Natural variation in the extent of phosphorylation of bone phosphoproteins as a function of *in vivo* new bone formation induced by demineralized bone matrix in soft tissue and bony environments

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Implants of allogenic demineralized bone matrix were placed in distinct *in vivo* environments, i.e. calvarial (bony) and subcutaneous (soft tissue) sites. Detailed analyses of the biochemical components were performed. Quantitative levels of osteopontin (OPN), bone sialoprotein (BSP) and calcium phosphate (Ca-P) deposition within each implant environment varied as a function of new bone formation, and were substantially different in samples from calvarial and subcutaneous sites. Quantification of the extent of phosphorylation of affinity-purified OPN and BSP from such implants indicated that: (i) the number of mols of phosphoserine (P-Ser)/mol of affinity-purified OPN or BSP varied as a function of implant time and bone formation within both implant sites, and (ii) the 'effective P-Ser concentration' provided by the total OPN and BSP within each implant site varied and increased as a function of time, being approx. 5-fold higher for BSP in calvarial compared with subcutaneous implants. Peak levels of mols of P-Ser/mol of BSP coincided with maximum rates of Ca-P deposition in calvarial implants. Levels

of OPN phosphorylation from both calvarial and subcutaneous implants also indicated fluctuations as a function of bone formation. Hence the present study, for the first time, provides direct evidence of natural variation in the extent of phosphorylation of both OPN and BSP as a function of time of mineralized tissue formation. Further evaluation of the data provides the first evidence of a direct and linear relationship between the rate of Ca-P deposition and the ratio of P-Ser-BSP/P-Ser-OPN for calvarial implants. Data for subcutaneous implants failed to provide such correlation. Overall, the present work demonstrates that the natural biological progression of the process of biomineralization follows strict criteria consistent with the anatomical location. Biomineralization fails to proceed in the same way in a soft tissue environment.

Key words: calvarial defect repair, DBM implants, osteopontin, bone sialoprotein.

INTRODUCTION

There has been tremendous interest, developed over recent decades, regarding the biological functions of the extracellular matrix (ECM) non-collagenous proteins of both normally and pathologically mineralizing tissues. This is probably a consequence of the postulation, and continual accumulation of evidence, that these ECM components may play a major role in a wide range of biological functions during mineralized tissue formation. A series of studies focusing on specific components of the mineralizing tissues such as bone, dentin, cartilage, tooth enamel and tendon, highlighted the possible importance of the ECM phosphoproteins present in these tissues [1–5]. In these studies it was postulated that the covalently bound phosphate groups, and the ECM components containing such moieties, are important elements which independently and/or in combination with collagen may play a critical role in orchestrating a well-defined and finely controlled biomineralization process. While the precise identities of the specific phosphoprotein components were not known 20 years ago, more recent studies have resulted in identification of the specific phosphoproteins of bone such as osteopontin (OPN) and bone sialoprotein (BSP) [6–9]. Extensive series of experimentation with both OPN and BSP revealed some

of their possible biological roles during mineralized tissue formation. These included their involvement in the mechanism/regulation of calcification in normally mineralizing tissues [10–16], and in pathologically mineralizing tissues [17–21], cell motility, cell adhesion [8,22–24], and participation in bone resorption and remodelling [25,26]. Despite extensive studies, however, the precise roles of these phosphoproteins, and especially their extensive post-translational modifications such as phosphorylation, remain to be clearly defined. Studies *in vitro* using BSP and OPN indicated that while BSP appears to induce calcium phosphate (Ca-P) apatite formation [13,15,27], OPN lacks this property or is inhibitory [12,13,21]. Such studies have been extended to *in vivo* evaluation of BSP during reparative dentinogenesis [15,16]. Furthermore, studies utilizing OPN and BSP in their phosphorylated and dephosphorylated states indicated the importance of the phosphate groups on these proteins during osteoclast cell attachment [23], and the phosphorylation dependent inhibitory action of OPN on calcification of vascular smooth muscle cells [21].

Clearly, if the covalently bound phosphate groups are important in biological functions of these bone ECM phosphoproteins, then biochemical factors or processes that can affect the state of phosphorylation of these proteins become biologically

Abbreviations used: ECM, extracellular matrix; OPN, osteopontin; BSP, bone sialoprotein; Ca-P, calcium phosphate; mCKII, microsomal casein kinase II; DBM, demineralized bone matrix; P-Ser, phosphoserine; CBB, Coomassie Brilliant Blue R-250; ALP, alkaline phosphatase; PBST, PBS containing 0.05% (v/v) Tween 20.

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significant. Hence the protein kinases, for example, that are involved in their phosphorylation prior to secretion into the ECM become important regulators of the ECM phosphoprotein functions. Previous work from this laboratory indicated that the microsomal casein kinase type II (mCKII) was responsible for the phosphorylation of bone ECM phosphoproteins [28–30]. Further detailed analysis defined the degree of both *in vivo* naturally occurring phosphorylation of bovine BSP and OPN, and the extent to which they can be phosphorylated maximally *in vitro* by using a series of purified protein kinases [30]. This study showed that the extent of *in vivo* phosphorylation was partial and ranging from 60–85% for BSP and OPN. The complete topographical distribution of the precise sites of phosphorylation of chicken bone OPN was identified and each of the ten sites on OPN were phosphorylated to a variable (30–100%) degree [31]. The identified phosphorylated sites and peptide regions were predominantly recognition sequences for mCKII [31], confirming the above *in vitro* studies. These studies were followed by an *in vivo* model for bone repair, using cranium critical size defect in rats induced to heal by demineralized bone matrix (DBM) implants, and evaluating the interrelationship between Ca-P, OPN, BSP and mCKII [32].

In the present study we have, for the first time, performed extensive quantitative analyses of several biochemical components during *in vivo* new bone formation in two anatomically distinct environments. We have evaluated: (a) the state of phosphorylation of affinity-purified OPN and BSP molecules, deposited at different times during new bone formation induced by implants of DBM in calvarial, and subcutaneous tissue sites, at intervals of 2, 5, 7, 10, 14, and 21 d after implantation, (b) total phosphoamino acid content as a function of new bone formation induced by DBM implants in both bony and soft tissue environments, and (c) the temporal expression patterns, accumulation and rate of accumulation of OPN, BSP, Ca-P and mCKII activities, and compared these components in two distinct anatomical tissue environments.

MATERIALS AND METHODS

Rat DBM

Rat DBM was prepared by acid demineralizing (0.5 M HCl) bone particles (75–250 μm) of tibial and femoral diaphyses of 3–6-month-old male and female Sprague–Dawley rats, utilizing modified methods of Reddi and Huggins [33].

Implantation

Male Sprague–Dawley rats, 32–36-day-old, were anaesthetized by intramuscular injection of ketamine and xylazine. Cranial defects (8 mm diameter) were produced with a dental burr and rinsed with lactate Ringer's solution (50 g/l dextrose, 3.1 g/l sodium lactate, 6 g/l sodium chloride, 300 mg/l potassium chloride, 200 mg/l calcium chloride) to clear debris. Six rats were used per time point (six time points, 2, 5, 7, 10, 14, and 21 d), resulting in a total of 36 rats. The rats were randomly allocated and were not littermates. Each rat received simultaneously one calvarial and one thoracic subcutaneous pouch implant, using rat DBM as the implant. The day of implantation was designated as day 0. The implants for morphological study were harvested together with the adjacent host beds. The implants for biochemical study of the calvarial site were excised under a dissecting scope to avoid harvesting host bone. Implants from six animals were evaluated at each time point, and the series of biochemical analysis for calvarial and subcutaneous samples were performed simultaneously.

Calcium content and alkaline phosphatase (ALP) activity

Calcium and ALP activity were determined as described previously [32].

Isolation and assays of mCKII from the implants

Isolation of subcellular mCKII was carried out as previously described [29,32] and the assays were performed with [^{32}P]ATP radiolabelling and HPLC techniques using casein kinase II specific commercial peptide substrate, RREETEEE [29–31] (LC Laboratories, Woburn, MA, U.S.A.). ^{32}P counts of aliquots from HPLC radiolabelled substrates were calculated in terms of total ^{32}P (d.p.m.) incorporated into 20 μg of substrate. These data were converted into ^{32}P (d.p.m.) per mg of protein in the enzyme preparation used for the assays, and plotted as a function of the time elapsed during repair.

ELISA for OPN and BSP

OPN and BSP were extracted from the DBM implants by sequential homogenization for 2 min (four times for 30 s with 1 min standing on ice between homogenizations) in 1 ml of 0.1 M HCl [containing the covalent proteinase inhibitors phenylsulphonyl fluoride and 2,2'-dipyridyl disulphide (0.5 mM each), and the phosphatase inhibitor levamisole (0.5 mM)], before standing for 1 h at 22 °C. Aliquots of 0.23 ml of these homogenates were saved for total component analyses (soluble + insoluble proteins, containing both the collagenous and non-collagenous proteins) including: (i) total protein by amino-acid analysis, (ii) total collagen by amino-acid analysis, and (iii) phosphoamino-acid analysis. The remaining 0.77 ml was centrifuged at 7000 rev./min using a bench top microcentrifuge. The supernatant was saved and the pellet was resuspended in 1 ml of 50 mM Tris buffer, pH 7.6, (containing the covalent proteinase inhibitors as described previously), homogenized and centrifuged as described previously. The HCl extracts (first supernatant from the 0.77 ml), after adjusting the volume to 2 ml with de-ionized water, was dialysed against 50 mM Tris buffer, pH 7.6, freeze dried and combined with the second supernatant from the Tris buffer extracts. These combined samples of total soluble extracts (containing only the soluble non-collagenous proteins) were adjusted to 1 ml total volume. Aliquots of 0.1 ml from each sample were measured for total soluble components, including: (i) total soluble non-collagenous proteins by amino-acid analysis, and (ii) total phosphoamino-acid analysis. The remaining total soluble extracts were used for OPN and BSP ELISA assays and affinity purification of OPN and BSP (see below). The ELISA technique used was as described previously using specific polyclonal antibodies generated against native bone OPN and BSP in our laboratory [32]. Standard calibration curves containing 14 different concentrations were prepared from purified bovine OPN and BSP. The standard curve was analysed by a '4 parameter equation' to calculate the OPN and BSP contents in the implants.

Protein content of implants

(i) The total soluble protein concentrations in the implants were determined by using bicinchoninic acid (Sigma), a modified Lowry's method [35]. Aliquots of the same respective samples prepared for OPN, BSP and mCKII analysis were used for the protein determinations. (ii) The total protein contents of all the samples for which phosphoamino-acid analysis was carried out were directly calculated from their total amino acid content, determined by complete acid hydrolysis in 6 M HCl at 110 °C

for 24 h using Waters HPLC system and pico-tag column for picomolar quantification, as described previously [30].

Evaluation of the total phosphoamino acid content per total protein (soluble only, and soluble + insoluble) as a function of new bone formation in cranial and subcutaneous implants

The samples of cranial and subcutaneous implants retrieved were analysed for phosphoamino acids, P-Ser, P-Tyr and P-Thr as a function of new bone formation. Samples of 0.23 ml, in triplicate for each time point, and for both calvarial and subcutaneous implants from the 0.1 M HCl first homogenate before centrifugation (which contained total soluble + insoluble components, i.e. non-collagenous proteins + collagen), were hydrolysed by 6 M HCl in sealed tubes at 110 °C, before phosphoamino acid and total amino acid analyses. These phosphoamino-acid and amino-acid analyses were carried out as described previously in this laboratory [30]. These results were used to calculate the total phosphoamino acids/1000 residues as well as moles phosphoamino-acids/mg of protein.

Preparation of specific solid-phase antibody-affinity columns for the purification of microgram quantities of OPN and BSP

We prepared the respective specific solid-phase affinity columns using specific polyclonal antibodies raised in rabbits against purified OPN and BSP [32], cross-linked to an agarose gel matrix to purify microgram quantities of OPN and BSP from the total extracts of the implants. The polyclonal antibodies were separately immobilized by covalent cross-linking to a solid phase of CNBr-activated agarose gel (Sigma). A 1 ml aliquot of each OPN and BSP polyclonal antibody (0.8–1.5 mg/ml) was used with 5 ml of CNBr-activated agarose gel per antibody in the presence of 5 ml of sodium acetate buffer, pH 4.0. The reaction was allowed to proceed overnight at 22 °C with continuous shaking. The gel was then packed into a glass column (1 × 10 cm), washed free of unreacted reagents and equilibrated with PBST buffer [PBS containing 0.05 % (v/v) Tween 20], pH 7.2.

Determination of phosphoamino acid contents of affinity purified OPN and BSP from the implants of cranial and subcutaneous environments as a function of new bone formation/mineralization

An OPN-antibody affinity column and a BSP-antibody affinity column were used to purify milligram quantities of OPN and BSP from both implant sites, as a function of time, to perform accurate determination of the extent of phosphorylation of these proteins. A 0.8 ml aliquot of the total soluble extracts (combined Tris buffer, pH 7.6, and HCl extracts) at each time point for both calvarial and subcutaneous implants were then divided into two 0.4 ml aliquots; one was applied onto an OPN-affinity column and the other onto a BSP-affinity column. After each application, the extract was allowed to sit at 22 °C for 15 min in the column, followed by washing the column free of the unbound material using PBST buffer five times the column void volume. The bound OPN and/or BSP was eluted by 0.1 M glycine/HCl, pH 2.0. The original samples, unbound material, and the bound and eluted proteins were analysed for the absence and presence of OPN and BSP by Western blotting and X-ray visualization of the chemiluminescence.

Phosphoamino acid analyses of affinity purified OPN and BSP were carried out as described previously [30]. Six implant samples per time point were separately analysed for total amino acid and phosphoamino acid content by a single determination per sample. The data were calculated in terms of moles of P-Ser/mole of OPN

or BSP with the mean and standard deviation of six sample analyses.

Statistical analyses of the biochemical assays of the implants

The data obtained from six rats (with each rat having one calvarial and one subcutaneous implant) at each time point were treated for statistical variation. The results from implants from six animals at each time point were used to calculate the mean and the standard deviation (data points and vertical bars respectively on the Figures). Since each of the sets of biochemical analysis (Ca²⁺, OPN, BSP, P-Ser content etc.) at each time point were represented by a mean and standard deviation, when divisions were performed by another numerical number which was also represented by mean and standard deviation, a formula was used to calculate the propagation of the errors [34]. Further statistical analyses were performed, using the two-sample Student's *t* test with ten degrees of freedom, on the data in Figures 1, 2, 5 and 6. This determined the statistical significance of the results between the calvarial and subcutaneous implants, as well as between the data from one time point to another within a given implant site.

SDS/PAGE and Western blotting

SDS/PAGE was carried out as described previously [30] using aliquots (0.1–0.2 µg, as determined by total amino-acid analysis at pmole level, see above) of affinity purified OPN and BSP. Each protein sample was electrophoresed in duplicate, one set was then stained with Coomassie Brilliant Blue (CBB) while the other set was transferred to nitrocellulose and the lanes containing OPN and BSP were immunodetected using specific polyclonal antibodies raised in this laboratory [32].

RESULTS

Assessment of OPN and BSP content of DBM

The contents of OPN and BSP in DBM prior to implantation were found to be approx. 0.3 µg/15 mg of DBM, and approx. 0.6 µg/15 mg of DBM respectively using the ELISA method.

Cumulative deposition of Ca²⁺, OPN, BSP and mCKII activity during new bone formation in subcutaneous implants

The comparative observations of Ca²⁺, OPN and BSP deposition are presented in Figure 1. The total amount of mineral which accumulated in the calvarial implants increased very rapidly during the first 10 days, and slightly less rapidly from day 10–21. Ca-P, BSP and OPN deposition occurred earlier, more rapidly and the amounts were far greater in the cranial implants than in the subcutaneous implants (Figures 1A–1C). Statistical analysis, using the two-sample Student's *t* test with ten degrees of freedom, was carried out (see Figure 1 legend). The data for OPN and BSP in the subcutaneous environment were determined in the present work (solid lines) and compared with the corresponding values for the cranial implants reported previously from this laboratory (dotted lines [32]). Throughout the whole implant period (days 2–21), the above components for the subcutaneous implants were about 10 % that of the same components, and at the same corresponding time period, in cranial implants. The total accumulation of OPN and BSP was approximately the same for subcutaneous implants throughout the implant period, whereas for the cranial implants this was only true between days 2–14. During the final week (days 14–21) the accumulation of BSP was significantly greater than that of OPN (Figures 1B and 1C). All

Figure 1 Time-dependent changes in total cumulative Ca^{2+} , OPN, BSP and mCKII activity of DBM implants between implant days 0 and 21 in subcutaneous tissue (Subc.) and calvarial defects

(A) Ca^{2+} accumulation, (B) BSP accumulation, (C) OPN accumulation, and (D) mCKII activity. Solid lines represent present work and dotted lines represent previously reported results [32]. The mCKII activities were measured using commercial casein kinase II synthetic peptide substrate, RRETEEE. The data points are mean values determined for six different implant samples and the vertical bars are standard deviation. The paired *t*-test with ten degrees of freedom indicated that the cumulative Ca^{2+} , BSP and OPN data for calvarial versus subcutaneous implants at each time point (from days 5 to 21) were different and statistically significant at levels $P < 0.001$.

of the above ECM components showed a steady increase correlating with those of the mCKII activities (Figure 1D).

Rate of accumulation of Ca^{2+} , BSP and OPN in subcutaneous implants

In addition to measuring the total cumulative content of OPN, BSP and Ca-P in the bone repair tissue as a function of time, we also calculated the mean rate/d at which these components were accumulated during specific time periods, i.e. 0–2, 2–5, 5–7, 7–10, 10–14, and 14–21 d. The rate of accumulation of Ca-P, OPN and BSP as a function of time was distinctly different from the patterns of the total cumulative content in the tissue with time, and provided a more effective evaluation of the data in terms of the interrelationships between Ca^{2+} , OPN and BSP during new bone formation and mineralization. The rates of accumulation of these components were clearly different and variable in the two implant sites, with much lower levels in those of the subcutaneous implants, (Figure 2). In the calvarial implants the rates of BSP and OPN deposition were variable with rapid increases and decreases, and such occurrences were not so prominent in subcutaneous implants (Figures 2B and 2C). Statistical analysis of these data using two-sample Student's *t* test are recorded in the legend of Figure 2. A note of interest is that while the time intervals for most of the data were in the range 2–4 d, for the last time point the interval was 7 d. Since the values of the rates represent an average rate/d (over a given time interval), then analysis at more frequent intervals for this time point may have provided somewhat lower and higher rates

Figure 2 Time-dependent changes in the rate of deposition per day of the cumulative matrix components Ca^{2+} , BSP and OPN in subcutaneous DBM implants between days 0 and 21 in subcutaneous DBM implants and in calvarial defect implants

The data points are mean values determined for six different implant samples and the vertical bars are standard deviation. (A) Rate of Ca^{2+} deposition. The two sample Student's *t* test indicated that the difference in the rate of Ca^{2+} deposition within the calvarial implants was statistically significant for days 2 versus 5 ($P < 0.001$), 5 versus 7 ($P < 0.001$), 7 (or 10) versus 2 ($P < 0.001$), 7 (or 10) versus 5 ($P < 0.001$), 7 (or 10) versus 14 ($P < 0.01$), 7 (or 10) versus 21 ($P < 0.01$), and 10 versus 14 ($P < 0.01$). The rates at day 7 (maximum rate of Ca^{2+} deposition) and day 10 were statistically the same, but both of these were statistically different from those at days 2 ($P < 0.001$), 5 ($P < 0.001$), 14 ($P < 0.01$), and 21 ($P < 0.01$). The rates of Ca^{2+} deposition within the subcutaneous (Subc.) implants were statistically approximately the same. The rates of Ca^{2+} deposition for calvarial versus subcutaneous implants were analysed statistically as pairs at each time point and the results of such analyses are recorded directly on the Figure. (B) Rate of BSP deposition. The rates of BSP deposition for calvarial versus subcutaneous implants were analysed statistically as pairs at each time point and the results of such analyses are recorded directly on the Figure. (C) Rate of OPN deposition. The rates of OPN deposition for calvarial versus subcutaneous implants were analysed statistically as pairs at each time point and the results of such analyses are recorded directly on the Figure. Shaded bars represent present work and open bars represent previously reported results [32].

Figure 3 Time-dependent changes in the total phosphoamino acid content of total soluble fraction, and total soluble + insoluble fraction per total respective protein content of each fraction of implants during new bone formation in calvarial and subcutaneous DBM implants

(A) Total phosphoamino acid analysis of the soluble fractions (which contains only the non-collagenous proteins) from both calvarial and subcutaneous (Subc.) implants between days 0 and 21. (B) Total phosphoamino acid analysis of the whole implant (soluble + insoluble fraction) expressed as nmoles per mg total protein present (including collagen). (C) Total phosphoamino acid analysis of whole implant (soluble + insoluble fraction) expressed as nmoles per mg total non-collagenous protein determined to be present in the whole sample. The data points are mean values determined for six different implant samples and the vertical bars are standard deviation.

than the actual average rate/d we observed by analysing at the end of a 7 day interval. The impact of this to the overall essence of our conclusions is minimal, since the rates/d determined in the present work for the subcutaneous implants vary very little over the whole implant period (0–21 d). In the calvarial implants the defect was almost 70% healed by 14 d (as assessed by

Figure 4 SDS/PAGE and Western-blot analysis of affinity purified OPN and BSP from calvarial and subcutaneous implants

Panel 1 represents data from subcutaneous implants (at day 10): lanes a and c, CBB stain of affinity purified (approx. 70 ng) BSP and OPN respectively; lanes b and d, corresponding Western blots using polyclonal antibodies towards BSP and OPN respectively. Panel 2 represents data from calvarial implants (at day 10): lanes a and c, CBB stain of affinity purified (approx. 250 ng) BSP and OPN respectively; lanes b and d, corresponding Western blots using polyclonal antibodies towards BSP and OPN respectively. Arrows indicate the OPN fragments.

X-irradiation and histology), consistent with the decrease in the rate of Ca-P deposition at days 14 and 21, indicating that more frequent intervals between day 14 and 21 would provide a rate of Ca-P deposition/d comparable with the actual average rate/d determined. However, for the rates of deposition of BSP and OPN, more frequent analysis over the 7 day interval (days 14–21) may have provided rates that were both lower and higher than the mean rate/d observed in the present work.

Phosphoamino acid analysis of the total implants and the affinity-purified OPN and BSP from both cranium and subcutaneous implant samples as a function of time and new bone formation

Total phosphoamino acid content per total 'soluble' and 'soluble + insoluble' protein content of implants during the new bone formation in calvarial and subcutaneous implants

Figure 3(A) shows total phosphoamino acid analysis of the soluble fraction (which contains multiple non-collagenous proteins) from both calvarial and subcutaneous implants. The content of phosphoamino acids of these samples are predominantly P-Ser with no detectable P-Tyr and minimal P-Thr ($\leq 5\%$ that of P-Ser). The pattern of nmoles of P-Ser per mg of total soluble non-collagenous protein shows a consistent rapid increase in P-Ser for calvarial implants, closely following the cumulative Ca-P deposition, OPN and BSP accumulation in these implants (Figure 3A). The subcutaneous implants contained much lower levels of P-Ser with steady but slowly increasing amounts. Figure 3(B) shows phosphoamino acid analysis of the whole implant material (soluble + insoluble) where the data for P-Ser content is expressed as per mg of total protein (soluble + insoluble), and per mg of total non-collagenous protein content, (Figure 3C).

State of phosphorylation of OPN and BSP affinity purified from the calvarial and subcutaneous implants as a function of time and new bone formation

The affinity purified BSP and OPN (at submicrogram, $\leq 1 \mu\text{g}$) from both calvarial and subcutaneous implants at all time points, contained predominantly P-Ser residues with no P-Tyr and minimal P-Thr ($\leq 5\%$ that of P-Ser content). Figure 4 shows SDS/PAGE and Western blot of affinity purified OPN and BSP, demonstrating that our approach was effective and there was no

Figure 5

Figure 5 Time-dependent changes in the absolute phosphorylation state in moles of P-Ser/mol of affinity purified BSP and 'effective P-Ser concentration in the whole implant result of all of BSP present' during new bone formation and maturation induced by DBM in calvarial and subcutaneous environments

The data points are mean values determined for six different implant samples and the vertical bars are standard deviation. (A) Phosphorylation state of BSP in mol of P-Ser/mol of BSP. Statistical two sample Student's *t* test analysis on the extent of phosphorylation of BSP within the calvarial implant group indicated that these were variable and the differences were statistically significant for days 2 (or 5) versus 7 ($P < 0.01$), 2 (or 5) versus 10 ($P < 0.001$), 2 (or 5) versus 21 ($P < 0.001$), 7 versus 14 ($P < 0.001$), 10 versus 14 ($P < 0.001$), 10 versus 21 ($P < 0.001$), 14 versus 21 ($P < 0.01$). Similar statistical analyses within the subcutaneous implant group for BSP revealed that the extent of phosphorylation of BSP were different and statistically significant for days 2 versus 5 ($P < 0.05$), 2 versus 7 ($P < 0.01$), 10 versus 14 ($P < 0.01$), and 14 versus 21 ($P < 0.01$). (B) Effective P-Ser concentration in the whole implant on total BSP present. Statistical two sample Student's *t* test analysis on the effective P-Ser concentrations for BSP within the calvarial implant groups provided *P*-values of: days 2 versus 5 ($P < 0.0001$), 5 versus 7 (0.0027), 7 versus 10 (0.0001), 10 versus 14 (0.0048), 14 versus 21 (0.0001); and for the subcutaneous implants days 2 versus 5 (0.049), 5 versus 7 (0.0001), 7 versus 10 (0.0001), 10 versus 14 (0.2394), and 14 versus 21 (0.0003). The *P*-values above the pairs of data on the Figures at each time point represent statistical analysis for calvarial versus subcutaneous implants.

contamination by other tissue proteins as judged by lack of any visible CBB staining. The lack of CBB staining for OPN and BSP corresponding to Western blot bands was expected since the amounts purified were 1 μ g or less (as determined by total amino-acid analysis) and these proteins stain very weakly by CBB. Also, total amino-acid analysis on the affinity purified samples at pmole level provided the correct and expected amino acid compositions corresponding to those of rat OPN and BSP

Figure 6 Time-dependent changes in the absolute phosphorylation state in moles of P-Ser/mol of affinity purified OPN and 'effective P-Ser concentration in the whole implant result of all of OPN present' during new bone formation and maturation induced by DBM in calvarial and subcutaneous environments

The data points are mean values determined for six different implant samples and the vertical bars are standard deviation. (A) Phosphorylation state of OPN in mol of P-Ser/mol of OPN. Statistical two sample Student's *t* test analyses of the OPN results within calvarial implants indicated that the results were statistically significant and different for days 2 versus 5 ($P < 0.01$), 2 versus 10 ($P < 0.001$), 2 versus 21 ($P < 0.001$), 5 versus 10 ($P < 0.05$), 5 versus 21 ($P < 0.001$), 7 versus 10 ($P < 0.001$), 7 versus 21 ($P < 0.001$), 10 versus 14 ($P < 0.05$) and 14 versus 21 ($P < 0.001$). Similarly the data within subcutaneous implants indicated the extent of phosphorylation of OPN were statistically significant and different for days 2 (or 5) versus 7 ($P < 0.001$), 2 (or 5) versus 10 ($P < 0.001$), 2 (or 5) versus 14 ($P < 0.001$), 2 (or 5) versus 21 ($P < 0.001$), 7 versus 14 ($P < 0.01$), and 14 versus 21 ($P < 0.001$). (B) Effective P-Ser concentration in the whole implant on total OPN present. Statistical two sample Student's *t* test analysis on the effective P-Ser concentrations for OPN within the calvarial implant groups provided *P*-values of: days 2 versus 5 ($P < 0.0064$), 5 versus 7 (0.6847), 7 versus 10 (0.0052), 10 versus 14 (0.3176), and 14 versus 21 (0.0007); and for the subcutaneous implants days 2 versus 5 (0.5058), 5 versus 7 (0.0001), 7 versus 10 (0.0102), 10 versus 14 (0.462), and 14 versus 21 (0.0001). The *P*-values above the pairs of data on the Figures at each time point represent statistical analysis for calvarial versus subcutaneous implants.

(results not shown). The presence of minor lower-molecular-mass fragments (approx. 30 kDa and 40 kDa) of the affinity purified OPN which were present in samples from both implant sites (Figure 4, panel 1 lane d, and panel 2 lane d), was expected since OPN is susceptible to rapid fragmentation (while BSP is not), and such fragments always exist in bone environment.

Figures 5 and 6 show accurate and direct identification of amount (mol) of P-Ser/mol of purified BSP and OPN, respectively. The

Figure 7 Direct and linear correlation between rate of Ca^{2+} deposition versus ratio P-Ser (BSP)/P-Ser(OPN) and alkaline phosphatase

(A) Rate of Ca^{2+} deposition versus ratio of P-Ser (BSP)/P-Ser(OPN); ($\diamond$) data for calvarial implants where solid line represents best fit regression line with correlation coeff. = 90%, and ($\blacksquare$) data for subcutaneous implants where the solid lined curve is a polynomial fit. (B) Rate of Ca^{2+} deposition versus ALP activity; ($\diamond$) data for calvarial implants where solid line represents best fit regression line with correlation coeff. = 87% and ($\blacksquare$) data for subcutaneous implants where solid line represents best fit regression line with correlation coeff. = 87%. (C) Ratio of P-Ser(BSP)/P-Ser(OPN) versus ALP activity; ($\diamond$) data for calvarial implants where solid line represents best fit regression line with correlation coeff. = 88% and ($\blacksquare$) data for subcutaneous implants where solid line represents best fit regression line with correlation coeff. = 85%.

data were calculated in terms of mols of P-Ser/mol of BSP using: (i) the total amino-acid analysis data which provided amounts of BSP or OPN in μg , (ii) the determined P-Ser content, and (iii) the respective molecular weights of BSP and OPN based on the amino-acid composition deduced from cDNA. The results were analysed by two sample Student's *t* test to show statistical significance (*P* values are recorded in the legend of Figure 5). These analyses indicated that the extent of phosphorylation of BSP within the calvarial implant group, as well as for calvarial versus subcutaneous implants, were variable and the differences were statistically significant. Since the actual protein levels of BSP also varied substantially within each implant environment and between those found in the calvarial versus subcutaneous implants, it was of interest to evaluate these data by taking into account the absolute total amount of BSP present and the mols of P-Ser present per mol of BSP at each time point. Such calculations provided the effective P-Ser concentration in the whole implant as a result of total BSP present, at each time point. Figure 5(B) shows that the total effective P-Ser concentrations in the subcutaneous implants were minimal when compared with those of calvarial implants. Also, in the subcutaneous implants the effective P-Ser concentration increased slowly but continuously from approx. 7–21 d, whereas those of calvarial implants showed significant rapid increases (e.g. day 10 and day 21), with a decrease at day 14.

Similar evaluations and analysis were carried out for affinity purified OPN isolated from both calvarial and subcutaneous implants. Figure 6(A) shows the determined mols of P-Ser/mol of OPN which significantly change as a function of time in both calvarial and subcutaneous environments, the values for subcutaneous OPN samples (ranging from 1 to 11 mol of P-Ser/mol of

OPN) being higher than those of calvarial OPN samples (ranging from 1.5 to 6.3 mol of P-Ser/mol of OPN). The highest extent of phosphorylation for OPN samples from calvarial implants occurred at days 10 and 21, while in the subcutaneous implants these occurred at days 7, 10 and 21. Statistical two-sample Student's *t* test analyses of the results of the extent of phosphorylation of OPN in both implant sites, at each time point indicated that the results were statistically significant (Figure 6A). Figure 6(B) shows the calculated total effective P-Ser concentration as a result of total OPN present, within the whole implant, with peak concentrations occurring at day 21 for both implant environments.

ALP activity

ALP activities for both calvarial and subcutaneous implants determined in the present work are presented as plots of: (i) rate of Ca-P deposition versus ALP activity (Figure 7B), and (ii) ratio of P-Ser (BSP)/P-Ser (OPN) versus ALP activity (Figure 7C). The presentation of the data in this form was based on the general trends observed in the context of the present work.

DISCUSSION

The biological importance of the ECM phosphoproteins during calcified tissue formation (bone and dentin for example), nucleation/regulation of Ca-P mineral phase, and modulation of cellular activity have been extensively documented (see the Introduction). There is a general understanding that these phosphoproteins appear to be important as a consequence of their intimate relationship with the mineral deposition/phase. However, one property unique to these proteins is their extensive

phosphorylation. To date there has been no study or direct evidence, provided from either *in vivo* or *in vitro* studies, to define the degree to which these proteins are phosphorylated within a given mineralized tissue as a function of time, biomineralization and developmental stage. Based on previous work from this laboratory, we have hypothesized that the ECM bone phosphoproteins are subject to natural variation in their state of phosphorylation as a function of time of bone formation, developmental stage and the anatomical tissue environment. That is, within a given population of molecules (isolated from bone of a particular age) heterogeneity exists with respect to the extent of phosphorylation. This may be attributed to changes in factors such as the rate of phosphoprotein synthesis, the mCKII activity, the available ATP concentrations, and some degree of dephosphorylation while these proteins reside within the ECM. To provide direct evidence in support of such conceptual developments, we have utilized *in vivo* new bone formation models in two anatomically different environments. Bone formation was induced in calvarial defect (bony), and subcutaneous (soft tissue) environments by implantation of DBM. In the former model, the formation of new bone induced by DBM implantation proceeded through direct bone formation by differentiation of the primitive mesenchymal/stem cells into osteoblasts which form the new bone (results not shown), for more details see [32,36]. However, in the subcutaneous soft tissue environment the same implant material (DBM) induces initial formation of cartilage which mineralizes, resorbs and becomes replaced by the new bone (results not shown), for more details see [36].

Interrelationship between accumulation and rate of accumulation of BSP, OPN, Ca-P and mCKII activity in bony and soft tissue environments

The occurrence of predominant cartilage and mineralized cartilage in subcutaneous implants up to approx. 14 days, permitted, for the first time, the evaluation *in vivo* of the properties of a series of biochemical components important in mineralized tissue formation involving chondrocytes. In addition, we were able to assess how the biochemical parameters varied within this tissue as a function of tissue development, and compare these with those of the direct bone formation through osteoblastic activity. This is relatively easy during the first 14 days of subcutaneous implants, however, around this time and beyond there is overlap with the resorption and new bone formation through osteoblastic activity. Bone formation in the calvarial sites proceeded much more rapidly than in subcutaneous site implants, consistent with higher (approx. 5–10-fold) quantitative levels of OPN, BSP and Ca-P throughout the experimental time points studied (2–21 d). In addition, these components were deposited at variable rates within each implant environment as a function of time during mineralized tissue formation, which became apparent only when rates of accumulation for the cumulative components were calculated (Figure 2). The rates of accumulation of OPN, BSP, and Ca²⁺ were calculated to derive and highlight hidden functional interrelationship between these matrix cumulative components. Such analyses showed correlation between these components within each implant site, with substantial differences between calvarial versus subcutaneous implants (Figure 2). In the subcutaneous environment it was not until beyond day 14, when significant osteoblastic activity ensued, that the accumulation of the typical bone cumulative components began to increase. In a series of possible permutations of plots of rate of Ca-P or cumulative Ca-P deposition versus cumulative BSP, OPN (or their rate of accumulation), or ratio of BSP/OPN

we observed no linear correlation for both calvarial and subcutaneous data (plots not shown).

Biological significance of the extent of phosphorylation of bone phosphoproteins during mineralized tissue formation in bony and soft tissue environments

The present study provided direct evidence, for the first time, in support of the concept of the natural variation in the extent of phosphorylation of ECM phosphoproteins and its biological significance during *in vivo* mineralized tissue formation. The extent of phosphorylation of OPN and BSP in the two anatomically distinct sites were substantially different and varied as a function of time of mineralized tissue. Our findings signify variation between approx. 1–14 mols of P-Ser/mol of OPN or BSP as a function of time during new bone formation and the divergence in the P-Ser content between the bony and soft tissue derived phosphoproteins (Figures 5 and 6). These results, in turn, signify the important role that mCKII may play during mineralized tissue development.

The calculated 'effective P-Ser concentrations' were significantly higher (approx. 5-fold) for the calvarial implants compared with those of subcutaneous implants throughout the implant period studied (Figure 5B). Figure 6(B) shows the corresponding data for OPN with only marginal (approx. 1.6-fold) difference between the calvarial and subcutaneous samples. Although it is not easy to interpret precisely, the occurrence of low and high levels of phosphorylation of these proteins may reflect different regulatory effects during biomineralization. The relatively higher level of OPN phosphorylation in subcutaneous implants may have been expected from the high ratio of mCKII activity to that of OPN in these implants relative to those of the calvarial implants. The results also indicate that the chondrocytes and osteoblasts secrete OPN and BSP with different extents of phosphorylation, and this was more prominent for OPN. It is interesting to note that other studies with normally non-mineralizing tissues have shown that, with the exception of milk [37], OPN was secreted in its almost totally unphosphorylated form [38]. The distinction between the present work and the reported predominantly unphosphorylated OPN in other soft tissues, is that the data obtained from subcutaneous implants are derived from a tissue which was stimulated by DBM to form differentiated bone cells such as chondrocytes and osteoblasts. Hence, OPN secreted from such soft tissue environment may have been expected to be synthesized in its highly phosphorylated state, unlike OPN from other general soft tissues.

Direct and linear correlation between the 'coupled' state of phosphorylation of BSP and OPN, 'rate' of Ca-P deposition and alkaline phosphatase activity

In a large number of possible permutations of graphical analysis, e.g. plots of 'rate' of Ca-P or 'cumulative' Ca-P deposition versus P-Ser-BSP, or P-Ser-OPN we observed no direct (linear) correlation for both calvarial and subcutaneous data (plots not shown). However, importantly, plots of 'rate' of Ca-P deposition versus the ratio of P-Ser-BSP/P-Ser-OPN or ALP activity, and a plot of ratio of P-Ser-BSP/P-Ser-OPN versus ALP activity for calvarial implants showed direct and linear correlation (Figures 7A–7C). The linear relationship between the rate of Ca-P deposition and the ratio of P-Ser-BSP/P-Ser-OPN for calvarial implants clearly demonstrated hidden and important facets of the factors that control mineral deposition, and the coupling of the P-Ser contents of BSP and OPN in this process. Figure 7(A) shows that the rate of calcium deposition was maximal when the ratio of P-Ser (BSP)/P-Ser(OPN) was approx. 3–4, represented

by calvarial implant days 7–10. Furthermore, there is a limiting value of approx. 1.2 for the ratio of P-Ser(BSP)/P-Ser(OPN) at or below which the rate of calcium deposition approaches zero. The corresponding data for the subcutaneous implants (Figure 7A) showed no such direct correlation. In this Figure, four of the six time points fall below the value of 1.5 for the ratio P-Ser(BSP)/P-Ser(OPN), whereas two time points, at around the value 2.5, correspond to an approx. zero rate of Ca-P deposition (but these are the earliest implant times of 2 and 5 d where there is very little calcification). There was, however, direct and linear correlation between the rate of Ca-P deposition versus ALP activity (Figure 7B) and between the ratio of P-Ser (BSP)/P-Ser(OPN) versus ALP activity (Figure 7C). The latter relationship, however, was the inverse of the calvarial implants. The precise mechanism of how ALP participates in the overall biomineralization is not clearly known and may not be precisely discussed in the present work.

Overall the results indicated that the process of finely controlled *in vivo* biomineralization is critically dependent upon the delicate balance of the ratio of total P-Ser present on BSP to that of OPN, and not merely on the presence of BSP and OPN protein per se or indeed the presence of any arbitrary amount of P-Ser on these proteins. These results pose a series of uncertainties on the interpretation of the results in the literature based on studies analysing mineralized tissue formation as a function of time, and determining the mRNA levels of these proteins or even the actual protein. These uncertainties are also applicable to experiments performed by addition of pure protein samples in functional assays without clear and precise knowledge of their state of phosphorylation. Such considerations become even more complex when one considers the possibility that the gross state of phosphorylation may not be sufficient, since the phosphorylation on different regions and sites of these proteins may provide different biological functions. The biological implication of the present study is that the process of biomineralization and the biological functions of bone phosphoproteins cannot be dissociated from their state of phosphorylation, including coupling of the specific extent of phosphorylation on BSP and OPN. In addition, there is a broader significance of the present findings to studies in other biological fields which involve OPN, BSP and Ca-P deposition, as is the case in soft tissues with pathological calcification such as atherosclerosis and kidney stones, or in the cancer field where prostate and breast tumours frequently show calcification.

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