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ORIGINAL ARTICLE

Biochemical Mechanism of Titanium Fixation into Living Bone: Acid Soluble Phosphoproteins in Bone Binds with Titanium and Induced Endochondral Ossification *in vivo*

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SYNOPSIS
In 2014, we discovered that bone phosphoproteins, which are collectively called SIBULING family of proteins, are equipped with titanium-binding ability. Furthermore, the titanium implant devices which were coated with titanium-bound SIBLING induced more than 100 times faster bone formation of early stage when implanted into rat calvaria. These findings led us to an explanation why titanium implants could be fixed into living bone. Several other phosphoproteins including, phosvitin, caseins and phosphophoryn (a dentin phosphoprotein) were also found to bind with titanium by use of a chromatographic column packed with titanium beads. In this study we demonstrated that a typical phosphoprotein, phosvitin lost its titanium-binding ability in a time-dependent manner by the reaction with λ-protein phosphatase. The fact confirmed that certain specific phosphoserines λresidues in this protein were responsible for the titanium-protein interaction. For an additional confirmation of SIBLING-titanium interaction, we extracted bone and dentin proteins with a new and simple method of acidic condition and applied them to the chromatographic column packed with titanium beads. The results showed that definite portions of the acid soluble proteins from both bone and dentin were retained in the column. Electrophoretic analysis showed the retained fractions were Stains-all positive, indicating that both bone and dentin contain multiple phosphoproteins which have affinity with titanium. The titanium-bound fraction of acid extract was again coated on the titanium device and implanted into rat calvaria. After one week, histology showed that in addition to definite pattern of bone formation, process of endochondral ossification was clearly observed. In the control implant of uncoated titanium device, only collagenous tissues were observed, without any cartilage nor bone formation. Based upon these findings we reconfirmed that the core biochemical mechanisms underlying the strong bond between the titanium and living bone is based upon the interaction between the implanted titanium and multiple bone phosphoproteins in the host tissue.

***Key words**: Titanium implants, titanium-binding proteins, titanium-chromatography, phosphoproteins, phosvitin, bone phosphoproteins*

Introduction

The phenomenon of the strong affinity between titanium and living bone was accidentally discovered by Brånemark [1-2] and is now applied worldwide in clinical applications in dental and orthopedic fields[3-5]. However, the biochemical mechanism behind this unique phenomenon has not been fully elucidated. We hypothesized that the core biochemical mechanisms may reside in the interaction between certain proteins in the host tissues and the implanted titanium. In previous studies to confirm the interaction between titanium and proteins, we chose the technique of chromatography using spherical beads of titanium packed into a column. We showed that whilst most of the albumin and lysozyme eluted with the breakthrough peak, indicating practically no affinity for titanium, on the other hand, α-casein, phosvitin, phosphophoryn and bone phosphoproteins, which are collectively called SIBLING family proteins exhibited a distinct retained peak separate from the breakthrough peak [6-7]. When we coated the titanium implant devices, a titanium web (TW) (Zellez, Hi-Lex-Funakoshi, Japan) with the titanium-bound SIBLING fractions and implanted them into rat calvaria, it was found that more than 100 times faster bone formation occurred within a week after implantation [7].

From these findings, we concluded that phosphate groups (mainly those of phospho-serine) in the phosphoproteins is the key factor in the binding of these proteins with titanium. Furthermore, bone phosphoproteins (SIBLINGS) are well known to contain a cell adhering amino acid sequence (Arg-Gly-Asp) that recruits osteoblast precursors. Together with the potent hydroxyapatite inducing ability of numerous phosphate groups, SIBLING proteins immobilized on the surface of implanted titanium may induce rapid bone formation there, which eventually create a strong bond between titanium and living bone.

To confirm above theory, in this study we investigated whether the Ti-binding SIBLING proteins are in a relatively free soluble state or in a bound state with insoluble matrix, as shown in our previous report [7]. The acid soluble proteins in bovine bone and dentin were extracted by a new and simple method using 0.01 M HCl (decalcifying solution). It was found that a small fraction of acid extracted proteins bound to the titanium in column chromatography. SDS polyacrylamide gel electrophoresis (PAGE) analysis of the titanium-binding fractions from both bone and dentin indicated that they contained phosphoproteins judging from their staining behavior with Stains-all [8]. Furthermore, the acid soluble titanium-binding proteins (AS-TiBP) from bone showed bone enhancing function, when implanted with TW into rat calvaria. Different from previous report, histology showed that in addition to definite bone formation, local endochondral ossification was clearly observed. In the control implants of uncoated titanium device, only collagenous tissues were observed, without any cartilage nor bone formation.

2. Materials and Methods

1) Extraction of extracellular matrix proteins from bovine bone and dentin

Total soluble components of bone and dentin matrix were extracted by a modified method reported previously[8-10]. Briefly, fresh bovine metatarsal bones were cleaned of soft tissues and pulverized in liquid nitrogen to obtain powders, with a particle size smaller than the opening of a 60-mesh sieve[8, 9]. Bovine dentin powders were prepared as described[10]. Both bone and dentin powders were decalcified with 0.01 M HCl, maintained at pH 2 by continuous addition of 6 M HCl at 5ºC, and decalci-

fied and extracted in the same at pH 2. The acid extracts from bone and dentin were immediately desalted by dialysis against distilled water at 5ºC until the pH of the aliquot reached 5, and then lyophilized. The acid extracts and the samples eluted following titanium chromatography were analyzed by SDS-PAGE electrophoresis.

2) De-phosphorylation of phosvitin by λ-protein phosphatase

Ten milligrams each of phosvitin (chicken egg yolk, Sigma-Aldrich, Tokyo, Japan) were incubated with 4,000 units of λ-protein phosphatase (Cell Signaling Technology, Tokyo, Japan) in 0.05M Tris-HCl/1M NaCl/2mM MnCl2 at 30 ºC, for 2.5 and 6 h.

3) Titanium-beads chromatography

Method for titanium-beads chromatograhy was reports previously[6,7]. Briefly, pure titanium beads with an average diameter 45 µm were obtained from a commercial supplier (Osaka Titanium Technologies, Hyogo, 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 2 M urea in Dulbecco's physiological buffered saline (PBS) at a flow rate of 120 ml/h using a peristaltic pump (SJ-1211, Atto, Tokyo, Japan). Samples for analysis dissolved in 2 M urea/PBS were applied to the column, which were first eluted with 60 ml of 2 M urea in PBS, then with a straight gradient system made by using 2 M urea/PBS and 25 mM NaOH/2 M urea (100 mL of each). Elution was monitored by spectroscopy using an automatic UV monitor system at 254 nm (AC 5100, Atto). Samples applied to the column were: 10 mg of phosvitin (chicken egg yolk, Sigma-Aldrich), 10 mg of the same protein which had been treated with λ-protein phosphatase, and 40 mg each of the lyophilized acid extracts from bovine bone and bone.

4) SDS polyacrylamide gel electrophoresis (PAGE)

Ninety micrograms of each sample were dissolved in SDS sample buffer and applied to a 15% polyacrylamide gel containing 0.1% SDS or a 5-20% pre-cast gradient gel (Atto). Pre-stained standard molecular weight markers (Odyssey, Protein Molecular Weight Marker, Li-Cor, NE, USA) and a standard pepsin-treated type I collagen from bovine skin (I-PC, Koken Co., Tokyo, Japan) were also applied on the gel. The electrophoresed gel was stained with 0.0025% Stains-all[11], 0.25% Coomassie Brilliant Blue (CBB) R-250 (Takara Bio Co., Ltd., Shiga, Japan). For Western blotting of the gel was done using anti-N-terminal (90-111) of DMP1 antibody (Takara Bio).

5) Implantation into rat calvaria

Discs form of (2 x 3 mm) of titanium webs (TW), (Zellez®, Funakoshi, Japan) were immersed in a solution of acid soluble titanium-binding proteins (AS-TiBP) in PBS (1 mg/500 mL). The TW/ AS-TiBP composites were air dried in a clean room and store at 5ºC. Eight-week-old male rats (Wistar, 200-230) were anesthetized with pentobarbital solution (3.6 mg body weight, Nembutal R, Dainippon Sugimitomo Pharma, Osaka, Japan). The TW/AC-TiBP composite were implanted into the holes of calvaria created by diamond bur and trepan bur. After implantation, periostea and skin were carefully placed back and sutured. The protocol of the animal experiments was approved by the Committee for Animal Experiments in Kanagawa Dental College. It was carried out in accordance with guide-

lines proposed by the Institutional Animal Care and Use Committee. One piece of either the TW/AC-TiBP composite or TW alone was implanted into one rat calvaria. After one week and one month, the samples were removed for examination as described previously[4,7].

6) Histological observation

Excised pieces of cranial bone, which contained implant, were fixed in 10% neutral formaldehyde, embedded in polyester resin (Rigolac, Showa Denko, Tokyo, Japan), cut and polished into 80 μm section by Maruto Systems (Rigolac). Sections were stained with Cole's hematoxylin and eosin and analyzed histologically [4, 7].

RESULTS

1) Effect of de-Phosphorylation of phosvitin

Figure 1 shows titanium-beads chromatography of intact phosvitin (10 mg) and the same amount of the proteins which were de-phosphorylated by λ-phosphatase for 2.5 h and 6 h. It was clearly shown that the second peaks which had been retained in the titanium column were decreased as the reaction periods were increased, while the breakthrough fractions which were not bound to the titanium beads under this chromatographic condition increased as shown by thinner lines in the chromatogram.

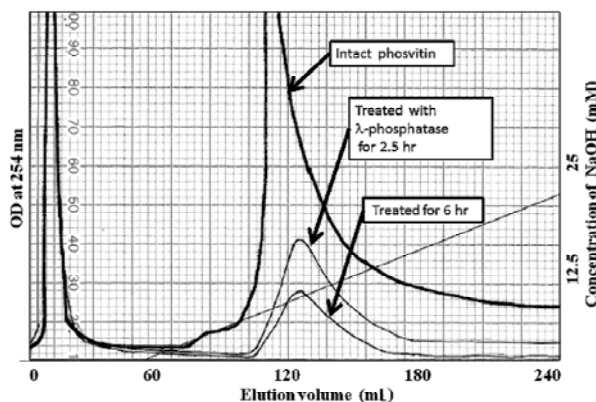


Figure 1 Comparative titanium chromatograms of 10 mg of phosvitin (a), the same protein treated with λ-protein phosphatase for 2 h (b) and 6 h(c).

2) Binding of bone and dentin proteins on titanium column

Results of titanium chromatography of the bone extracts, SDS PAGE profiles their fractions stained with Stains-all and the same profiles stained with CBB are shown in figures 2A, 2B and 2C, respectively. Most of the acid soluble proteins from bone applied to the column eluted at the breakthrough fraction, indicating that they were not adsorbed to the titanium. However, small secondary peaks were observed after the gradient was elevated to about 10 mM NaOH with the proteins from both tissues, which indicates that these protein fraction adsorbed to titanium with a certain affinity.

The SDS PAGE profile of the crude extracts of bone which was stained with Stains-all showed a broad band around 50-60 KDa (lane 2 in figure 2B). The breakthrough fraction (lane 3 in figure 2B) similarly showed broad bands ranging from 10 to 70 kDa. But in the titanium-binding fraction (lane 3 in figure 2B), two faint bands which were stained blue by Stains-all of about 40 to 70 kDa and several blue-stained bands of smaller molecular weight less than 20 kDa were discernible.

As shown in figure 2C, the pattern of SDS PAGE of the crude extracts of bone (lane 2 in Fig. 2C) which was stained with CBB showed a distinct double band at an approximate molecular weight of 100 kDa, which coincide with the α-chains of standard bovine collagen (lane 5 in figure 2C), and diffused bands starting from 50 kDa to 20-25 kDa were observed (lane 2 in figure 2C). The breakthrough fraction likewise shows a double band at 100 kDa and diffused bands starting from 50 kDa to 20-25 kDa (lane 3 in figure 2C). However, in the titanium-binding fraction (lane 4 in figure 2C), a new clear band appeared at a slightly higher molecular weight than 50 kDa.

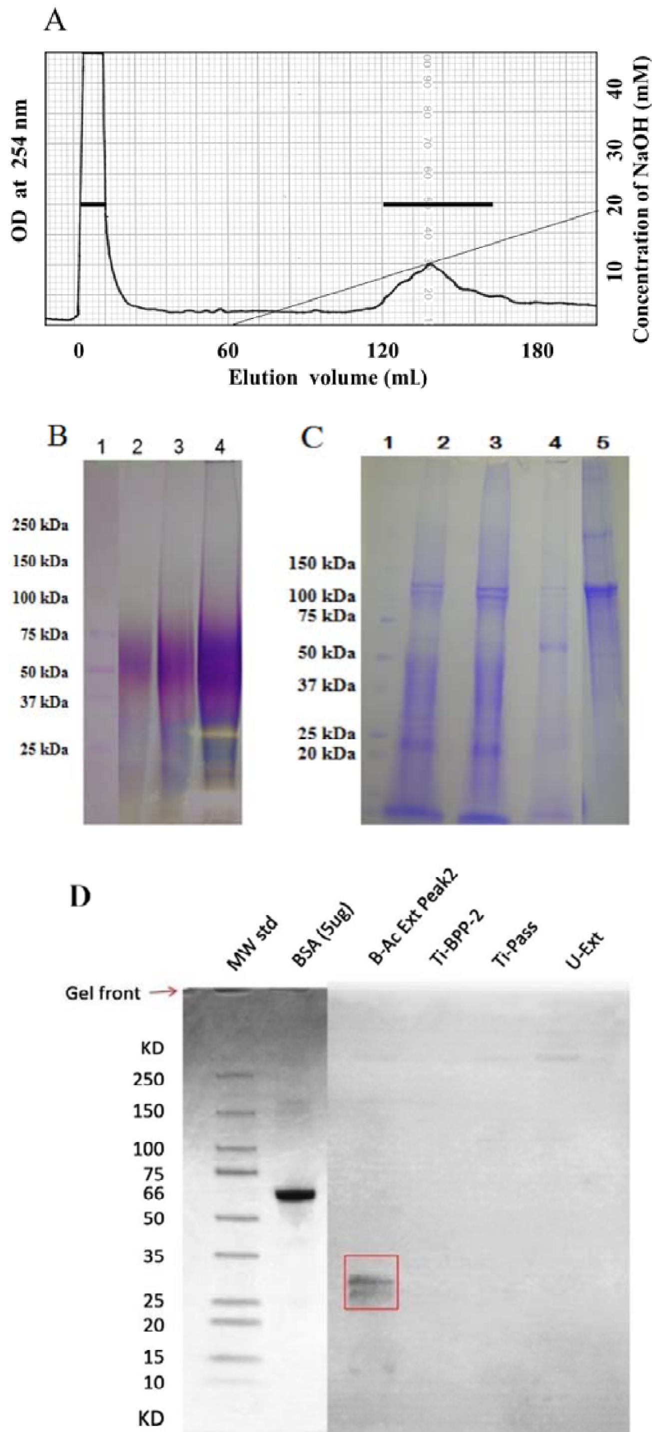


Figure 2 Titanium chromatography of acid soluble protein fractions from bovine bone (A), their SDS PAGE profiles stained with Stains-all (B), the same profiles stained with CBB (C). DMP-1 was detected on nitrocellulose membrane with anti-DMP1 antibody by Western blotting (D, red box).
Lane 1 in Fig. 2BC, molecular mass standard, with their kDa in the left side; lane 2, the extracted crude proteins; lane 3, the break-through fraction indicated by bar in A; lane 4, titanium-binding fractions indicated by bar in A; lane 5, standard bovine type I collagen.

Titanium chromatography, SDS PAGE profiles stained with Stains-all and the same profiles stained with CBB of acid soluble proteins from bovine dentin were shown in Figures 3A, 3B and 3C respectively. Result of the chromatography was similar to that of bone proteins (Fig. 2A), but in the dentin extract, relative amount of the retained fraction (titanium-binding) to break-through (non-binding) fraction seems to be larger than that of bone.

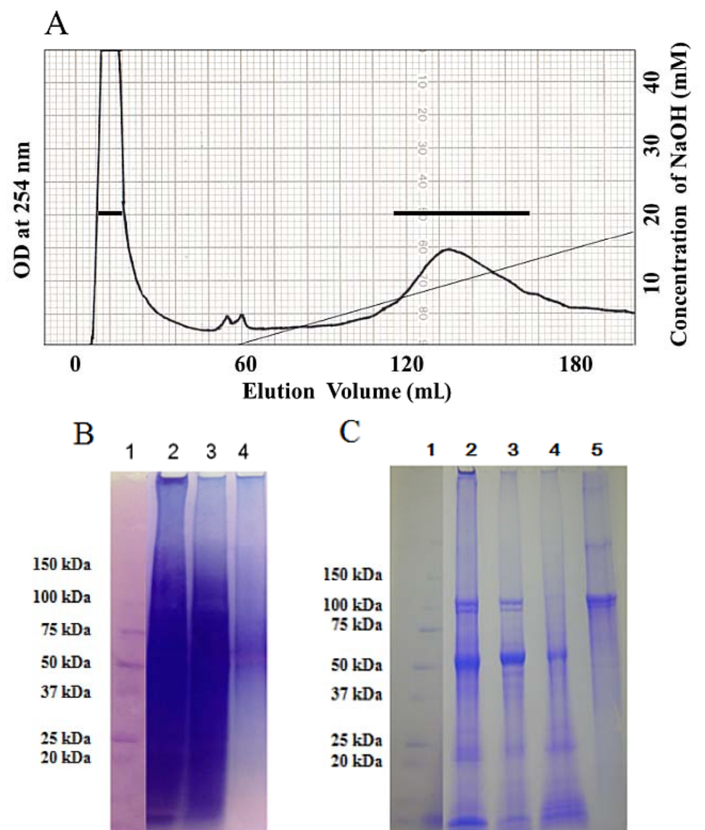


Figure 3 Titanium chromatography of acid soluble protein from bovine dentin (A) and their SDS PAGE profiles stained with Stains-all (B) and the same profiles stained with CBB (C).
Lane 1 in Fig. 3B and C, molecular mass standard, with their kDa in the left side; lane 2, the extracted crude proteins; lane 3, the break-through indicated by bar in A; lane 4, titanium-binding fractions indicated by bar in A; lane 5, standard bovine skin collagen.

The results of SDS PAGE of dentin proteins stained with Stained-all (Fig. 3B) show that the crude extract and break-through fraction show quite broad distribution of blue-staining zone, the typical electrophoretic patterns observed in dentin phosphoproteins. In the titanium-binding fraction (lane 4 in figure 3B) however, two or three discrete bands around 50 kDa were observed.

The results of the SDS PAGE of dentin proteins stained with CBB (Fig. 3C) show that the crude extract and break-through fractions exhibit a similar pattern with a double band at about 100 kDa which corresponds with collagen (lane 5 in figure 3C), a distinct band at slightly higher molecular weight than 50 kDa, and a lower molecular weight band at 20-25 kDa. In the titanium-binding fraction (lane 4 in figure 3C) however, collagen bands disappeared, but the distinct band at slightly higher molecular weight than 50 kDa and a diffused band at 20-25 kDa bands still remained.

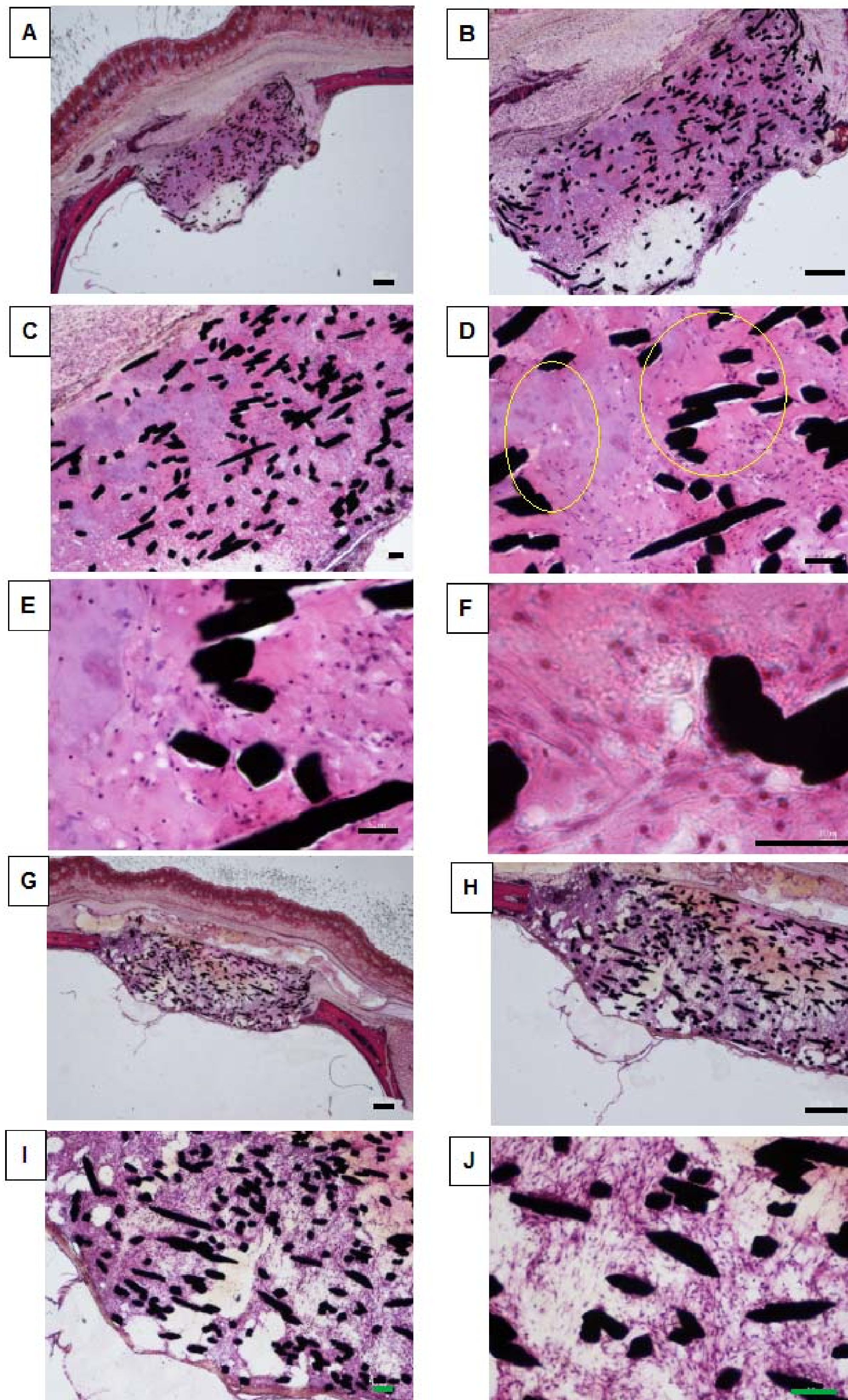
3) Bone-enhancing effect in the AS-TiBP by histological observation

As shown in Figs 4A-F, the titanium webs (TW) which were coated with acid soluble titanium-binding proteins (TW/AS-TiBP) induced active bone and cartilage in the whole areas of TW, except a corner part of TW. In enlarged images (Fig. 4C-E), clear distinction between cartilage (left side) and bone (right side) were observed. At the highest magnification (Fig. 4F), many longitudinal sections of capillaries were seen near the titanium fibers. It was noted that cartilage formations were limited in the area where the density of titanium fibers were relatively low, while the bone formation with capillaries are located in the area where density of titanium wires were constantly higher density. Effect of bone enhancement by the AS-TiBP-coating on TW was estimated histometrically. Average ratio of the areas occupied by bone and cartilage to the total TW area in the AS-TiBP-coated group (65%) was 6.5 times higher than those of the uncoated TW group (10%).

DISCUSSION

Phosvitin is a highly phosphorylated egg yolk protein, with 40 amino acid residues, nearly half of which are phosphorylated serine[12]. We have previously shown that phosvitin binds to the titanium column and assumed that the phosphate groups of this protein were responsible for the binding. In order to confirm the mechanism, we attempted de-phosphorylation of this protein in this study. Figure 1 indicates almost certainly that the binding can be attributed to phosphate groups of phosvitin, although direct chemical confirmation may be still necessary.

Mature bone tissue of vertebrate animals contains various non-collagenous matrix proteins, in addition to bone collagen and hydroxyapatite as the two major chemical components. It is well established that many of these matrix proteins are phosphorylated and play important roles in bone formation and metabolism[3,9,13]. Since our hypothesis is that phosphoproteins in bone may bind to titanium and function to promote bone formation on the surface of the implanted titanium biomaterial, the first step of the verification may be detection of titanium-binding phosphoprotein in bone. For this purpose, we chose an easily extractable fraction of the proteins, which is the 0.01 M HCl soluble fraction, because they may be more easily interact with implanted titanium biomaterials, rather than more insoluble bone matrix proteins, which are only solubilized with dissociating solvents such as 4 M guanidine HCl. So, we have previously shown the presence of titanium-binding proteins which enhanced bone formation in 2M urea soluble fraction from bone [7].



Figures 4 Histological observation of the composites of TW coated with acid soluble titanium-binding proteins (TW/AS-TiBP) (Figs. 4A-F) and their uncoated control TW (Figs. 4G-J) retrieved one week after implantation

TW/AS-TiBP composites (Figs. 4A-F) induced active new bone (indicated by the ellipse at right side of Fig. 4D) and cartilage formation (indicated by the ellipse at left side in Fig. 4D). In contrast, uncoated TW control (Fig. 4G and H) induced no bone nor cartilage formation. Titanium fibers (50 micron in width) in the TW were seen black structures. Burs indicate 1 mm in Figs. 4 A, B, G and H; and 100 μm in Figs. C, D, I and J, and 50 μm in Figs. E and F.

Figure 2A and 2B show there is a small amount (10-20% of total acid soluble proteins) of titanium-binding acid soluble proteins in bone, which were stained positively with Stains-all, indicating the proteins are phosphorylated. A similar situation was observed in acid soluble dentin proteins. From these results it is reasonable to assume that the titanium-binding protein at least partly contain phosphoprotein, judging from their staining behavior with Stains-all. It should be noted that the distinct bands with approximate molecular weight of 50 kDa were found in both bone (lanes 4 in figure 2C) and dentin extracts (lanes 4 in figure 3B and 3C).

Dentin matrix protein 1 (DMP1) was first identified by cDNA cloning using rat odontoblast mRNA[14] and later detected in bone. Recent study on the post-translational proteolytic processing of DMP1 showed that there are two fragments of DMP1 with 37 and 57 kDa fragments, the former being the N-terminal and the latter C-terminal part of DMP1, respectively[15]. Their study suggested us that one of the candidates for the titanium-binding proteins with approximate molecular weight of 50 kDa in lanes 4 in figures 2C, 3B and 3C may include DMP1. Furthermore, immunological staining of this 50 kDa band with anti-DMP antibody revealed clear double bands, as shown in Fig. 2D, confirming the identification. Detailed identification of other titanium-binding proteins in bone and dentin is still ongoing.

The group of non-collagenous phosphoproteins was categorized as members of a family known as SIBLINGs (Small Integrin-Binding Ligand N-linked Glycoproteins)[16-17], which include osteopontin (OPN), bone sialoprotein (BSP), dentin sialophosphoprotein (DSPP), dentin matrix protein 1 (DMP1) and matrix extracellular phosphoglycoprotein (MEPE). It should be noted that all of these phosphoproteins have the cell-adhesive functions through their integrin-binding sequence RGD, and the functions in mineralization through their phosphate groups [6, 9]. To our knowledge, there are little or no systematic study of bone and dentin proteins from the view-point of titanium-binding ability. This study gives the first evidence that there are considerable amounts of titanium-binding phosphoproteins in the acid soluble form (AS-TiBP) in bone and dentin. In addition, when the AS-TiBP coated titanium device (TW) was implanted into the rat calvaria, 6.5 times higher amount of bone was observed than the control implantation of uncoated TW. This bone-enhancing effect is comparably high with the previous results when we observed on the TiBP which was extracted with 2 M urea from bone.

CONCLUSION

Enzymatic dephosphorylation of phosvitin by using λ-protein phosphatase clearly reduced the titanium-binding ability of this phosphoprotein in a reaction-time dependent manner. The phenomenon clearly verifies the cause of titanium binding ability of phosphoprotein are phosphate group of the phosphoserine in phosvitin.

Not only in the 2 M urea extracts as shown in the previous reports[7], but also in the acid decalcification solution, titanium-binding phosphoproteins were detected by titanium chromatography and SDS-electrophoresis (notably DMP1), showing the presence of relatively free state of titanium-binding SIBLING (AS-TiBP) which are ready to bind with implanted titanium. Furthermore, AS-TiBP showed a remarkable bone- enhancing effect when implanted with titanium device. Both results confirmed the definite role of titanium-binding phosphoproteins in titanium implant fixation in bone.

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