface of implanted Ti and the other is the presence of a cell-adhering amino acid sequence (Arg-Gly-Asp), which may recruit osteoblasts. Both properties may accelerate rapid bone formation on the surface of Ti, which eventually creates a strong bond between Ti and living bone[3-5)]. To create such a new synthetic molecule which equips above two functions, we focused attention on chitin and collagen, two major extracellular matrices in the animal kingdom, and attempted to provide the Ti-binding ability by phosphorylating chitin and collagen, since we already know that Ti-binding function of various phosphoproteins attributes to their multiple phosphate groups of serine or threonine residues. In the present study, we found that the phosphorylated chitin (P-chitin) has the remarkable, Ti-biding ability, and the phosphorylated collagen (P-collagen) significantly increased its Ti-biding ability.

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Materials and Methods

Chemicals

Fujifilm-Wako Co., Japan, provided Chitin. Koken Co., Japan provided Type I acid-soluble collagens, and pepsin treated collagen. We prepared insoluble bovine skin collagen by the method reported previously[8,9)].

Phosphorylation of chitin and collagen

As a method of phosphorylation of chitin and collagen, we used

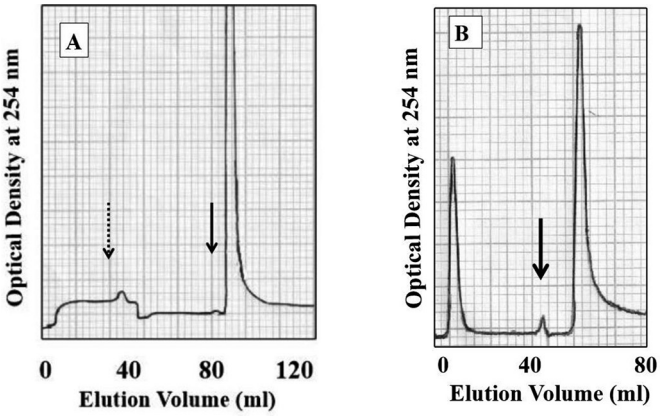
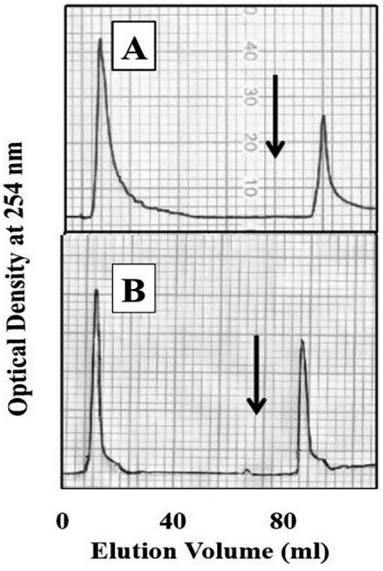


Figure 1. Ti beads chromatography of phosphorylated chitin (P-chitin) immediately after dialysis (A) and after lyophilization (B). In Fig. 1A, the dialyzed solution of P-chitin (approximately 20 mg in 40 ml of distilled water) was first eluted with distilled water. After 32 ml of distilled water was eluted, PBS was started to elute and after 80 ml, 25 mM NaOH was eluted. The dotted arrow indicates the starting point of PBS elution, and solid arrow indicates 25 mM NaOH. The pass-through and the PBS-eluted fraction (50 ml) complied with about 30 % (the fractions without Ti-affinity), and the NaOH eluted fraction complied with about 70% (Ti-binding fraction) of the total charged P-chitin. In Fig.1B, the lyophilized P-chitin samples of approximately 10 mg in diluted HCl (pH 4) was applied on the Ti column and first eluted with HCl. After the fraction without Ti-binding ability (51%) eluted, 25 mM NaOH washed out the remaining Ti-binding fraction (49%).



Figures 2. Ti-chromatography of the bovine skin collagen before (A) and after heat phosphorylation (B). In the intact bovine skin collagen, the ratio of Ti-bound fraction per total charged collagen was 31.4% (A), which increased to 38.6% (B) after phosphorylation. Solid arrows indicate starting point of 25 mM NaOH elution.

phosphorus pentoxide (P_2O_5). We dissolved P_2O_5 in dimethyl formamide (DMF), and added methane sulfonic acid as a catalyst[10,11]. Two gram of chitin powders were mixed in 40 ml of DMF, in which 4.5 g of P_2O_5 and 1.5 ml of methane sulfonic acid were dissolved beforehand. The molar ratio of phosphorous pentoxide against acetyl glucosamine was about 3:1. Stirring the mixture continued for 6 h at 70 ~ 80°C. The reaction was stopped by the addition of acetone to obtain a final concentration of 60 vol %. The reaction product was collected by filtration and rinsed by acetone several times and air-dried. We dissolved the dried product in 50 ml of distilled water followed by dialysis for 48 h against distilled water in a refrigerator at 10°C and lyophilized.

For the collagen samples, we suspended insoluble bovine skin collagen (50-100 mg) or acid-soluble bovine skin collagen (30~60 mg) in the solutions, which was composed of 30 ml of dimethyl sulfoxide (DMSO), 30 ml of 0.5 M K_2HPO_4, 1.5 ml of methane sulfonic acid, and 0.35 g of P_2O_5, which was adjusted as pH 4. The reaction mixture was stirred for 6 h at 20-27°C at constant pH 4. The reaction was stopped and precipitated by the addition of acetone to obtain a final concentration of 60 vol % at ice-cold (5°C). The precipitate was dialyzed extensively against distilled water for 72 h in a refrigerator at 10°C and lyophilized.

Quantitative analysis of the degree of phosphorylation

Analysis of phosphorus in the phosphorylated chitin and collagen was made by molybdenum blue method developed initially by Fiske and Sabbarow[12] after hydrolyzing the samples. Phosphorylated chitin or collagen (5-10 mg) each were suspended in a mixture of 1.5 ml of perchloric acid and 0.5 ml of sulfuric acid in Kjeldahl flasks, which were set on the micro Kjeldahl distillation apparatus, (Model ME-6, 6-unit, electric heating, Shibata Science, Tokyo, Japan). We heated them under a constant gentle boiling until the contents became transparent solution, which takes 30-50 min. The hydrolyzed samples were diluted quantitatively by distilled water for further analysis. We mixed aliquot of the sample solutions (3 ml) and 0.5 ml of the ammonium molybdate reagent and then 0.5 ml of ascorbic acid reagent (1 g of ascorbic acid in 5ml of distilled water). The ammonium molybdate reagent contained 0.5 g of hexa-ammonium hepta-molybdatetetrahydrate: $(NH_4)_6Mo_7O_{24}4H_2O$, 20 mg of potassium antimonyl tartrate and 14 ml of 4.5 M sulfuric acid mixed to obtain a volume of 20 ml, The absorption values determined the phosphorus contents at 720 nm, referring to a standard curve made by KH_2PO_4 as an authentic sample.

Analysis of binding ability with Ti of the P-chitin and P-collagen

To analyze the binding ability of materials with Ti, we chose the chromatography method using the column packed with Ti beads, which we have reported previously[3-5]. Briefly, a commercial supplier (Osaka Ti Technologies, Co., Japan) provided pure Ti beads with an average diameter of 45 μm. We carefully removed finer particles by repeated decantation from the suspension in distilled water. We packed the Ti-beads into a commercial glass chromatography column (XK16/20, GE Health Care, Tokyo, Japan) to obtain a bed volume of 16 x 50 mm. We quilibrated the column with diluted HCl at pH four or phosphate-buffered saline (PBS). A flow rate was of 160 ml/h using a ceramic pump (VSP-3200W, Eyela, Japan) at 15°C, and monitored at 254 nm by ATTO Bio-Mini-UV monitor (Atto Co., Japan). After applying a phosphorylated chitin or collagen (10 mg/3 ml), the column was eluted first by diluted HCl at pH four or PBS. The initial group of peaks indicates the fractions without Ti-binding ability, which appeared around the brake-through elution point. After these peaks appeared and the baseline recovered, the 25 mM NaOH was eluted to wash out all of the remaining sample, which indicates the Ti-binding fraction. We previously proved that the 25 mM NaOH elutes all of the chitin-related substances from the Ti-column. Peak areas in the chromatogram were measured using Image-J.

Table 1. The percentage of the Ti-binding fraction to the total samples

Ti-binding fraction / Total samples (%)	
Phosphorylated-chitin	69.2 ± 2.2
Acid soluble collagen	31.4 ± 2.1 *
Phosphorylated collagen	38.6 ± 2.4 *

The mean ± SD (n=3) , * $p<0.05$

Percentage of Ti-binding fraction of the phosphorylated collagen was significantly higher than that of the intact acid-soluble collagen, by using Student's t-test. A p-value of <0.05 was considered to be statistically significant.

Results

Evidence of Ti-binding ability of phosphorylated chitin

The most important result of this study is the discovery of the Ti-binding ability of P-chitin and increased Ti-binding capacity of P-collagen (Figs. 1 and 2, Table 1). Fig. 1 is an example of Ti-chromatogram of the water-soluble P-chitin (20 mg /40 ml). We charged the sample on the column immediately after dialysis without the lyophilization process.

In Fig. 1A, we can see a low plateau peak which appeared from the breakthrough point (6 ml of elution) and continued to 45 ml of elution, where the plateau ended. On this plateau peak, a tiny peak appeared, soon after the change of the elution from distilled water into PBS (indicated by dotted arrow). This small fraction was retained on the column under the water environment but released under the PBS environment, having a relatively weaker affinity to the Ti-column. The rest of the charged sample eluted out with 25 mM NaOH at 80 ml (indicated by solid arrow). This second peak indicates the Ti-binding fraction, which complied approximately 70% of the total charged amount of the sample. The first group of peaks with no or weaker affinity to Ti met 30%.

Fig. 1B shows typical Ti-chromatographic profile of the phosphorylated chitin sample after lyophilization, which demonstrated a definitely large amount of the Ti- binding fraction (peak eluted around 60 ml). The average ratio of the Ti-binding fraction to the total P-chitin was 69.2 ± 2.2% (Table 1).

Increase of Ti-binding ability of collagen by phosphorylation

Figs. 2A and B show the Ti-beads chromatographic patterns of collagen before (2A) and after phosphorylation (2B). We found that the ratio of Ti-bound fraction per total charged collagen was about 31.4 ± 2.1%, which increased to 38.6 ± 2.4% after phosphorylation (Table 1).

Efficiency of phosphorylation

Results of phosphorus content analysis of P-chitin indicated that average 1.28 mole of phosphorus per one residue of N-acetyl glucosamine was detected. And in the P-collagen, an average 1.36 mole phosphorus per 1 mole of collagen molecules were detected, provided a molecular weight of collagen is 300,000.

Discussion

Several laboratories including ours[10,11,13-17] reported chemical attempts to phosphorylate chitin and chitosan, but none of the researchers have tried and demonstrated the Ti-binding ability of P-chitin. In this study, we were able to show for the first time the Ti-binding ability of P-chitin, and increase of the Ti-binding ability of P-collagen. We have already shown that the phosphorylated proteins including SIBLINGs bind with Ti[3-5]. In this study, for the first time, we demonstrated that not only protein but also a sort of carbohydrate derivative, chitin binds with Ti, after phosphorylation.

Fig. 1A showed the first Ti-chromatography of P-chitin, which was dialyzed against distilled water after the reaction and directly charged on the column. This result indicates the first definite evidence that the phosphorylated chitin bound with the Ti-column and retarded in the Ti-chromatography. About 70% of the charged sample retained in the Ti-column and eluted only after 25 mM NaOH elution (indicated by solid arrow in Fig. 1A). The reason why we avoid lyophilization is that this process apt to render the sample partially insoluble. When we analyze the lyophilized samples of P-chitin, in the routine works at the later works, about Ti-binding fraction was about 70% of the total charged P-chitin (Table 1). These Ti-binding ratios of p-chitin are remarkably high. Because we know that even the typical phosphoproteins, such as casein and phosvitin, exhibited that Ti-binding ratios was about 70~75% of the total proteins charged on the Ti-column[3].

Since chitin is highly insoluble in common solvents, our group[10,11] and Yokogawa et al.[13,14] have attempted various methods to increase the efficacy of phosphorylation, including a high concentration of urea in the solvent and high temperature of 110-155°C[13,14], which might inevitably result in some degradation of the substrate. After the numerous trials, we concluded that non-proton solvent such as DMSO, DMF, and hexane are suitable for chitin phosphorylation[10,11]. One of the advantages of non-proton solvents such as DMSO, DMF and hexane as the solvents for chitin seems to give homogenous emulsification in the reaction mixture, which probably due to higher affinity of hexane with chitin molecule.

Our goal was to create a substitute for the implant protein, SIBLING, which can bind with the Ti device and accelerate bone formation on the surface of Ti devices. We have designed two steps: one was the creation of molecules that binds with Ti, and the second step was to provide the substance with functions to induce bone formation on the surface of Ti-device. The first step was achieved in this study by P-chitin, which bound with the Ti device. Now, the second step is to show the P-chitin coated Ti device for bone formation. For such a Ti-device, we have already developed an ideal three-dimensional porous Ti-device called the titanium web (TW)[18-22].

Recently, however, Wang et al.[15] reported that a soluble P-chitin itself in culture media expressed a particularly positive effect on osteoblast growth and differentiation. This work encourages us to test the P-chitin coated TW for bone formation *in vivo* and *in vitro*[17-18]. Using the TW as the insoluble scaffold, we have obtained a highly favorable results in bone formation *in vitro*[18]. Because the immobilizing the SIBLING proteins upon Ti device was the central idea of our implant protein hypothesis, the P-chitin coated TW, and soluble P-chitin in culture media are fundamentally different functions for bone formation. Our latest studies demonstrated that the implantation of the P-chitin coated TW into rat calvaria remarkable increase of bone formation[17-18].

We have already shown that phosphoprotein such as casein, phosvitin, and SIBLING bound with Ti-beads column[2]. Therefore, we can describe empirically phosphoproteins bind with the Ti beads column, in general, more or less. However, the only exceptional protein was collagen, which generally is not classified to be phosphoprotein[16]. The present study showed that 31.4% of natural collagen bound with Ti (Fig. 2A, Table1). After phosphorylation, the ratio of Ti-bound fraction per total charged collagen increased to 38.6% (Fig. 2B, Table 1). According to Qui et al.[16], phosphorylation of collagen might increase structural stability. Thus, p-collagen will be another potential material for Ti-related bone reconstruction.

In conclusion, the most important demonstration of this study may

be the Ti-binding ability of P-chitin and the increased Ti-binding capacity of P-collagen. Chitin is the most abundant exoskeletal matrix molecule of carbohydrate, and collagen is the most abundant protein in the animal kingdom. Once, we can utilize P-chitin coated Ti devices, and accelerate the binding between living bone and Ti, it will be an excellent contribution for the needy patients, who want to rapid fixation of Ti-device in dental implants and orthopedic reconstruction of bone and cartilage.

Acknowledgments

Authors appreciate valuable advice given by Dr. Arthur Veis, Professor Emeritus, North Western University, USA. This study was supported in part by a Grant-in-Aid for Challenging Exploratory Research (23659907) and a Grant-in-Aid for Research (C) (15K05554) from the Ministration of Education, Culture, Sports, and Technology in Japan.

Conflict of Interest

The authors have declared that no COI exists.

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