Chapter 1

Biomimetic coatings for bone tissue engineering of critical-sized defects

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doi:10.1098/rsif.2010.0115.focus
Published online 19 May 2010
ABSTRACT

The repair of critical-sized bone defects is still challenging in the fields of implantology, maxillofacial surgery and orthopedics. Current therapies such as autografts and allografts are associated with various limitations. Cytokine-based bone tissue engineering has been attracting increasing attention. Bone-inducing agents have been locally injected to stimulate the native bone-formation activity, but without much success. The reason is that these drugs must be delivered slowly and at low concentration to be effective. This then mimics the natural way of cytokine release. For this purpose, a suitable vehicle was developed, so called biomimetic coating, which can be deposited on metal implants as well as on biomaterials. Materials that are currently used to fill bony defects cannot by themselves trigger bone formation. Therefore, biological functionalization of such materials by the biomimetic method resulted in a novel biomimetic coating onto different biomaterials. Bone morphogenetic protein 2 (BMP-2) incorporated biomimetic coating can be a solution for a large bone defect repair in the fields of dental implantology, maxillofacial surgery and orthopedics. Here we review the performance of the biomimetic coating both in vitro and in vivo.

Keywords: Biomimetic; biphasic calcium phosphate coating; Bone morphogenetic protein-2 (BMP-2); bone tissue engineering.

INTRODUCTION:

Critical-sized bone defect
Adequate volume of bone tissue is of paramount importance to obtain the excellent restoration of maxillofacial aesthetics and musculoskeletal functions. The repair rate of a bone defect is mainly dependent on the bone wound size [1]. When the defect size is greater than the healing capacity of osteogenic tissues, the fibrous connective tissue, depending upon the faster migration mechanism of fibroblasts than osteoblasts, regenerates faster than bone tissue and becomes dominant in the bone defect [1-4]. Critical-sized bone defect (CSBD) is defined as the smallest size intraosseous wound that will not spontaneously heal completely with bone tissue, or the defects will heal by connective tissue during the lifetime of the animal [1-2]. The CSBD may therefore be considered to be the
prototype of discontinuity defects, as a condition of failed osteogenesis for overcoming the threshold of physiological repair processes.

The need for an appropriate bone substitute is significantly increasing: in 2004 alone, more than 1,100,000 surgical procedures involving the partial excision of bone, bone grafting, and inpatient fracture repair were performed in USA, at an estimated total cost of more than $5 billion [5]. And the United Nations and the World Health Organization has endorsed the years 2000–2010 as the Bone and Joint Decade [6-7] to recognize the global burden of musculoskeletal diseases. Although tremendous efforts have been made to solve the problem, current strategies encounter a variety of limitations and leave the effective healing of CSBD as an unsolved problem in implantology and orthopedics.

**Autografts and allografts:**

Autografts are still regarded as the ‘gold standard’ of bone repair because it can provide all the necessary osteogenic elements for bone regeneration such as osteoconductive 3-dimensional scaffolds, osteogenic cells and osteoinductive growth factors [8]. However, autografting is associated with a series of limitations such as the pain and morbidity of the donor site [9-10], prolonged surgery, and limited available volume [5]. Besides, when used to repair the defects on specific sites (e.g. orbital bone defects) where the restoration of the complicated anatomic structure is indispensable, autografts are hardly manipulated to reproduce the curvature of the local site [11-12]. Furthermore, resorption rates for endochondral bone have been reported up to 75% [13] and rates of 20%-30% [14] reported for membraneous bone grafts. The uncontrollable and variable spontaneous resorption of autografts may compromise the precision of the restoration of orbital volume and thus potentially lead to the asymmetry of eye globes [15].

The conventional alternative to an autograft is an allogeneic bone graft that is obtained either from cadaveric donors or from donors undergoing total hip arthroplasty [16-17]. Although it provides a good, natural, and bony scaffold, allogeneic bone carries certain risks such as disease transmission, [18] toxicity associated with sterilization, [19], variable host immune response [20] and limited supplies [8]. Also in some areas of the world, the practice of allogenic bone transplantation is culturally unacceptable.
Bone tissue engineering

Bone tissue engineering is an interdisciplinary field that combines the knowledge and technology of material engineering and biological factors to regenerate damaged bone tissues. It includes gene-, cell- and cytokine-based therapies and has been resorted by researchers to substitute autologous and allogeneic bone grafts [21]. Although gene- and cell-based therapies are attracting increasing attentions [22-26], they are still in their infancy regarding the safety and efficacy for humans [27]. In contrast, cytokine-based therapy is advantageous in safety, feasibility and practical potential for nearest clinical application over the gene- and cell-based therapies [28-29]. In the field of cytokine-based bone engineering, a consensus has been achieved that an appropriate carrier to deliver cytokines sustainably and in a physiological level is of paramount importance for osteogenic efficacy of the cytokines [29-31].

An ideal carrier should have the following properties:

1) not only be optimal in delivering cytokines but also be biocompatible to minimize host inflammatory reactivity;
2) be properly biodegradable to eliminate a second surgery within certain time;
3) be 3-dimensionally structured and surface-osteoconductive to support vascularization and bone ingrowth [21].

Although many efforts to develop this “ideal scaffold” have been made during the past decades, the criteria were far away from being satisfied by the current carrier strategies.

Compared with autologous and allogeneic grafts, either naturally derived mineral phase and organic matrix such as deproteinized bovine bone [32] and demineralized bone collagen [33] or synthetic polymers such as PLGA (co-polymer of lactic and glycolic acid), Polyactive (copolymer of polyethylene oxide and polybutylene terephthalate) were not limited in available volume and have been widely accepted for the reconstruction of a bone defect. However, they are neither highly osteoconductive to facilitate the adequate migration of osteogenic cells nor are they intrinsically osteoinductive. These are the two main stumbling blocks for their applications in CSBD.

This dilemma could be overcome by a process of consolidation, viz., by coating the synthetic polymers with a layer of calcium phosphate (CaP) [34].
Biomimetic coatings
The technology whereby calcium-phosphate layers are deposited upon a substratum has been so greatly improved during recent years as to render the process possible at physiological [35-36] rather than at grossly unphysiological temperatures (> 1’000 ºC) [37-39]. Moreover, the structure of the crystals formed (carbonated apatite) is more akin to that of bone mineral than are those of hydroxyapatite and tri- or tetracalcium phosphate [38], which are produced at exceedingly high temperatures.

Bioactive coatings
The beauty of the so-called biomimetic coating technique is that it can be rendered osteoinductive through the co-precipitation of an osteogenic agent and consequently, it is incorporated into the crystalline latticework of biomimetic coating [40-41]. Because such agents are proteinaceous and so inactivated at high temperatures, formerly they could be only superficially adsorbed onto a preformed biomimetic calcium phosphate coating [34]. When exposed to a physiological milieu, such a superficially adsorbed depot of an osteogenic agent is released so rapidly as to be exhausted within a few hours, and the transient high local concentration of the drug that is thereby generated may compromise osteogenic activity [42-43]. In contrast, a coating-incorporated depot of such an agent is liberated gradually [42] and in a cell-mediated manner over a period of several weeks [44]. Under these conditions, osteogenic activity can be efficaciously induced and sustained at both ectopic and orthotopic sites in animal models [42-43].

1. Deposition of biomimetic coating on bone substitutes
The concept of biomimetic mineralization was introduced by Kokubo and his colleagues in 1990 [36]. The layer of calcium phosphate that is produced can reduce fibrous encapsulation [45] and promote the differentiation of bone-marrow stromal cells into osteoblasts [46], as well as enhance the ingrowth of osseous tissue and its direct contact with the implanted materials [47-49]. This coating technique can be applied to various types of materials, including bioceramics [50], metals [51] and organic polymers [52]. However, using the original biomimetic technique, the successful coating of the materials can be achieved only on the surfaces with active chemical groups [53-55], that serve as nucleation sites for mineralization [55-57]. Active chemical groups such as...
dihydrogen phosphate (H$_2$PO$_4$) or carboxylic acid (COOH) have been shown to be highly conducive to biomimetic mineralization, and those composed of methyl groups (CH$_3$) to be unpropitious for the process [57]. But since many of the bone substitutes available on the market, especially the polymeric materials which lack active chemical groups on their surfaces, attempts have been made to attach functional anionic ones covalently [56]. However, most of the bone substitutes that are in clinical use are either not amenable to such manipulation, or, if they are, the modifications carry the risk of compromising the physicochemical and biological properties of the material. Moreover, not only the surface chemistry but also the surface geometry and the three-dimensional structure of a polymer can influence its biomimetic mineralization [58]. Different kinds of bone defect also resulted in the requirement of bone substitutes with different physiochemical properties: the voluminous bone defect needs block- or granule-structured bone substitutes, whereas the laminar bone defect needs membrane-structured ones. The variety of bone substitute makes it very difficult to modify their surface by mineralizing them. To overcome this dilemma, we refined a biomimetic coating technique which comprises two basic steps (Fig. 1) [59-60].
Fig. 1 Graph depicting the procedure of the biphasic biomimetic calcium phosphate coating. Step 1: the biomaterial is immersed in a 5-fold-concentrated simulated body fluid for 24 hours at 37°C. A fine, dense layer of amorphous calcium phosphate (ACaP) thereby formed serves as a seeding substratum for the deposition of a more substantial crystalline layer. Step 2: After freeze-drying, the crystalline layer will produced by immersing the ACaP-coated biomaterials in a supersaturated calcium phosphate solution for 48 hours at 37°C. The samples are then freeze-dried. If the crystalline layer is to be functionalized by the incorporation of bioactive agents such as bone morphogenetic protein-2 (BMP-2), then these agents are introduced into the latter medium at a certain concentration.

Using this biphasic biomimetic coating technique, not only titanium implants [40, 61-62] but also deproteinized bovine bone [63] and four polymeric bone substitutes [64-65] (seen in table 1) with different physiochemical properties can be biomimetically coated with a layer of calcium phosphate without any additional surface-modification (Fig. 2). The morphological and physicochemical properties of coatings were independent on either the surface chemistry, or the surface geometry or the three-dimensional structures of the underlying materials [65]. The consistency of the result is to be laid at the merits of the amorphous seeding layer. Previously, when this biphasic biomimetic coating technique was applied to titanium-alloy implants [42], the “adhesion” thereto of the amorphous layer of calcium phosphate was attributed to the affinity of Ca²⁺ and HPO₄⁻² ions.
for titanium [66-67]. But this chemical affinity may not be involved here since such an amorphous layer of calcium phosphate can also be precipitated on polymers with diverse surface chemistries in our studies. A more likely explanation is that the tiny particles of calcium phosphate - which are formed under the nucleation-inhibitory influence of Mg²⁺ [66] and HCO₃⁻ [68] – can be captured and immobilized on the substratum by a process of mechanical gomphosis. These particles then serve as seeding centres for the subsequent growth of a crystalline latticework of calcium phosphate under conditions that are conducive to nucleation [69]. A series of findings indicated that our biphasic biomimetic CaP coating technique has the potential to mineralize a broader-spectrum of bone substitutes without the need for any additional surface-modification.

<table>
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<th>Brand name</th>
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<td>Integra, USA</td>
<td>Collagen bovine deep flexor tendon</td>
<td>porous</td>
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<tr>
<td>Polyactive®</td>
<td>IsoTis B.V., Bilthoven, the Netherlands</td>
<td>70% polyethylene oxide (PEO) and 30% polybutylene terephthalate (PBT)</td>
<td>porous</td>
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<tr>
<td>Ethisorb™</td>
<td>Johnson &amp; Johnson, USA</td>
<td>g lactin 910 and P-dioxanon</td>
<td>fibrous</td>
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<td>PLGA</td>
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<td>poly(lactic-co-glycolic acid)</td>
<td>fibrous</td>
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<td>Bio-Oss®</td>
<td>Geistlich Biomaterials, Inc., Switzerland</td>
<td>deproteinized bovine bone (DBB)</td>
<td>porous</td>
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Table 1 The information of the biomaterials functionalized by BMP-2 incorporated biomimetic calcium phosphate coating.
Fig. 2 Scanning electron micrographs of the geometry at low magnification (first vertical panel) and the topography at high magnifications of six biomaterials in the native condition (second vertical panel), and after coating first with an amorphous seeding layer of calcium phosphate (third vertical panel) and then with a crystalline one, which was co-precipitated with bovine serum albumin [(BSA) fourth vertical panel]. The six materials include: one metallic material titanium plates; one bovine-derived mineral material Bio-Oss® granules (deproteinized bovine bone, DBB); one bovine-derived organic sponge Helistat® (collagen); and three synthetic polymeric membrane Polyactive®, Ethisorb™, and PLGA® (from up to down). Bars = 200μm (first vertical panel) and 5μm (second, third and fourth vertical panels). (Part of this figure was reproduced under the kind permission of the journal of Tissue Engineering and the publisher of Mary Ann Liebert, Inc. New Rochelle, NY)
In bone tissue engineering, a 3-dimensional scaffold needs the interconnective porosity to ensure the ingrowth of osteogenic cells and vascular cells. However, current direct coating techniques either biomimetic or unphysiological failed to mineralize on the deep surfaces of a 3-dimensional bone substitutes with complicated structures. This can be a major concern for scaffolds with small pores and the apatite on the pore surface can decrease the original scaffold pore size and even block some of the tiny pores. Li and coworkers reported an indirect coating preparation [70]. Apatite was first coated on the surface of paraffin spheres of the desired sizes. The coated paraffin spheres were then molded into foam. PLGA/pyridine solution was cast into the interspaces among the coated paraffin spheres. After the paraffin spheres were dissolved and removed by solvent, apatite was left on the pore surfaces inside the scaffold. However, the risk of some residual solvent, which is harmful to transplanted cells or host tissues [71-72], may increase dramatically because critical measure could not be taken to remove them under the presence of cytokines. Another possibility is to apply a dynamic perfusion technique to prepare coatings [58]. Dynamic conditions allowed for the production of thicker apatite layers as consequence of higher mineralizing rates, when comparing with static conditions. Micro-CT analysis clearly demonstrated that flow perfusion was the most effective condition in order to obtain well-defined apatite layers in the inner parts of the scaffolds.

2. Functionalization, coating thickness and incorporation rate of proteins

Although biomimetic calcium-phosphate coatings improve the osteoconductivity of metal implants, they do not confer on them the osteoinductivity. This is a feature of promoting the differentiation of immature progenitor cells down an osteoblastic lineage. To overcome this problem, one possible approach is to integrate functional biological agents, such as growth factors, into the biomimetic coating [73-75]. Growth factors are proteins that serve as signalling agents for cells and stimulate cellular differentiation, proliferation, migration, adhesion, and gene expression [76-79]. Bone tissue regeneration is an orchestrated process including angiogenesis, osteogenesis, and remodeling which can be the targets of various growth factors.

One of the most important growth factors is the sulfate-linked dimeric bone morphogenetic proteins (BMPs) which belong to the transforming growth factor-
β (TGF-β) superfamily and it can specifically induce ectopic bone formation. BMP-2 has been shown to be one of the most potent members of the BMP family for the induction of bone formation in vivo [42-43, 62, 80-82]. Other osteogenic BMPs, such as BMP-4 [83-84] and BMP-7 [85], were also shown to be potent in inducing bone regeneration. Very recently, we also described the functional characteristics of BMP-2/7 heterodimer (more potent BMP forms) during osteoblastogenesis in comparison with BMP-2 and BMP-7 homodimers. BMP2/7 showed a significantly lower effective-concentration range than the respective homodimers and thus it facilitated a promising application potential in tissue engineering [86]. Apart from the osteoinductive growth factors, angiogenic growth factors such as basic fibroblast growth factor (bFGF) [87], vascular endothelial growth factor (VEGF) [88] and platelet derived growth factor (PDGF) [89-91] were also shown to promote significantly bone regeneration in vivo since angiogenesis is an essential pre-requisite for bone survival and regeneration [92]. Furthermore, bone regeneration can also be promoted by other bioactive factors such as osteoclast-suppressive bisphosphonate [30] and cathepsin inhibitors, etc.

There is a general consensus that to maximize their osteoinductive efficacy, BMPs needs to be carried and released in a controlled and sustained way rather than in a burst. [21, 93]. Many control release systems including polymers, ceramics and hybrid materials have been developed and they have been review elsewhere [94-95]. In this review, we focus mainly on the materials functionalized by BMP-2-incorporated biomimetic calcium phosphate coatings. Using conventional coating methods, osteogenic agents can be deposited only superficially upon preformed coatings, either by adsorption [96-98], or by binding to biofunctional proteins [99] or by chemical treatment [100]. Although high protein incorporation efficiency (nearly 100%) is obtained with this method, the disadvantage of such attachment is that the biologically active molecules are released too rapidly to be effective upon exposure to a physiological environment [42-43]. Using the biomimetic technique, osteogenic agents can be incorporated into the crystal latticework of the coatings [101]. Evidence for the phenomenon is furnished elsewhere [42, 61, 101] and was confirmed in our recent study by Fourier-transform infrared spectroscopy [65]. The incorporated proteins can be released gradually and steadily at a low pharmacological level; not rapidly as in a single high-dose burst [42].
During the biomimetic approach, the osteoinductive protein is added to the modified-simulated body fluid (mSBF) solution and co-precipitated with the CaP coating onto the substrate material [41, 62, 102]. One disadvantage of this method is that the protein incorporation rate reported is relatively low, where approximately 3–15% of the proteins in the SBF were incorporated into the CaP coating [62, 101, 103]. This results in a waste of the extremely expensive osteoinductive proteins which gives a substantial burden on patients. Improvement in biomimetic coating technique to enhance the protein-incorporation rate is therefore needed to broaden their clinical applications. Yu, et al., attempted to improve the protein incorporation rate by carefully adjusting the substrate surface-area to SBF volume ratio (SSA/SV ratio) [104]. A low volume of SBF was employed to increase the SSA/SV ratio to assure that most of the calcium and phosphate ions in the SBF solution contribute to the coating formation. They also tried to optimize the protein loading efficiency by varying the specific configurations of the coating system such as solution height and container selection for the SBF. The loading efficiency of a model protein bovine serum albumin (BSA) was as high as 90% was achieved when the ratio of the substrate surface area to modified SBF (m-SBF) volume was as high as 0.072. The achievement of such a high incorporation rate is very important for decreasing the cost of growth factors that are used in biomimetic coatings. However, in this work, the final coating thickness was not clarified. Because a certain coating thickness is important for restoring an adequate amount of bioactive agents and ensuring an adequate length of time for the drug delivery to support bone formation [42]. Decreasing the volume of the coating solution will decrease the total content of calcium and phosphate and thus will definitely decrease the coating yield and thickness. So whether the decreased coating thickness could survive long enough to sustain the new bone formation is largely unknown. Moreover, when this method is applied to mineralize porous bone substitutes whose surface-area could be a dozen times larger than a plain titanium strip and the coating thickness could be further decreased.

The essential reason for increasing the incorporation rate of protein is to decrease the volume of the coating solutions and thus the total dose of proteins. In our recent study, another way was tried to decrease the solution volume without compromising the coating thickness. We significantly increased the concentration of calcium and phosphate in the coating solutions to 5 times higher
than the conventional ones we used. The preliminary results showed that a coating of a certain thickness can be achieved by using a dramatically smaller volume of the highly concentrated coating solution than the in a conventional one (unpublished data). However, the reaction condition still needs to be optimized since a slight change both in pH value of coating solution and in chemical components will either result in the failure of the coating or the changes of coating properties [105-106].

3. Release mechanisms of coating-incorporated BMP-2

The slow release kinetics of proteins can be obtained by many delivery modes such as entrapping agents into the carrier materials (e.g. polymers and compounds of calcium phosphates and gelatin), or binding proteins to titanium implants following chemical modification of the metal surface, etc [81, 107-108]. The mechanisms of slow delivery of growth factors by carriers can either be (i) diffusion-controlled, (ii) chemical and/or enzymatic reaction-controlled, (iii) solvent-controlled, or (iv) controlled by combinations of these mechanisms [85, 109]. The slow release of incorporated proteins into biomimetic coatings was also orchestrated by the combination of several mechanisms. Initially, a burst release (higher release rate) of incorporated proteins can be detected when exposed to an in-vitro release system-phosphate buffer saline (PBS) with a pH value of 7.4 [65]. This release lasts 3 to 5 days in a very high release rate and it can be attributed to the diffusion-controlled release which is governed by the solubility and diffusion coefficient of the protein, protein partitioning between the aqueous medium and material of the delivery system as well as the protein loading and the diffusional distance [110]. This release pattern always occurs to the adsorbed proteins and influenced by the three dimensional structures for steric hindrance. A typical example of such a release is the release of BMP-2 from porous scaffolds that was regulated via adjustment of pore size [111] or particle size [112]. In accordance with this research in our study, the influence of 3-dimensional structures in the burst release rate was also shown by the initial burst release rates of coating-incorporated protein from the two porous polymers (11% per day) which were significantly higher than those from the two fibrous polymers (6-8% per day). The existence of a larger depot of entrapped and incidentally-adsorbed proteins in association with the porous structure than with the fibrous structures would account for the phenomenon [65]. Burst release of
BMP-2 may cause a transient local high concentration of BMPs which may potentially over-stimulate the osteoclastic activity [113], and compromise early osteogenic activities [86]. Therefore, further efforts should be made to reduce the ‘burst release’.

Subsequent to the diffusion-controlled release, a slow release profile of incorporated proteins was exhibited over a monitoring span of 5 weeks after which around 65-75% for the two porous carriers and 30-40% for the two fibrous carriers of the total protein loading remained [65]. The protein release rates for the four carriers were similar during this slow release period and the release could be attributed to a chemical reaction-controlled process viz., coating dissolution [65]. In contrast, the adsorbed protein only exhibited a burst release for 3-5 days and then a complete clearance [65].

Until now, investigations of release kinetics were performed by incubating protein-containing biomaterials in physiological solutions such as cell culture media or simulated body fluid, thus addressing the passive, noncell-mediated release of the proteins from the carrier only. In vivo, however, inflammatory cells such as macrophages/foreign body giant cells (FBGCs, multinucleated giant cells which mediate foreign body reaction and eliminate foreign bodies) and osteoclasts may interact with the biomaterial. Therefore, apart from the aforementioned mechanisms, the release of incorporated proteins can also result from the interaction of host tissues. When coatings were implanted in vivo, multinucleated cells such as FBGCs and osteoclasts could resorb the coatings and form a resorption lacuna on the lattice work of coatings [42]. It is reasonable to assume that cell-mediated protein liberation significantly influences the temporal bioavailability of growth factors within a biomaterial implantation site. This suggestion is supported by a recent in-vitro study by Lee et al. [114] who found a significant increase in the release of BSA from polymer scaffolds in the presence of rat vascular smooth muscle cells when compared with the passive release of the protein into DMEM culture media.

**Osteoclast-mediated release of growth factors from natural bone matrix**

The biomimetics of BMP-2-incorporated CaP coating lies in that coating degradation and the release of the growth factor also partially follows the principles of natural bone remodeling (Fig. 3). With the natural bone formation, a large amount of growth factors such as BMPs, insulin-like growth factor (IGF) I and II and transforming growth factor (TGF)-β are pre-embedded in the bone...
inorganic mineral phase. Osteoclasts resorb bone by secreting hydrions and cathepsin K into specialized extracellular hemivacuole formed at the interface between cell and bone [115]. While hydrions dissolve bone mineral, Cathepsin K can degrade native collagen and dissolves the organic matrix [116]. When the matrix was resorbed by osteoclast, the pre-embedded growth factors could be excavated with matrix dissolution, and activated by the prevailing acid pH in the resorption lacuna of osteoclast. Finally the growth factors will be released and are thus becoming available to act upon osteoblast precursors to promote bone formation. The theory was proposed by Baylink, Howard and colleagues [117] as a pathway of a local ‘coupling factor’ linking bone resorption to subsequent formation.

**Cell-mediated release of the incorporated proteins from the biomimetic coating**

Interestingly in our technique the growth factors such as BMP-2 were also pre-embedded in the mineral phase-CaP coating. When the titanium implants with BMP-2-incorporated coatings were implanted subcutaneously in rats, multinucleated cells such as FBGCs and osteoclasts were found on the surface of coatings and formed resorptive lacuna on the coatings [42]. By the fifth week, the mild acute inflammatory response was almost completely quelled, but the resorption of coatings by FBGCs and osteoclasts continued.

The release of coating incorporated proteins mediated by osteoclasts was corroborated by our previous study [44]. In biphasic calcium phosphate (BCP), ceramics adsorbed with [\(^3\)H]-labeled BSA ([\(^3\)H]-BSA) the release kinetics were not modified by the presence of monocytes/macrophages or osteoclasts, showing that the release of the protein was induced exclusively by passive mechanisms. Superficial adsorption does not provide the possibility of modulating the liberation of proteins by means of cell-mediated release. When [\(^3\)H]-BSA was co-precipitated to BCP ceramics, bone marrow cell (BMC)-derived osteoclasts markedly influenced the liberation of [\(^3\)H]-BSA inducing a sustained, cell-mediated protein release. The cell-mediated release was found exclusively with BMCs’ differentiated toward osteoclasts. In contrast, BMC’s differentiated toward monocytes/macrophages did not modify the release kinetics of [\(^3\)H]-BSA. Although the cell-mediated release of coating incorporated proteins by osteoclasts was proved by others’ and our studies, whether such release mode can also be mediated by FBGCs remains unclear. Although FBGCs are considered to originate from the fusion of monocyte-macrophage lineage cells and have been
induced in vitro from human blood monocytes as the Langhans’ giant cell and the osteoclasts, molecular and cell biology studies have shown that the FBGC has distinctly different functional and phenotypic characteristics [118]. In vivo, FBGCs often occupied portions of the coatings that were not covered with bone [42, 63], which did not show a coupling manner.

In summary, BMP-2 incorporated into biomimetic coatings exhibited three release mechanisms: initial diffusion controlled burst release, release chemically controlled by coating dissolution, and cell (osteoclast)-mediated release.

Fig. 3 Graph depicting both osteoclastic release of preembedded growth factors in bone matrix during bone remodeling (A1, A2) and the osteoclast-mediated release of growth factors incorporated into the calcium phosphate coatings prepared on substrates (B1, B2).

4. **In vivo degradation of biomimetically coated substrate**

The basic function of bone substitutes is to provide a 3-dimensional scaffold for the migration and proliferation of osteogenic and angiogenic cells. After the establishment of a bone and vascular system, the substitute should be completely degraded and eventually replaced by natural bone [21]. Therefore, a certain degradation rate of a bone substitute is important if it is to fulfill its function: a too rapid degradation of bone substitutes will result in the disorder of osteogenic
activities and the dominance of connective tissue healing; on the other hand, a too slow degradation will hinder the replacement by natural bone. Many efforts have been made to modulate the degradation rate of bone substitutes [21, 119-120]. As a methodology to improve the osteoconductivity of and to confer the osteoinductivity to bone substitutes, BMP-2-incorporated CaP coating furnishes the substitutes with a layer of calcium phosphate of a certain thickness and induces a series of biological activities, which can also modulate the degradation rate of biodegradable bone substitutes. The modulating effect of the BMP-2-incorporated CaP coating on the degradation rate of the bone substitutes is complicated with many factors such as biocompatibility and biodegradability of bone substitutes, protein incorporation and the subsequent osteogenic activities.

The biomimetic coating prepared by our technique is consistent in chemical composition and physical morphology with the substitutes of different physiochemical properties [65]. After implantation, it is the coating instead of the bone substitutes themselves that interact directly with the host tissues and determine the formation of FBGCs. The intrinsic biodegradability of the five kinds of biomaterials selected in our experiments was examined in subcutaneous sites after a 5-week implantation. The highest degradation rate occurred to collagen which was completed degraded within the monitoring span. The lowest degradation occurred to the Bio-Oss® and Polyactive® in which no significant degradation could be detected. Degradation rates of PLGA and Ethisorb™ fell in between. The degradation rates of each kind of bone substitute that bore an adsorbed depot of BMP-2 were similar to the respective untreated ones. This suggested that the adsorbed BMP-2 of the amount used in our study did not drastically affect the degradation rate of bone substitutes. Accordingly, the volume densities of the FBGCs within the bone substitutes with adsorbed BMP-2 were also similar to those of the respective untreated bone substitutes. The finding was contradictory to previous studies in that adsorbed BMP-2 was shown to increase the degradation of neutralized glass ceramic [121]. This inconsistency may result from the relatively lower amount of BMP-2 and thus the subsequent smaller bone volume induced by the adsorbed BMP-2 in our experiment.

The volume of remaining collagen biomimetically coated was significantly higher than the untreated Helistat®. However, for the other three polymers and the deproteinized bovine bone, we did not find such a significant difference. The results indicated the protraction effect of coating on the degradation rate of bone
substitutes can only be apparent for the relatively highly degradable material such as Helistat®. It is of great interest that the volume of remaining materials such as Helistat®, PLGA and Ethisorb™ that were functionalized with BMP-2-incorpororated CaP coating were significantly lower than respective ones that with CaP coating only. For PLGA and Ethisorb™, the volume of remaining material was also significantly lower than with the other three groups respectively. Since the BMP-2 alone (adsorbed) did not alter the degradation of bone substitutes, it must be the BMP-2-induced osteogenic activities that contribute to such an increase in degradation rate of Helistat®, PLGA and Ethisorb™. What is even more interesting is that the volume density of FBGCs was found to be lower surrounding the bone substitutes with coating incorporated BMP-2. The mechanism accounting for the relatively higher degradation rate of the bone substitutes with coating-incorporated BMP-2 is still a mystery to be discovered.

5. Osteogenic activities induced by coating-incorporated BMP-2

BMP-2 has already been intensively investigated in recent years to enhance bone formation. Hitherto, there is a general consensus that to maximize their osteoinductive efficacies, growth factors such as BMPs need to be released in a controlled and sustained rather than in a burst manner [21, 93, 122-123]. Using our technique of biomimetic production of BMP-2- incorporated CaP coatings, BMP-2 of a pharmacologically low dose is thereby incorporated into the inorganic crystal latticework and released gradually in a cell-mediated, physiological-like manner. This mode of BMP-2 delivery is more conducive to sustain osteogenic activity in comparison with the adsorbed BMP-2. Significantly higher volume densities of newly formed bone tissue were consistently induced by coating incorporated BMP-2 rather than by the adsorbed BMP-2 of similar amounts for metallic implants [42-43], deproteinized bovine bone [63] and four kinds of polymers [64] respectively (Fig. 4).
Scarlet newly regenerated bone tissue (pointed by black arrow ↑) was extensively deposited along the surface of the two materials when they were functionalized by coating-incorporated BMP-2; tremendous amount of bone marrow tissue (black flower * ) filled in the interstitial space. In contrast, bone tissue was sporadically deposited along the surface of the two materials that were with an adsorbed depot of BMP-2; bone marrow tissue was rarely found in this group.

Besides, the 3-dimensional structure of bone substitutes is also a determining factor on bone regeneration. In the current concept of bone tissue engineering, the 3-dimensional geometry of the bone substitutes can be optimized to enhance their osteoconductivity for rapid bone ingrowth. A design standard like interconnective porous structure with pore size >300µm and porosity <90% was
recommended due to enhanced new bone formation and the formation of capillaries [124]. However, when functionalized by our technique, the bone substitute like Polyactive®, that bore such a typical structure as recommended, resulted in a significantly lower volume density of bone tissue than the other three polymers [64]. Furthermore, for the bone substitutes that are functionalized by the technique of BMP-2-incorporated biomimetic coating, the volume density of newly regenerated bone after 5-week implantation correlated proportionally to the surface-area density of polymers at time 0 [64]. Hence, for polymers that bore a BMP-2-functionalized calcium-phosphate coating, the surface-area density of the polymer itself overrode the influence of the other characteristics on bone formation, such as chemical composition or macroscopic form (fibrillar or sponge-like). The mechanism accounting for this phenomenon is not yet clarified. One explanation is that the higher surface area-density can provide an even slower release profile of BMP-2 in vivo and the relatively lower tissue permeation also extended the release of BMP-2. Another possibility is that the compacted fibrous structures could provide a relatively stable 3-dimensional environment and a good support for newly generated bone [125]. This hypothesis can be supported by our data that showed that among the four polymers with adsorbed BMP-2, volume density of bone tissue was also the highest within Ethisorb™ which bore a compacted fibrous structure and a highest surface-area density [64]. However, these mechanisms are still insufficient to explain the proportional correlation between surface-area density and volume density of newly formed bone tissue. In a very recent study, we mapped the spatial characteristics of osteogenic activities within the functionalized Ethisorb™ patch. These findings gave us some clues for the correlation. When BMP-2 was adsorbed to the Ethisorb™, newly formed bone occurred in the interval connective tissue (connective tissue-located intramembraneous ossification) and do not directly contact with Ethisorb™ fibers. In contrast, when BMP-2 was delivered by coating-incorporation, apart from the connective tissue-located intramembraneous ossification, a characteristic ossification type—“BMP-2 incorporated coating-originated intramembraneous ossification” could be found within the functionalized Ethisorb™ (unpublished data). This type of ossification originated directly on the surface of BMP-2-incorporating coating that was deposited on Ethisorb™ fibers and formed an “ossification ring”. In the primary stage of this type of ossification, no ossification could be found in the
surrounding connective tissue. This suggested that the coating-immobilized BMP-2 could also exert the osteoinductive effect and enrich osteoblasts directly on the interface between coating and surrounding tissues. After the formation of “ossification rings”, osteotoid formed centering on the ossification rings with scattered calcified points. The more calcified points could be found in the closer area to the original ossification rings. The calcified points eventually joined and formed a calcified woven bone surrounding the original ossification rings. These specific spatial characteristics suggested that this type of ossification was initiated and motivated by the coating-incorporated BMP-2. Within the inner space of BMP-2 incorporated coating functionalized Ethisorb™ discs, the “BMP-2 incorporated coating originated intramembraneous ossification” was the main type of bone formation and the unique mechanism for the advantages of functionalized Ethisorb™ discs in bone regeneration over the Ethisorb™ discs with adsorbed BMP-2.

This ossification may provide an explanation for the dependence of bone formation on the surface-area density of the functionalized materials. The technique thus changed the current concept in tissue engineering where the pore size and porosity are heavily emphasized [124].

With the process of bone regeneration, bone marrow tissues can also be induced by both coating-incorporated BMP-2 and adsorbed BMP-2. The interstitial space of Bio-Oss®/polymers with BMP-2-incorporated CaP coating and newly formed bone were filled with a significantly higher amount of bone marrow than Bio-Oss®/polymers with adsorbed BMP-2 [63-64]. This phenomenon can be observed within all the selected substrates after a 5-week implantation. The regeneration of bone marrow tissue is of great importance because it plays critical roles in many biological functions such as nutritious resources. Marrow stromal cells (MSCs) comprise a heterogeneous population of cells, including reticular endothelial cells, fibroblasts, adipocytes, and osteogenic precursor cells that play a role in the regulation of hematopoiesis [126-128]. The occurrence of tremendous bone marrow tissue indicated that the coating-incorporated BMP-2 not only brought massive bone but also conferred the complete biological functions to the tissue.
6. Inflammation-modulating factors

Host reactions following the implantation of biomaterials include injury, blood–material interactions, provisional matrix formation, acute inflammation, chronic inflammation, granulation tissue development, foreign body reaction, and fibrosis/fibrous capsule development [118, 129-130]. All foreign materials for bone substitute, like polymers, deproteinized bovine bone or ceramic, trigger an inflammatory response in which macrophages and foreign-body giant cells participate (Fig. 5) [131-132]. The pro-inflammatory cytokines that are released by migrant T-lymphocytes can suppress bone formation [133]. This osteoinhibitory cytokine signal far outweighs the potentially osteoinductive one that emanates from macrophages in the form of BMP-2 release. As a result of this inflammatory reactivity, the polymeric scaffold becomes ensheathed by a capsule of dense, fibrous connective tissue. The walling-off of the implanted material impedes its osseointegration with the surrounding tissue. This situation can be turned over from pro-fibrogenic to pro-osteogenic by using an osteogenic agent such as BMP-2.

In our studies, due to the highly aseptic operation, there is not a persistent acute inflammation to the materials and thus we focused mainly on the long-term host foreign body reaction to implants including FBGCs and dense fibrous capsulation which may lead to the failure of implants. To mimic the pro-fibrogenic environment in large volume or large area critical-sized bone defect, a subcutaneous ectopic bone induction model was adopted. After a 5-week implantation, significantly lower volume densities of FBGCs were found within the polymers with coating-incorporated BMP-2 than the controls such as the polymers with adsorbed BMP-2, polymers with coating alone. The phenomenon could be found for three polymers such as Polyactive®, PLGA and Ethisorb™ [64], except for collagen which degraded so fast that no collagen remained after a 5-week implantation and thus no FBGCs reactions could be detected in the two groups without coatings. A consistent result was also found for metallic materials [42] and deproteinized bovine bone [63]. The findings indicated that the BMP-2 incorporated biomimetic coating not only induced and sustained bone formation with a higher efficiency but also reduces the host inflammation such as foreign-body giant cell formation. The mechanisms accounting for the phenomenon remain unclear. However, past research provide some clues for the possible pathways.
Fig. 5 Light micrographs of cross sections through discs of the two polymer types (A: Polyactive®, B: PLGA) in the group of unfunctionalized uncoated polymer Polymer only), 5 weeks after subcutaneous implantation in rats and stained with McNeil’s Tetrachrome, basic Fuchsin and Toluidine Blue O. Bars = 30µm.

(A) The surface of pink Polyactive® (labeled by black pentagons) was tightly covered by a deep-blue spindle-like foreign-body giant cell with several nuclei (pointed by black arrows) and ruffled edges. (B) Light-blue elliptic or round cross-sectioned PLGA fibers (labeled by black pentagons) sporadically distributed in connective tissues. In the middle of the graph, a PLGA fiber was embraced and tightly covered by a deep-blue foreign-body giant cell with more than 10 nuclei (pointed by black arrows).

Firstly, FBGC formation is dependent on the surface properties of implants. When cells approach an implant material, it is unlikely that they will make direct contact with its surface. Rather, the rapid adsorption of proteins from blood (or
serum) effectively translates the structure and composition of the foreign surface into a biological language [134]. The apatite coating can reduce fibrous encapsulation, [45] promote bone ingrowth, enhance direct bone contact, [49] and has also been shown to promote differentiation of bone marrow stroma cells along osteogenic lineage [46]. This may be partially due to the enhanced adsorption of both fibronectin and vitronectin on calcium phosphate, compared with titanium, corresponding to a significant increase in osteoblast precursor attachment [135]. In our very recent study, after a 14-day implantation when the coating still remains, the volume densities of FBGCs, those in the groups of Ethisorb™ coated with or without incorporated BMP-2, were significantly lower than those in the groups of Ethisorb™ uncoated with or without adsorbed BMP-2 (unpublished data). However, the influence of coatings on the foreign body reaction can only be apparent before the complete degradation after which the biocompatibility of underlying substrates becomes dominant in determining the foreign-body reaction. This could account for the phenomenon that volume densities of FBGCs in the groups of coated polymer or deproteinized bovine bone were similar to those in the groups of polymer or deproteinized bovine bone either alone or with adsorbed BMP-2 after a 5-week implantation [64].

The foreign body reaction was found to be significantly reduced in the presence of coating-incorporated BMP-2. In contrast, the BMP-2 adsorbed onto the substrates, even though in the same or similar amount as the coating-incorporated one, failed to do so [63-64]. The findings suggested that the biomimetic coating and its incorporated BMP-2-induced osteogenic activities instead of BMP-2 itself accounted for the significantly reduced inflammatory reaction. In our very recent study, the advantage of coating incorporated BMP-2 in suppressing FBGC reaction was not apparent before the vigorous regeneration of bone tissue. Therefore, the BMP-2 induced osteogenic activities should account for the phenomenon (unpublished data). The mechanism may lie in both the blocking of the antigen presentation and the biochemical depression of the inflammatory reaction by the newly formed bone tissue. The coating can significantly improve the osteogenic cells adhesion and thus expedited the deposition of bone tissue which can shield the antigen-presentation of the overlying substrates. On the other hand, osteopontin (CD44 ligands) regenerated during BMP-2-induced bone formation may combine competitively with the CD44 surface receptors on macrophages, and thus inhibit their multi-nucleation process [136] which is
indispensable for the formation of FBGCs [137]. In our study for Bio-Oss®, the volume densities of dense fibrous capsule surrounding the Bio-Oss® with coating was significantly lower than those surrounding the Bio-Oss® with adsorbed BMP-2 or untreated. And the dense fibrous capsule could be further significantly reduced when BMP-2 was incorporated. Enhanced vascularization during BMP-2-induced osteoinductive activities [138] was shown to reduce fibrotic activity [139]. The effect may be mediated partially by the increased access of plasma fibronectin, which could significantly reduce the thickness of fibrous capsules [140]. Moreover, newly formed bone marrow tissue (Fig. 4) may also suppress the inflammation. In bone marrow, a population of nonphagocytic and fibroblast-like adherent cells termed the mesenchymal stem cell, [141-144] can suppress activation of T cells, modulate the immune function of the major cell populations involved in alloantigen recognition and elimination, including antigen presenting cells, and natural killer cells [145]. MSCs are capable of mediating the immunosuppressive effect and decreasing the production of inflammatory cytokines [146-147], which may partially account for the reduced inflammatory reaction such as FBGCs and fibrous capsules in our experiment.

7. CONCLUSION:
The repair of critical-sized bone defects remains a formidable challenge in the fields of implantology, maxillofacial surgery and orthopedics. The traditional bone fillers such as autografts and allografts are associated with various disadvantages. As alternatives, both synthetic and xenograft materials are developed. However most of these materials are neither intrinsically osteoinductive nor highly osteoconductive. Cytokine-based tissue engineering is a promising strategy to solve the problem. Various slow control release systems of bioactive cytokines have been developed to promote bone regeneration basing on different scaffolds. Compared to some other techniques, the biphasic biomimetic calcium-phosphate coating exhibited a very broad applicability to the biomaterials that were of different 3-dimensional geometries, surface topographies, and surface chemistries. The biomimetic coating enabled a controlled, local and slow release of BMP-2 in three mechanisms: initial diffusion controlled burst release, release chemically controlled by coating dissolution, and cell (osteoclast)-mediated release. The biomimetic coating can protract the in vivo degradation of biomaterials of a high but not low
degradability. The coating-incorporated BMP-2 induced the vigorous regeneration of bone and bone marrow tissues in a significant highly efficiency than the adsorbed BMP-2. Meanwhile, the host foreign body reaction to the implanted biomaterials could be significantly reduced through many pathways. Further studies of this technique can focus on several aspects such as the coating preparation in a voluminous three dimensional scaffolds, the mechanisms of immunosuppression and the adoption of more potent BMP forms like BMP2/7 heterodimers, etc.

The biomaterials that are functionalized using the biomimetic calcium phosphate coating technique can be a promising therapy for the bone engineering of critical-sized bone defects.
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Chapter 2

Biomimetic coating of organic polymers with a protein-functionalized layer of calcium phosphate: The surface properties of the carrier influence neither the coating characteristics nor the incorporation mechanism or release kinetics of the protein

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doi:10.1089/ten.tec.2009.0588
Published online 20 Apr 2010.
ABSTRACT:

Polymers that are used in clinical practice as bone-defect-filling materials possess many essential qualities, such as mouldability, mechanical strength and biodegradability, but they are neither osteoconductive nor osteoinductive. Osteoconductivity can be conferred by coating the material with a layer of calcium phosphate, which can be rendered osteoinductive by functionalizing it with an osteogenic agent. We wished to ascertain whether the morphological and physicochemical characteristics of unfunctionalized and bovine-serum-albumin (BSA)-functionalized calcium-phosphate coatings were influenced by the surface properties of polymeric carriers. The release kinetics of the protein was also investigated. Two sponge-like materials (Helistat® and Polyactive®) and two fibrous ones (Ethisorb™ and PLGA) were tested. The coating characteristics were evaluated using state-of-the-art methodologies. The release kinetics of BSA was monitored spectrophometrically. The characteristics of the amorphous and the crystalline phases of the coatings were not influenced by either the surface chemistry or the surface geometry of the underlying polymer. The mechanism whereby BSA was incorporated into the crystalline layer and the rate of release of the truly incorporated depot were likewise unaffected by the nature of the polymeric carrier. Our biomimetic coating technique could be applied to either spongy or fibrous bone-defect-filling organic polymers, with a view to rendering them osteoconductive and osteoinductive.

Key words: Bone; Biomimetic coating; Polymeric scaffolds

INTRODUCTION

Since large (critical-sized) osseous defects do not heal spontaneously, clinical intervention is necessary to restore the tissue. Due to the limited availability of autologous and allogeneic bone, as well as to the drawbacks that are associated with the transplantation of such tissue [1-4], synthetic bone substitutes have been sought [5, 6]. Ideally, the material should be mechanically strong, biocompatible, biodegradable and osteoconductive. In addition, it should be accommodative to
functionalization with an osteogenic agent, which would confer it with the property of osteoinductivity [7]. Various types of material have been tested, none of which are optimal: ceramics, although osteoconductive, are deficient in other respects [5], whereas synthetic polymers satisfy [to a greater or lesser degree] each of the requirements except osteoconductivity. This dilemma could be overcome by a process of consolidation, viz., by coating the synthetic polymers with a layer of calcium phosphate [8].

The technology whereby calcium-phosphate layers are deposited upon a substratum has been so greatly improved during recent years as to render the process possible at physiological [9, 10] rather than at grossly unphysiological temperatures [> 1\’000 °C] [11-13]. Moreover, the structure of the crystals formed [carbonated apatite] is more akin to that of bone mineral than is the hydroxyapatite or tri-/ tetracalcium phosphate [11], that is produced at exceedingly high temperatures. One of the main assets of the so-called biomimetic coating technique is that it renders possible the co-precipitation of an osteogenic agent and, consequently, its incorporation into the crystalline latticework of the mineral layer [14-16]. Formerly, such agents, being proteinaceous and thus inactivated at high temperatures, could be only superficially adsorbed onto a preformed mineral layer [17]. In a biological milieu, a depot of an osteogenic agent that has been merely adsorbed onto a preformed calcium-phosphate coating is released so rapidly as to be exhausted within a few hours, and the transiently high local concentration of the drug that is thereby generated is not conducive to sustained bone-formation activity [18]. On the other hand, a coating-incorporated depot of such an agent is liberated gradually and in a cell-mediated manner over a period of some weeks [19]. Under these conditions, bone-formation activity can be efficaciously induced and sustained at both ectopic and orthotopic sites in animal models [18, 20].

The purpose of the present study was to ascertain whether the morphological and physicochemical characteristics of unfunctionalized and bovine-serum-albumin[BSA]-functionalized calcium-phosphate coatings, as well as the release kinetics of the protein, were influenced by the surface properties of the underlying polymeric material. For this purpose, we tested four polymers: two with a sponge-like structure [Helistat\textsuperscript* (Collagen) and Polyactive\textsuperscript* (a co-polymer of ethyleneoxide and butylene terephthalate)] and two with a fibrous one
[Ethisorb™ (a co-polymer of glactin and \(\rho\)-dioxanon) and PLGA (a co-polymer of lactic and glycolic acids)].

MATERIALS AND METHODS

Experimental design
In this study, four polymers (Helista®, Polyactive®, Ethisorb™ and PLGA) with different surface geometries and chemistries were coated according to a two-step biomimetic procedure with a biphasic layer of calcium phosphate, which was either unfunctionalized or co-precipitated with the model protein BSA. The polymers and the coatings were characterized by scanning electron microscopy, histomorphometry (polymers only), Fourier-transform infrared spectroscopy and X-ray diffractionometry. The pattern of distribution of the amorphous seeding layer of the coating (labelled with Rhodamine), and of the BSA [conjugated with fluorescein isothiocyanate (FITC)] that was co-precipitated with the crystalline one, were mapped by confocal laser-scanning, dual-channel-fluorescence microscopy. The release kinetics of coating-incorporated and of adsorbed (control) FITC-BSA was monitored spectrophotometrically in vitro.

Polymeric materials
Of the four polymers tested, three are synthetic (Polyactive®, Ethisorb™ and PLGA) and one is natural (Helista®). Helista® (Integra, USA) is a sponge-like material, which is manufactured from natural collagen. Polyactive® (IsoTis B.V., Bilthoven, the Netherlands) is likewise sponge-like in appearance, but is a synthetic co-polymer of ethyleneoxide terephthalate and butylene terephthalate. Ethisorb™ (Johnson & Johnson) and PLGA (undergoing clinical trials by, and received as a gift from, Smith and Nephew, UK) are both fibrous in nature, the former being a co-polymer of glactin and \(\rho\)-dioxanon, and the latter one of lactic and glycolic acids.

Characterization of the surface geometry and chemistry of the four polymers
To reveal the surface geometries of the four polymers, these were examined in a scanning electron microscope (XL 30, Philips, the Netherlands). For this purpose, 1-cm-diameter discs of the material were mounted on aluminium stubs and sputtered with gold particles to a thickness of 10-15 nm. The surface-area density and the porosity of each polymer type were estimated histomorphometrically. 1-cm-diameter discs of the four different materials were
dehydrated in ethanol and embedded in methylmethacrylate according to a standard protocol [21]. By applying a systematic random-sampling strategy [22], ten 600-µm-thick vertical sections, 1 mm apart, were prepared from each disc using a diamond saw (Leica, Switzerland). The slices were mounted on plexiglass holders, polished down to a thickness of 80 µm, and surface-stained with McNeal’s Tetrachrome, basic Fuchsin and Toluidine Blue O [21]. The sections were examined in a Nikon-Eclipse E-1000 light microscope. By applying a systematic random-sampling strategy, 23-30 fields were selected and photographed in colour at final magnifications of approximately x 400 (Helistat®), x 200 (Polyactive®) and x 300 (Ethisorb™ and PLGA). By applying stereological principles, these prints were used to estimate the surface-area density and the porosity (volume density of the internal space) of each polymer. The former parameter was estimated using a cycloid test system [23] and the latter by point counting [24].

The chemical groups of which each material is composed were revealed by Fourier-transform infrared spectroscopy (Spectrum 1000, Perkin-Elmer, Germany). For this analysis, 1-cm-diameter discs of the material were frozen in liquid nitrogen for 10 minutes, pulverized, and then mixed with powdered potassium bromide for compression into pellets. The phase composition of each material was analyzed by X-ray diffractionometry (X’Pert PRO, PANalytical, the Netherlands) using a scanning range [20] of 2.00-60.00°, a scanning speed of 2.00° per minute and a scanning interval of 0.02°.

Coating procedure
1-cm-diameter discs of each polymer [with thicknesses of 0.6 mm (Ethisorb™), 1 mm (Helistat®) and 2 mm (Polyactive® and PLGA)] were immersed in 20ml of 5-fold-concentrated simulated body fluid (684mM NaCl, 12.5mM CaCl₂·2H₂O, 5mM Na₂HPO₄·2H₂O, 21mM NaHCO₃) under high-nucleation conditions, viz., in the presence of 7.5mM MgCl₂·6H₂O, to inhibit crystal growth, for 24 hours at 37°C. The fine, dense layer of amorphous calcium phosphate thereby formed serves as a seeding substratum for the deposition of a more substantial crystalline layer. This was produced by immersing the samples in 10 ml of a supersaturated solution of calcium phosphate (40mM HCl, 4mM CaCl₂·2H₂O, 136mM NaCl, 2mM Na₂HPO₄·2H₂O), which was buffered to pH 7.4 with 50mM TRIS, for 48 hours at 37°C [15, 25]. If the crystalline layer was
to be functionalized by the incorporation of BSA, then this protein was introduced into the latter medium (0.1 mg/ml). A portion of the samples that bore only an amorphous layer of calcium phosphate were reserved for analytical purposes. These samples, as well as those that bore either an unfunctionalized or a BSA-functionalized bilayer of calcium phosphate, were freeze-dried for at least 24 hours prior to their characterization.

Characterization of the coatings

Analogous to the native polymeric materials, the amorphous seeding layer of calcium phosphate, as well as the unfunctionalized and the BSA-functionalized bilayers, with which they were coated, were analyzed by scanning electron microscopy, Fourier-transform infrared spectroscopy and X-ray diffractometry. Scanning electron microscopy was conducted in conjunction with an energy-dispersive X-ray analysis, which revealed the relative densities of calcium and phosphorus that were present, from which data the calcium-to-phosphorus ratios were calculated.

Distribution of the coating material and of coating-incorporated BSA

By appropriate labelling of the amorphous seeding layer of calcium phosphate and of the depot of BSA that was co-precipitated with the crystalline one, the distribution of the coating material and of the incorporated protein can be mapped by confocal laser-scanning, dual-channel-fluorescence microscopy. To this end, Rhodamine B (0.1 mg/ml) was introduced into the 5-fold-concentrated simulated body fluid (see section 2.3), and FITC-conjugated BSA (Sigma) into the supersaturated solution of calcium phosphate (see section 2.3). For the analysis in a confocal laser-scanning microscope that was equipped for fluorescence imaging (Zeiss LSM 510 META with LSM 510 acquisitions software and images 3D software), freeze-dried samples (see section 2.3) were embedded in methylmethacrylate. 600-μm-thick sections were prepared from the embedded material, affixed to Plexiglass holders, and ground down to a thickness of 80μm. The distribution of the amorphous layer of calcium phosphate was revealed by a red fluorescent signal; that of the BSA and, by implication, that of the crystalline layer, was disclosed by a green one.

Release kinetics of BSA in vitro

The release kinetics of coating-incorporated depots of protein was spectrophotometrically monitored over a 35-day period in vitro using samples that had been functionalized with FITC-BSA. For the purpose of comparison,
FITC-BSA was directly adsorbed onto a portion of native. This was achieved by applying a 10-μl-drop of a stock solution of FITC-BSA (5μg/ml) to each side of the 1-cm-diameter, freeze-dried discs of the coated polymeric materials in turn, allowing for complete evaporation at ambient temperature between the applications.

Each sample (n = 6 for each polymer type) was introduced into a 15-ml centrifuge tube containing 10 ml of 0.9% saline, which was buffered to pH 7.4 with 50mM TRIS. The tubes were incubated for up to 35 days in a shaking waterbath (60 agitations/minute), which was maintained at 37° C. Triplicate 200-μl aliquots of the medium (containing released FITC-BSA) were withdrawn for analysis after 3 hours, 6 hours, 9 hours, 1 day, 2 days, 3 days, 5 days, 7 days, 10 days, 14 days, 18 days, 23 days, 28 days and 35 days. Fluorescence density was measured in a spectrophotometer (excitation wavelength: 485 nm; emission wavelength: 519 nm).

To estimate the total amount of FITC-BSA that had been incorporated into the crystalline layer of calcium phosphate during the coating procedure, 6 samples of each polymer type were immersed in 2 ml of 0.5M ethylenediamine tetra-acetic acid (pH 8.0) and vortexed twice for 5 minutes. The final volume was made up to 10 ml with TRIS-buffered 0.9% saline, and triplicate 200-μl aliquots of this medium were withdrawn for its spectrophotometric analysis.

Fluorescence readings were converted to amounts of protein using a standard curve, which was generated by preparing a dilution-series of FITC-BSA in 10 ml of Tris-buffered 0.9% saline. The temporal release of FITC-BSA was expressed as a percentage of the total amount that had been incorporated into the crystalline layer of the calcium-phosphate coating.

**Statistical analysis**

The surface-area density and the porosity of each polymer type, as well as the kinetic data, are presented as mean values together with the standard deviation. Data pertaining to each group were compared using a one-way analysis of variance (ANOVA). The level of significance was set at p<0.05. SPSS statistical software (version 15.0 for windows) was used for this evaluation. Post-hoc comparisons were made using Bonferroni corrections.
RESULTS

Scanning electron microscopy
Scanning electron microscopy revealed Helistat® and Polyactive® to be sponge-like materials with a labyrinthine system of interconnecting pores (Fig. 1). Ethisorb™ and PLGA consisted of entangled fibres, which were distributed compactly in the former case and loosely in the latter (Fig. 1). At high resolution, the surface relief of continuous portions of Helistat® was flat, whereas that of Polyactive® was undulated (Fig. 1). The surface profiles of individual fibres of Ethisorb™ and PLGA were flat on a broad scale, but not smooth: in each case, the surface was scattered with approximately 0.5-μm-diameter particles (Fig. 1).

After the polymers had been coated with an amorphous seeding layer of calcium phosphate, the surfaces of the different materials appeared similar: they were covered with dense, non-crystalline material, which was deposited in the form of spherical particles (Fig. 1). Upon this amorphous seeding layer of calcium phosphate, a thicker crystalline one was deposited, either in the absence or in the presence of BSA. Irrespective of the absence or presence of the co-precipitated protein, or of the nature of the underlying polymer, the crystalline layers had a similar surface appearance in the scanning electron microscope: they were composed of densely-packed, small needle-like crystals (Fig. 1).
Fig. 1 Scanning electron micrographs of the surface of each polymer type (left to right) in the native condition at low (first horizontal panel) and high magnifications (second horizontal panel), and after coating first with an amorphous seeding layer of calcium phosphate (third horizontal panel) and then with a crystalline one, which was co-precipitated with bovine serum albumin [(BSA) fourth horizontal panel]. Bars = 200 µm (first horizontal panel) and 5 µm (second, third and fourth horizontal panels).

Surface-area density and porosity of the native polymers
The surface-area density of each native polymer [Helistat®: 43.2(±3.0)mm⁻¹, Polyactive®: 9.2(±0.3)mm⁻¹, Ethisorb™: 100.2(±9.4)mm⁻¹ and PLGA: 12.6(±1.5)mm⁻¹ differed significantly (p< 0.001) from that of the other three, except in the comparison between Polyactive® and PLGA (p> 0.05). The great difference between the surface-area densities of the two fibrous materials (Ethisorb™ and PLGA) reflects the compact (Ethisorb™: high value) versus loose (PLGA: low value) general organization and surface relief of the fibrous meshworks (Fig. 1). The porosity – viz., the volume density of the internal space – of each polymer [Helistat®: 88.2(±1.2)%, Polyactive®: 70.7(±5.5)%, Ethisorb™: 42.2(±3.8)%, PLGA: 96.8(±0.3)%] differed significantly from that of the other three in each case (p< 0.05). Once again, the great difference between the values for the two fibrous materials reflects the organization of the fibres, the volume density of the internal surface being much smaller for compactly (Ethisorb™) than for loosely (PLGA) arranged structures.

Confocal laser-scanning, dual-channel-fluorescence microscopy
In the confocal laser-scanning microscope, the Rhodamine-labelled amorphous seeding layer of calcium phosphate was revealed to follow the surface contours of each of the cross-sectioned polymer types and to be of uniform thickness (red signal in Fig. 2). The crystalline layer of calcium phosphate was co-precipitated with FITC-BSA, and the protein phase (green signal in Fig. 2) was presumed to co-locate with the mineral one. As was the case with the amorphous layer of calcium phosphate, the BSA-bearing crystalline one followed the surface contours of each of the cross-sectioned polymer types, and was likewise of uniform thickness in each case (green signal in Fig. 2). Indeed, merger of the two images (red and green signals) revealed the BSA-bearing crystalline layer to be deposited upon, and yet to be clearly demarcated from, the amorphous one (Fig. 2).
Fig. 2 Confocal laser-screening fluorescence micrographs of each polymer type (left to right) after coating first with an amorphous seeding layer of calcium phosphate [(labelled with Rhodamine: red signal) first horizontal panel] and then with a crystalline one, which was co-precipitated with bovine serum albumin (BSA) that had been conjugated with fluorescein isothiocyanate [(FITC: green signal) second horizontal panel]. The protein is presumed to co-localize with the crystalline layer. The two sets of images are merged in the third horizontal panel. Bars=10 μm.

**Fourier-transform infrared spectroscopy**

Fourier-transform infrared spectroscopy revealed each of the four polymer types to be characterized by a distinct spectrum (Fig. 3), which reflects its chemical composition. Helistat® (collagen) was characterized by bands at wavelengths of 1656cm⁻¹, 1536cm⁻¹ and 1334cm⁻¹, which correspond to amide-I, II and III groups, respectively. Ethisorb™ and PLGA are characterized by a band at 1456cm⁻¹ and 1434cm⁻¹, respectively, which corresponds to their CH₂ groups [26], and Polyactive® manifests one at 1717cm⁻¹, which represents its C=O groups [27].
After coating the different materials with an amorphous seeding layer of calcium phosphate, several new bands consistently appeared in the spectrum of each polymer type: two at wavelengths of 1047 cm\(^{-1}\) and 556 cm\(^{-1}\), respectively, which correspond to PO\(_4^{3-}\) groups [28], one at 871 cm\(^{-1}\), which corresponds to HPO\(_4^{2-}\) groups, and one at 1428 cm\(^{-1}\), which corresponds to CO\(_3^{2-}\) [29]. On the basis of these data, the amorphous seeding layer can be classified as carbonated calcium phosphate.

Additional bands appeared after the crystalline layer of calcium phosphate had been deposited, and these were likewise consistently associated with each polymer type. They included a twin band at 602 cm\(^{-1}\) and 563 cm\(^{-1}\), which corresponds to O-P-O bending, and a single one at 1027 cm\(^{-1}\), which corresponds to P-O stretching [30, 31]. These bending and stretching modes of the P-O group reflect the crystalline nature of this calcium-phosphate layer [32]. An additional band, the position of which varied between 1655 cm\(^{-1}\) and 1631 cm\(^{-1}\) according to the nature of the polymer, and which represents molecular water of the crystalline layer, underwent a shift to higher wavelengths when the latter was co-precipitated with BSA (Fig. 3) [33]. Also in the spectrum of BSA itself, a band was apparent at this position (1656 cm\(^{-1}\)), and, in the case of the protein, corresponds to C=O stretching in amide-I groups (\(\cdot\text{CO}\cdot\text{NH}_2\)). The shift to a higher wavelength position that occurred in the spectra of the polymers when the crystalline layer of calcium phosphate was deposited in the presence of BSA indicates that the protein was incorporated into the crystalline latticework via an interaction involving amide-I groups in the former case and molecular water in the latter.
Fig. 3 Fourier-transform infrared spectra for each polymer type in a native condition (A), and after coating first with an amorphous seeding layer (ASL) of calcium phosphate (A) and then with a crystalline one (CL) in the absence or presence of bovine serum albumin [(BSA) B].

**X-ray diffractionometry and energy-dispersive X-radiography**

X-ray diffractionometry revealed each of the four polymer types to be characterized by a unique spectrum (Fig. 4), with distinctive peaks at $2\theta = 21.35^\circ$ and $23.74^\circ$ for Helistat®, at $2\theta = 23.54^\circ$ for Polyactive®, at $2\theta = 22.08^\circ$ and $28.31^\circ$ for Ethisorb™, and at $2\theta = 21.29^\circ$, $21.88^\circ$ and $28.44^\circ$ for PLGA. Coating of the different polymer types with an amorphous seeding layer of calcium phosphate led to the appearance of a new broad peak at $2\theta = 28^\circ$-32$^\circ$ in the case of Helistat®, and to its superimposition upon an existing one in the cases of Polyactive®, Ethisorb™ and PLGA. The position corresponds to that of apatite, and the broadening of the diffraction peak reflects the amorphous nature of the material.

After the crystalline layer of calcium phosphate had been deposited, two major new peaks were introduced into the diffraction spectrum of each polymer type: one narrow band close to $2\theta = 26^\circ$ and a broader one at $2\theta = 32^\circ$. According to information published by the International Centre for Diffraction, these bands are characteristic of calcium-deficient apatite with a low crystallinity. The
incorporation of BSA into the crystalline layer of calcium phosphate elicited no profound change in the diffraction spectrum. The energy-dispersive X-radiographic analysis (which was conducted in conjunction with scanning electron microscopy) revealed the calcium-to-phosphorus ratios of the amorphous seeding layer of the four polymer types to range from 1.53-1.64. After the crystalline layer had been deposited, the calcium-to-phosphorus ratio was lower in each case, and fell within the range of 1.37-1.45. This finding confirms that of the X-ray diffraction analysis, namely, that the crystalline layer was poor in calcium.
Fig. 4 X-ray diffraction spectra for each polymer type in a native condition (a), and after coating first with an amorphous seeding layer of calcium phosphate (b) and then with a crystalline one in the absence (c) or presence of bovine serum albumin [(BSA) d].

Release kinetics of a coating-incorporated depot of BSA in vitro
The release kinetics of a depot of FITC-BSA that had been incorporated into the crystalline layer of calcium-phosphate coatings are represented in Fig. 5 for each polymer type. For the purpose of comparison, the release kinetics of an adsorbed depot of FITC-BSA are also depicted. As expected, the adsorbed depot of BSA was released rapidly from the coatings of each polymer type when the specimens were immersed in buffered physiological saline. The initial depot was depleted by more than 80% after 6 hours, and was completely exhausted after 3 days in all cases.

The release kinetics of a coating-incorporated depot of BSA followed a biphasic course in the case of each polymer type: an initial rapid, and a subsequent slower one. However, the rate of protein release during the initial rapid phase differed between the sponge-like and the fibrous polymer types. In the case of Helistat® and Polyactive®, both of which have a labyrinthine sponge-like structure (see Fig. 1), 54% of the coating-incorporated depot of BSA had been released by day 5 at a rate of nearly 11% per day in each instance. But in the case of Ethisorb™ and PLGA, both of which have a fibrous structure (see Fig. 1), only 28% (Ethisorb™) to 32% (PLGA) of the coating-incorporated depot of BSA had been liberated by the same juncture, at rates of 6% (Ethisorb™) and 8% (PLGA) per day.

During the subsequent slower phase (days 5-35), the rates of protein release were similar for each polymer type. By the end of the monitoring period (day 35), the initial coating-incorporated depot of BSA had been depleted by 68% and 74% for Helistat® and Polyactive®, respectively, and by 31% and 41% for Ethisorb™ and PLGA, respectively.

Our data indicate that the surface geometry of the underlying polymeric material influences only the initial rapid rate of release of a coating-incorporated depot of protein; not the subsequent slower one.
DISCUSSION

The concept of biomimetic mineralization was introduced by Kokubo et al. in 1990 [9]. The layer of calcium phosphate that is thereby produced is more conducive than conventionally-prepared ones to the differentiation of bone-marrow stromal cells into osteoblasts [34], as well as to the ingrowth of osseous tissue and its contact with the implanted material [35-37], which is less vigorously encapsulated with fibrous tissue [38]. Various types of material have been thus coated, including bioceramics [39], metals [40] and organic polymers [41]. Using the original biomimetic technique, the successful coating of polymers depends greatly upon the surface chemistry of the material [42-44], since this surface furnishes the active chemical groups that serve as nucleation sites for mineralization [43, 45, 46]. Monolayers of dihydrogen phosphate (H₂PO₄) or carboxylic acid (COOH) have been shown to be highly conducive to biomimetic mineralization, and those composed of methyl groups (CH₃) to be unpropitious to the process [46]. But since many of the polymers that are marketed for clinical
use are not equipped with active chemical groups on their surfaces, attempts have been made to covalently attach functional anionic ones [45]. Furthermore, a patterning of these functional groups permits a selective topographical localization and orientation of the mineral deposits, which in turn permits a control of cell binding and alignment [47]. However, most of the polymers that are in clinical use are either not amenable to such manipulation, or, if they are, the modifications carry the risk of compromising the physicochemical and biological properties of the material. Moreover, not only the surface chemistry but also the surface geometry and the three-dimensional structure of a polymer can influence its biomimetic mineralization [48].

Using the biphasic biomimetic coating technique that was refined in our laboratory [15, 16], the four tested polymer types could be coated with an amorphous and a crystalline layer of calcium phosphate, the morphological and physicochemical properties of each of which were independent of either the surface chemistry, the surface geometry or the three-dimensional structure of the underlying carrier. The consistency of the result is to be laid to the merits of the amorphous seeding layer. Previously, we had applied this biphasic biomimetic coating technique to titanium-alloy implants [20], and the “adhesion” thereto of the amorphous layer of calcium phosphate was attributed to the affinity of Ca$^{2+}$ and HPO$_4^{2-}$ ions for titanium [29, 49]. But since the data of the present study indicate that such an amorphous layer of calcium phosphate can be precipitated also on polymers with diverse surface chemistries, and without any change in its morphological or physicochemical properties, the deposition process is probably unrelated to the phenomenon of chemical affinity. A more likely explanation is, that the tiny particles of calcium phosphate – which are formed under the nucleation-inhibitory influence of Mg$^{2+}$ [50] and HCO$_3^{-}$ [29] – are captured and immobilized on the substratum by a process of mechanical gomphosis. These particles then serve as seeding centres for the subsequent growth of a crystalline latticework of calcium phosphate under conditions that are conducive to nucleation [28].

One might perhaps question whether an amorphous layer of calcium phosphate would not in itself suffice as an osteoconductive surface and, after its functionalization with an osteogenic agent, as an osteoinductive one. This question must be answered in the negative. An amorphous layer of calcium phosphate undergoes rapid dissolution [51]. Although this circumstance may not
interfere with the property of osteoconductivity, it renders the layer an unsuitable vehicle for the carriage of an osteogenic agent. To be efficacious at the target site, an osteogenic agent needs to be liberated gradually and steadily at a low pharmacological level; not rapidly in a single high-dose burst [20]. And therein lies the beauty of a crystalline latticework of calcium phosphate and the advantage that is afforded by the biomimetic co-precipitation of this layer with a protein, which is thereby incorporated into its structure. Evidence for the latter phenomenon is furnished elsewhere [15, 20, 52] and was confirmed in the present study by Fourier-transform infrared spectroscopy.

In vivo, the degradation of a crystalline coating of calcium phosphate is not a spontaneous, but a cell-mediated process [18, 20], and in this respect it resembles the resorption of bone mineral, which is mediated by osteoclasts. Indeed, when the crystalline layer of calcium phosphate is functionalized by the incorporation of an osteogenic agent [18, 20, 25, 52, 53], the analogy to physiological bone resorption is still closer: osteogenic growth factors are trapped within the mineralized matrix of bone and are released when it is resorbed by osteoclasts [45, 46]. These osteogenic growth factors then stimulate bone-formation activity. Hence, a biomimetic coating of calcium phosphate that has been functionalized by the incorporation of an osteogenic agent can facilitate bone formation along a course that resembles the physiological process of bone remodelling, which involves the resorption of old, and the formation of new bone [20, 52].

The release kinetics of a coating-incorporated depot of BSA (which served as a model protein) were influenced by the surface geometry and the three-dimensional structure of the underlying polymer only during the initial rapid phase (up to 5 days). The protein was liberated at a higher rate from polymers that had a chambered, sponge-like structure (Helistat® and Polyactive®) than from those that had an open fibrous one (Ethisorb™ and PLGA). Albeit so, the rate of release during this phase was, in each case, still markedly slower from a coating-incorporated than from an adsorbed depot of BSA (Fig. 5). During the subsequent slower kinetic phase (5-35 days), the rate of protein release was similar for each polymer type.

During the initial rapid kinetic phase, the BSA that was released in the coating-incorporated groups probably originated predominantly from an incidentally-adsorbed depot. The labyrinthine system of interconnecting pores that characterizes the sponge-like materials offers a greater potential for the
entrapment of fluid-borne protein (due to the formation of poorly diffusible “stagnation” pockets), and thus to its incidental adsorption during subsequent freeze-drying, than do the open and more freely diffusible spaces that surround the non-porous fibrous ones. The existence of a larger depot of entrapped and incidentally-adsorbed BSA in association with the sponge-like (Helistat® and Polyactive®) than with the fibrous polymers (Ethisorb™ and PLGA) would account for the higher rates and percentages of protein release from the former during the initial rapid kinetic phase. The BSA that was liberated during the slower kinetic phase (5-35 days) originated exclusively from the coating-incorporated depot, the rate of release of which was similar for each polymer type. This latter observation is consistent with the findings of the Fourier-transform infrared spectroscopy analysis: for each type of polymer, the incorporation of BSA into the crystalline layer of calcium phosphate elicited a comparable change in the spectrum of the latter, which indicates that the integration mechanism was similar in each case.

Clearly, BSA is not biologically active as an osteogenic agent. In this explorative study, it was used as an inexpensive alternative to BMP-2. Although BMP-2 is a much smaller molecule than BSA and has a different charge density, it can nevertheless be incorporated into a calcium-phosphate coating without impairing its osteoinductive efficacy, either in vitro [25] or in vivo [53]. BMP-2 that had been incorporated into a calcium-phosphate coating was previously shown not only to induce bone formation at both an ectopic and an orthotopic ossification site in rats [20] [53] and miniature pigs [18], respectively, but to do so potently, at a low pharmacological dose (1.7 µg/coating), and at a level that was sustained for some weeks. These data afford indirect evidence that the coating-incorporated depot of BMP-2 was liberated gradually and steadily in vivo, viz., along a temporal course that resembled the elution of a coating-incorporated depot of BSA in the present study.

It is important here to mention that once the functionalized coating has been degraded in vivo, the underlying carrier will be exposed to the host tissues. The biocompatibility and biodegradability of the carrier will undoubtedly influence the osteoinductive activity of the osteogenic agent that was incorporated into the coating. In an ongoing study, which is nearing completion, the four polymeric carriers that were the subject of the present work were coated with a BMP-2-functionalized layer of calcium phosphate and implanted at an ectopic
ossification site in rats. Parameters such as coating stability after implantation, the rates of coating and polymer degradation, foreign-body-giant-cell reactivity (a gauge of biocompatibility), and osteoinductive efficacy were histomorphometrically assessed for 5 weeks. These data, which do not fall within the scope of the present study, will be published shortly. Another elegant coating methodology has been investigated for the controlled release of biologically active molecules, namely, the sol-gel technique [54]. However, the methodology is more cumbersome than the biomimetic coprecipitation of a biologically active agent and a layer of calcium phosphate.

In summary, our data indicate that the biphasic biomimetic coating technique can be applied to polymers with diverse surface chemistries, surface geometries and three-dimensional structures without impacting the morphological and physicochemical properties of either the amorphous seeding layer of calcium phosphate or the crystalline one, or affecting the release kinetics of a protein depot that is incorporated into the latter. The technique could thus be applied to spongy or fibrous polymers that are used in clinical practice as bone-defect-filling materials, with a view to improving their osteoconductivity. Moreover, the technique can readily accommodate a process of coating-functionalization with a proteinaceous agent, whose release kinetics would be highly conducive to the efficacious induction and sustentation of bone-formation activity.

ACKNOWLEDGEMENTS:
The authors sincerely thank the generous gift of PLGA from Smith and Nephew.
REFERENCES


Chapter 3

The effect of a slow mode of BMP-2 delivery on the inflammatory response provoked by bone-defect-filling polymeric scaffolds

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Biomaterials (2010) 31, 7485-7493
doi:10.1016/j.biomaterials.2010.06.037
Published online 17 Jul 2010
ABSTRACT

We investigated the inflammatory response to, and the osteoinductive efficacies of, four polymers (collagen, Ethisorb™, PLGA and Polyactive®) that bore either an adsorbed (fast-release kinetics) or a calcium-phosphate-coating-incorporated (slow-release kinetics) depot of BMP-2. Titanium-plate-supported discs of each polymer (n = 6 per group) were implanted at an ectopic (subcutaneous) ossification site in rats (n = 48). Five weeks later, they were retrieved for a histomorphometric analysis of the volumes of ectopic bone and foreign-body giant cells (a gauge of inflammatory reactivity), and the degree of polymer degradation. For each polymer, the osteoinductive efficacy of BMP-2 was higher when it was incorporated into a coating than when it was directly adsorbed onto the material. This mode of BMP-2 carriage was consistently associated with an attenuation of the inflammatory response. For coated materials, the volume density of foreign-body giant cells was inversely correlated with the volume density of bone ($r^2 = 0.96$), and the volume density of bone was directly proportional to the surface-area density of the polymer ($r^2 = 0.97$). Following coating degradation, other competitive factors, such as the biocompatibility and the biodegradability of the polymer itself, came into play.

Key words: Biocompatibility; calcium-phosphate coating; controlled drug release; foreign-body giant cells; inflammation; osteogenesis.

INTRODUCTION

Fractures of the orbital, maxillary and zygomatic bones are common in both children [1] and adults [2], and these lesions often require surgical reconstruction. For this purpose, the grafting of autologous bone is still considered to be the optimal therapy [3]. However, such material is of limited availability, and its removal is associated with donor-site morbidity and pain [4-6]. The grafting of allogeneic bone is an alternative [7, 8], but the use of this material also has its drawbacks, which include the risk of transmitting infectious (viral) micro-organisms from the donor to the host [9, 10] and of triggering a host immune response [11]. Consequently, non-osseous scaffolding materials have been sought. Both natural and synthetic polymers have been considered and
tested in a clinical setting. The use of natural polymers, such as collagen [12], has not met with unqualified success, since these materials are potentially immunogenic and, moreover, are not readily amenable to fabrication in a stable, solid form. Synthetic polymers, on the other hand, being non-immunogenic and fully resorbable, are a popular option, and developments in this field have been rapid during the past few decades [13]. For the clinical repair of defects within laminar bone, several synthetic polymers are available. These include polydioxanone [14-16] and mixtures of either polylactate or polyglycolate [17-19] or polyethylene glycol and polybutylene terephthalate (Polyactive®) [20, 21]. Although these natural and synthetic polymers furnish a scaffold for the deposition of new tissue, they are not – indeed cannot be – intrinsically osteoinductive. During their biodegradation, they are replaced not by bone but by dense collagenous tissue [15, 22, 23]. Consequently, the mechanical stability of the repair tissue is sometimes insufficient. For example, when such polymers are used to fill large bony defects within the orbital floor, the repair tissue that is deposited cannot support the eyeball, thereby resulting in persistent enophthalmos or diplopia [14, 15].

The property of osteoinductivity can be conferred only by an osteogenic agent, such as BMP-2 (bone morphogenetic protein 2), which is a member of the transforming growth factor beta family [24]. BMP-2 can induce ossification at ectopic sites [25], and has been used extensively to enhance bone formation at orthotopic ones, both in experimental animals and in clinical trials [25-29]. However, the mode of delivery of BMP-2 to its potential site of action is a crucial determinant of its osteoinductive efficacy. This agent is water-soluble, and when it is administered topically by injection, it diffuses away too rapidly to be effective [30]. Attempts have been made to adsorb BMP-2 to polymeric bone-defect-filling materials. Although bone formation can be thereby triggered, the pharmacological dose that must be applied to induce ossification is exceedingly high: the adsorbed depot is released so rapidly [31, 32] that most of it diffuses away before it can take effect. Moreover, apart from the unnecessary cost of such wastage, the transiently very high concentrations of this growth factor can trigger undesirable side-effects. These include an over-stimulation of local bone resorption, an over-stimulation of bone formation at both local and distant sites, and an augmentation of local neuropathy [33, 34].
In previous studies, we have refined a technique for the biomimetic deposition of osteoconductive calcium-phosphate coatings, which can be co-precipitated with an osteogenic agent, such as BMP-2 [35]. Using this technique, BMP-2 can be truly incorporated into the inorganic crystalline latticework, and is subsequently liberated at a slow, steady rate [27, 36] in a cell-mediated manner. Using pharmacologically low loading doses, BMP-2-functionalized calcium-phosphate coatings have been shown to induce and sustain bone formation for several weeks around titanium implants that were inserted at both ectopic [26] and orthotopic sites [27] in experimental animals.

In the present study, we wished to apply these BMP-2-functionalized calcium-phosphate coatings to four polymeric bone-defect-filling materials that are characterized by different geometries and surface densities, and to compare their inflammation-provoking reactivities, their biodegradabilities and their osteoinductive efficacies at an ectopic (subcutaneous) site in rats. The polymers tested were collagen (Helistat®), a combination of polyglactin 910/poly-\(p\)-dioxanone fleece and poly-\(p\)-dioxanone foil (Ethisorb™), a co-polymer of glycolic and lactic acids (PLGA), and a co-polymer of polyethylene oxide terephthalate and polybutylene terephthalate (Polyactive®).

MATERIALS AND METHODS

Experimental design

The physical characteristics of the one natural polymer (collagen: Helistat®, Integra, Plainsboro, NJ, USA), and the three synthetic ones [Ethisorb™ (Johnson and Johnson, USA), PLGA (Smith and Nephew, UK) and Polyactive® (Genci, USA)], as well as those of the protein-functionalized layers of calcium phosphate with which they were coated and the protein-release profiles in vitro, are described elsewhere [37]. The purpose of the present study was to compare the inflammation-provoking reactivities, the biodegradabilities and the osteoinductive efficacies of the native polymers, with or without an adsorbed depot of BMP-2, and of the coated polymers, with or without an incorporated depot of BMP-2, at an ectopic (subcutaneous) site in rats, 5 weeks after implantation. The inflammatory response (gauged by determining the volume density of foreign-body giant cells), the degradation of the polymer, and the volume density of bone were evaluated histomorphometrically.
Biomimetic coating procedure

1-cm-diameter discs of each polymer type, which were of variable thickness (collagen: 1mm; Polyactive®: 2mm; Ethisorb™: 0.6mm; and PLGA: 2mm), were coated with a layer of calcium phosphate according to a standard biomimetic procedure [35, 38]. Initially, a thin (1- to 3-μm-thick) layer of amorphous calcium phosphate was deposited, which served as a seeding substratum for the growth of a more substantial crystalline one. The amorphous layer was produced by immersing the polymer discs in five-times-concentrated simulated body fluid (683.8mM NaCl, 12.5mM CaCl_2·2H_2O, 5mM Na_2HPO_4·2H_2O, 21mM Na_2CO_3), which contained 7.5mM MgCl_2·6H_2O to inhibit crystal growth. The samples were incubated in this solution for 24 hours at 37°C. After freeze-drying in an evacuated chamber for 24 hours, they were transferred to a supersaturated solution of calcium phosphate (4mM CaCl_2·2H_2O, 136mM NaCl, 2mM Na_2HPO_4), which was buffered with 50mM TRIS (pH 7.4). The samples were incubated in this medium (10ml per sample) for 48 hours at 37°C, and then freeze-dried in a vacuum. The entire coating procedure was conducted under sterile conditions.

Incorporation of BMP-2 into calcium-phosphate coatings

Human recombinant BMP-2 [derived from a Chinese Hamster Ovary (CHO) cell line (InductOs®, Wyeth®, Cambridge, MA, USA)] was introduced into the supersaturated solution of calcium phosphate during the coating procedure (see above) at a final concentration of 10μg/ml. It is co-precipitated with the inorganic components, and is thereby incorporated into the crystalline latticework [35]. The amount of BMP-2 that was incorporated into each coating (Table 1) was determined using an enzyme-linked immunosorbent assay (ELISA) kit (PeproTech EC, London, UK), as previously described [26].

Adsorption of BMP-2 onto uncoated samples

A 20-μl drop of a stock solution (500μg of BMP-2/ml) was deposited upon the upper and the lower surfaces of each polymer disc in turn, allowing for complete evaporation between the applications under sterile, ambient conditions. The amount of adsorbed BMP-2 was pre-determined at 10μg per sample for each polymer type (Table 1).
Table 1 Initial loading doses of BMP-2 (expressed in microgrammes per cubic millimetre of polymeric material). Mean values (n = 6) are represented together with the standard error of the mean (SEM).

<table>
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<th>Incorporated BMP-2</th>
<th>(µg/mm³)</th>
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<th>Ethisorb</th>
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<th>Polyaactive</th>
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</table>

Animal model of ectopic bone formation: experimental set-up

One experimental and three control groups were established for each polymer type: (i) coated polymer bearing an incorporated depot of BMP-2 (experimental group); (ii) uncoated polymer (negative control for the effects of a calcium-phosphate coating and of BMP-2); (iii) coated polymer (negative control for the effects of BMP-2); (iv) uncoated polymer bearing an adsorbed depot of BMP-2 (positive control for the effects of BMP-2). To neutralize the effects of irregular stress-fields generated within the dorsal skin and body muscles of the rats, each polymer was affixed with Vicryl 5-0 thread (Ethicon) to a titanium disc (10mm in diameter and 1mm in thickness; Ti Grade 4 medical, Fredec AG, Murten, Switzerland) via two 0.5-mm-diameter holes, one at each pole. Six samples per group were distributed amongst 48 rats. Under conditions of general anaesthesia, two discs were implanted per animal within the dorsal subcutaneous tissue, one on the left side and one on the right side. Each rat always received either BMP-2-containing samples or non-BMP-2-containing ones. This strategy was adopted to avoid the possibility of cross-reactivity. With this precondition, the samples were distributed amongst the animals according to a systematic random protocol. The samples were retrieved 5 weeks after surgery for the histomorphometric evaluation of the host inflammatory response (gauged by estimating the volume density of foreign-body giant cells), the degradation rate of the polymers, the volume density of bone and the osteoinductive efficacy of BMP-2. On the basis of our previous findings, we know that a coating-incorporated depot of BMP-2 in
the order of magnitude that was used in the present study can sustain bone-
formation activity for minimally 5 weeks [26].

**Histological processing**

The rats were sacrificed by administering an overdose of gaseous carbon dioxide, which induced cardiac arrest. The implanted polymers were retrieved together with a minimum quantity of the surrounding tissue, which embraced the capsule of fibrous connective tissue. The retrieved samples (as well as unimplanted controls) were chemically fixed by immersion in 10% formaldehyde solution for several days at ambient temperature. They were then rinsed in tap water, dehydrated in ethanol and embedded in methylmethacrylate. Applying a systematic random-sampling protocol (with a random start at the left-hand margin of the specimen) [39], each disc was cut perpendicular to its flat surface into 8-12 slices, 600µm in thickness and 1mm apart, using a diamond saw. The slices were mounted on plexiglas holders, polished, and surface-stained with McNeal’s Tetrachrome, basic Fuchsin and Toluidine Blue O [40]. Using this protocol, newly-formed bone stains deep red, cell nuclei blue, collagen fibres pink and the calcium-phosphate coating pale red. They were then examined in a Nikon-Eclipse E-1000 light microscope and photographed in colour at a final magnification of either x 200 (collagen and Polyactive®) or x 300 (Ethisorb™ and PLGA). Approximately 25 photomicrographs were collected per sample using a systematic random-sampling strategy. These prints were used for the histomorphometric analysis of the various stereological estimators.

**Histomorphometry**

**Total volume of subcapsular tissue: Reference volume**

The total volume of tissue that was embraced by the capsule of fibrous connective tissue (reference volume) was estimated using Cavalieri’s methodology [41]. This involves measuring the cross-sectional area of a defined number of tissue sections at a fixed distance apart through the reference volume. The cross-sectional area of each section was estimated using the point-counting technique [39]. The reference volume is calculated by multiplying the sum of the cross-sectional areas of the sections by the fixed distance between them.

**Surface-area density at time-point zero**

The surface-area density (the area of a surface per unit volume) of each polymer type was estimated at time-point zero using a cycloid grid, which was superimposed on light micrographs of the sectioned material [42].
Total volume of bone, of foreign-body giant cells and of the remaining polymeric material, 5 weeks after implantation

The total volume of bone, of foreign-body giant cells and of the remaining polymeric material were estimated 5 weeks after implantation from the volume densities (the volume proportion of the phase of interest within a reference volume) and the subcapsular reference volume (see above). The volume density of each estimator was determined stereologically from its area density on tissue sections by the point-counting technique [39].

**Osteoinductive Efficacy of BMP-2**

Five weeks after implantation, the coatings associated with each polymer type had been completely degraded. Hence, the amount of BMP-2 liberated was equivalent to the initial loading dose. The depot of BMP-2 that was adsorbed onto uncoated polymers is known to be completely exhausted within the first few days of implantation [32]. The osteoinductive efficacy of BMP-2 was estimated by dividing the total volume of bone that was deposited after 5 weeks by the total quantity of BMP-2 that was either incorporated into the coated polymers or adsorbed onto uncoated ones.

**Statistical analysis**

All data are presented as mean values together with either the standard deviation or the standard error of the mean. Data pertaining to each group were compared using a one-way analysis of variance (ANOVA). The level of significance was set at $p<0.05$. SPSS statistical software (version 11.0.4 for an Apple Macintosh computer) was used for this evaluation. Post-hoc comparisons were made using Bonferroni corrections.

**RESULTS**

Five weeks after implantation, each polymer disc was surrounded by a capsule of dense, fibrous connective tissue. Within the confines of this capsule, viz., within the subcapsular space, no bone tissue had been deposited on or around any of the polymeric materials that had not borne either a directly adsorbed or a coating-incorporated depot of BMP-2 (Fig. 1).
Fig. 1 Light micrographs of sections through discs of the indicated polymer types in the four groups [coated polymer bearing an incorporated depot of BMP-2 (Inc. BMP-2); uncoated polymer bearing an adsorbed depot of BMP-2 (Ads. BMP-2); unfunctionalized coated polymer (Coating only); and unfunctionalized uncoated polymer (Polymer only)], 5 weeks after implantation. Bone (B) was deposited only in association with polymeric materials (coated or uncoated) that had been functionalized with BMP-2. Foreign-body giant cells (FBGC) were encountered in each of the groups. P = polymeric material; F = fat tissue. The sections were surface-stained with McNeal’s Tetrachrome, basic Fuchsine and Toluidine Blue O. Bars = 200µm.

The volume density of bone that was associated with polymers bearing a calcium-phosphate coating into which BMP-2 had been incorporated was highest
for collagen and Ethisorb™, lower for PLGA, and lowest for Polyactive® (Fig. 2).

Fig. 2 Graph depicting the volume density of bone that was deposited 5 weeks after implantation within the subcapsular space (reference volume) of each polymer type. Each polymeric material was functionalized with BMP-2, which was either adsorbed directly onto the uncoated material or incorporated into a calcium-phosphate coating. Mean values (n = 6 for each group) are represented together with the standard deviation.

For each polymer type, the volume density of bone associated with the uncoated material bearing an adsorbed depot of BMP-2 was consistently lower than that associated with the coated counterpart bearing an incorporated depot of the drug (Fig. 2). The volume density of bone deposited after 5 weeks in association with polymers bearing a coating-incorporated depot of BMP-2 was proportional ($r^2 = 0.97$) to the surface-area density of the polymer (Fig. 3).
Inflammatory activity was gauged by estimating the volume of foreign-body giant cells (Fig. 4) within the fibrous capsule. The total volume of foreign-body giant cells was lowest in association with collagen, Ethisorb™ and PLGA, and highest in association with Polyactive® (Fig. 5). For collagen, the total volume of foreign-body giant cells was negligible in all groups except that in which the polymer bore an unfunctionalized coating. For Ethisorb™ and PLGA, the total volume of foreign-body giant cells was low and similar in each of the four groups. For Polyactive®, the total volume of foreign-body giant cells was lower in association with the polymer that bore a coating-incorporated depot of BMP-2 than in any of the other three groups, for which the values were similar. However, if the total volume of foreign-body giant cells is expressed relative to the subcapsular volume (Fig. 6), then a different picture emerges: the highest volume density of foreign-body giant cells was associated with coated, but unfunctionalized, collagen. Moreover, the negligible differences between the various groups for Ethisorb™ and PLGA were accentuated, revealing the lowest
values for the polymer that bore a coating-incorporated depot of BMP-2. The volume density of foreign-body giant cells was inversely proportional ($r^2 = 0.96$) to the volume density of bone that was deposited after 5 weeks in association with polymers bearing a coating-incorporated depot of BMP-2 (Fig. 7).
Fig. 4 Light micrographs of foreign-body giant cells that were associated with uncoated, non-functionalized discs of Poyactive® (A), Ethisorb™ (B) and PLGA (C) 5 weeks after implantation. These cells, which contain numerous nuclei (arrows), intimately follow the surface contours of the longitudinally (A), transversely (B, C) and obliquely sectioned (C) polymeric material (asterisks). (N.B.: By the 5th postoperative week, the uncoated, non-functionalized collagenous polymer had been completely degraded). The sections were surface-stained with McNeal’s Tetrachrome, basic Fuchsine and Toluidine Blue O. Bars = 30μm.

Fig. 5 Graph depicting the total volume of foreign-body giant cells that were found 5 weeks after implantation within the subcapsular (reference) space of each polymer type; the four different groups are indicated. Mean values (n = 6) are represented together with the standard deviation.
Fig. 6 Graph depicting the volume density of foreign-body giant cells within the subcapsular (reference) space, 5 weeks after implantation. Mean values (n = 6) are represented together with the standard deviation. *: p< 0.05 ; **: p< 0.01; ***: p< 0.001.
Fig. 7 Graph depicting the inverse linear relationship existing between the volume density of bone that was deposited after 5 weeks in association with polymers bearing a coating-incorporated depot of BMP-2 and the volume density of foreign-body giant cells that had accumulated after 5 weeks, likewise in association with polymers bearing a coating-incorporated depot of BMP-2. Mean values (n = 6) are represented together with the standard deviation. The correlation coefficient (r²) = 0.96.

The influence of functionalizing the coatings with BMP-2 on the degradation of the polymeric materials themselves was evaluated. Coated collagen, Ethisorb™ and PLGA underwent significant degradation during the 5-week implantation period, and functionalization of the coating with BMP-2 tended to accelerate this process, although the effect attained significance only for collagen and PLGA (Fig. 8). Coated Polyactive® underwent no significant degradation during the 5-week implantation period, and functionalization of the coating with BMP-2 had no influence on the process.

![Graph depicting the influence of functionalizing coated forms of each polymer type with BMP-2 on the degradation of the polymeric material itself.](image)

* : p< 0.05 ; ** : p< 0.01; *** : p< 0.001

On the other hand, the coatings of each polymer type underwent complete degradation during the 5-week implantation period. Hence, within this timespan,
all coatings into which BMP-2 had been incorporated had liberated their initial depot of the drug (see Table 1). Uncoated polymers bearing an adsorbed depot of BMP-2 were assumed, on the basis of existing evidence [32], to have been exhausted of their entire initial loading dose during the first few hours of implantation. The osteoinductive efficacy of BMP-2 in association with each polymer type was estimated by dividing the total volume of bone that was deposited after 5 weeks by the total quantity of BMP-2 that was initially either incorporated into the coated materials or adsorbed onto the uncoated ones (Fig. 9). For each polymer type, the osteoinductive efficacy of BMP-2 was higher when the drug was incorporated into a coating than when it was directly adsorbed onto the uncoated material (Fig. 9). The difference was least (non-significant) for Polyactive® and greatest for Ethisorb™ and collagen; PLGA held an intermediate position. Since the initial loading doses of BMP-2 were of the same order of magnitude for each polymer type (see Table 1), and since the patterns of coating deposition and protein incorporation have been previously shown to be the same [37], the different osteoinductive efficacies of BMP-2 in association with the four polymer types may reflect, either directly or indirectly, differences in their surface-area density, since, as aforementioned, this parameter was proportional to the volume density of bone deposited within the 5-week implantation period (Fig. 3).

![Graph](image_url)

Fig. 9 Graph depicting the osteoinductive efficacies of BMP-2 in association with each polymer type. Each polymeric material was functionalized with BMP-2, which was either
adsorbed directly onto the uncoated material or incorporated into a calcium-phosphate coating. Mean values (n = 6) are represented together with the standard deviation.

DISCUSSION

In the fields of dental, maxillofacial and orthopaedic surgery, non-immunogenic, biodegradable and osteoconductive materials that can be functionalized with an osteogenic agent are needed as viable options to autologous (or even allogeneic) bone for the reconstruction of voluminous osseous defects. Several synthetic polymers are commercially available and have been clinically applied for this purpose. However, like all foreign materials, these polymers trigger an inflammatory response in which macrophages and foreign-body giant cells participate [43, 44]. The pro-inflammatory cytokines that are released by migrant T-lymphocytes suppress bone formation [45]. This osteoinhibitory cytokine signal far outweighs the potentially beneficial osteoinductive one that emanates from macrophages in the form of BMP-2 release. As a result of this inflammatory reactivity, the polymeric scaffold becomes ensheathed by a capsule of dense, fibrous connective tissue. This walling-off of the implanted material impedes its osseointegration with the surrounding tissue.

Quite apart from the triggering of this osteoinhibitory inflammatory reactivity, the available polymeric scaffolds must be applied in conjunction with an osteogenic agent, such as BMP-2, to be osteoinductive. Hitherto, BMP-2 has been directly injected into the implantation site. But since the drug is water-soluble, and since marked inflammatory reactivity is associated with the scaffold, it must be applied at extremely high concentrations to be effective, with the undesirable consequences that bone is also formed within non-osseous tissues, such as muscle. More recently, attempts have been made to adsorb BMP-2 to the surfaces of polymeric scaffolds. But an adsorbed depot of the drug is released too rapidly to be locally effective. And if the loading dose is raised, then the aforementioned side-effects ensue.

We have refined a technique for the biomimetic production of BMP-2-functionalized calcium-phosphate implant coatings. Since the BMP-2 is thereby incorporated into the inorganic crystalline latticework, it is released gradually, at a low pharmacological dose, and in a cell-mediated, physiological-like manner. This mode of BMP-2 delivery is conducive to sustained bone-formation activity. In the present study, we wished to investigate the inflammatory responses to, and

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the osteoinductive efficacies of, four types of polymeric material (with different chemical compositions and surface characteristics) that bore either an adsorbed depot of BMP-2 (fast-release kinetics) or a calcium-phosphate coating into which the drug had been biomimetically incorporated (slow-release kinetics). For this purpose, we drew on a well-established ectopic ossification model in rats [46]. With respect to osteogenic activity, the findings of the present study confirm those of a previous investigation in which titanium implants rather than polymeric scaffolds served as a carrier for the BMP-2-functionalized calcium-phosphate coating [46]: This mode of BMP-2 delivery was more efficacious in inducing and sustaining bone formation over a 5-week period than was a directly-adsorbed depot of the drug (Figs 2 and 9).

One of the most striking and novel findings of the present study relates to the influence of the mode of BMP-2 carriage on the inflammatory response that is triggered by the polymeric materials. As gauged by the volume density of foreign-body giant cells within the subcapsular space, the inflammatory response was less severe when the polymeric material bore a BMP-2-functionalized calcium-phosphate coating than when it bore a directly-adsorbed depot of the drug. In a clinical context, this finding is of paramount importance, since it points to a means not only of curbing inflammatory reactivity but also of dramatically lowering the pharmacological dose of the applied osteogenic agent to a safe level. The relevant literature affords several clues that might help to account for our finding. In the first place, an augmentation of osteogenic activity has been shown to dampen inflammation by suppressing the participation of foreign-body giant cells. The effect is mediated by osteopontin, which, by occupying the CD44 surface receptors on macrophages, inhibits the multi-nucleation process [47] that is indispensable for the formation of foreign-body giant cells [48]. The presence of osteoprogenitor cells is also known to dampen inflammatory reactivity [49]. Secondly, the inflammatory response may be modulated by differences in the controlled, macrophage-activated degradation of the polymeric material itself, since the degradation products can further promote the recruitment and activation of macrophages. In this context, the nature of the polymeric material, as well as the absence or presence of a BMP-2-functionalized calcium-phosphate coating, will play a role. During the early post-implantation phase (up to 7 days), the presence of a calcium-phosphate coating would be expected to afford the host some protection against the pro-inflammatory effects of polymer degradation.
Indeed, during this phase, which was not investigated in the present study, the inflammatory response that is triggered by the calcium-phosphate coatings should not be influenced by the nature of the underlying polymer. However, after about 2-3 weeks, the coating will have undergone complete degradation, and the underlying material will be exposed. The degradation of the polymeric material will then provoke a secondary inflammatory response, which will vary according to the pro-inflammatory characteristics of the breakdown products. The influence of differences in the mechanical properties of the four materials could be excluded, since each polymer was stabilized by a titanium disc (to neutralize the effects of irregular stress-fields generated within the dorsal skin and body muscles of the rats). A direct correlation existed between the surface-area density of the polymeric material and the volume density of bone \( r^2 = 0.97 \) (Fig. 3), and an inverse one between the volume density of bone and the volume density of foreign-body giant cells \( r^2 = 0.96 \) (Fig. 7). Hence, for polymers that bore a BMP-2-functionalized calcium-phosphate coating, the surface-area density of the polymer itself overrode the contribution of other characteristics, such as chemical composition or macroscopic form (fibrillar or sponge-like), on bone formation. Nevertheless, the influence of competitive factors, such as biocompatibility and biodegradability, on the inflammatory response, and thus on bone formation, cannot be neglected at a later stage. During the 5-week monitoring period, the four types of polymer were degraded to varying degrees (Fig. 8), the two extremes being represented by Polyactive® (less than 10%) and collagen (more than 90%). These differences in degradation are reflected in the volume density of foreign-body giant cells that occurred within the subcapsular space (Fig. 6). Since the discs of collagen had been almost completely degraded by the 5-week juncture, the peak in foreign-body-giant-cell activity had passed. Discs of Polyactive®, on the other hand, had only just begun to undergo degradation at this time. Hence, foreign-body-giant-cell activity was still rising at the 5-week juncture, and the peak had not yet been attained. The biodegradability of a material will depend not only upon intrinsic factors, such as chemical composition, but also upon extrinsic ones, such as the local enzymatic apparatus, as well as upon co-operative ones, such as the biocompatibility of the material with the bodily compartment in which it is implanted. As aforementioned, the presence of a BMP-2-functionalized calcium-phosphate coating delays the biodegradation of the underlying polymer. In the case of collagen particularly,
which is characterized by a high pro-inflammatory response [50] and a high degradation rate, osteogenic activity is probably favoured by the delay in, and perhaps also the attenuation of, inflammatory reactivity (as being triggered at a later stage, viz., after the coating has been degraded and when bone-formation activity is well underway).

The amounts of BMP-2 that were incorporated into the calcium-phosphate coatings of the four different polymer types ranged from 0.1-0.7 μg/mm³ of polymeric material (Table 1). The amounts of BMP-2 that were adsorbed directly onto the surfaces of each polymeric disc ranged from 0.06-0.26 μg/mm³ of material (Table 1). Hence, the incorporated and the adsorbed depots of the drug that were associated with the four types of polymer lay within an order of magnitude of each other. Albeit so, the osteoinductive efficacy of BMP-2 varied greatly in association with the different materials, although, as aforementioned, this parameter was consistently higher for a coating-incorporated than for a directly-adsorbed depot of the drug (Fig. 9). The difference in osteoinductive efficacy between the two modes of BMP-2 delivery was least for Polyactive®. This finding is not surprising, since a higher volume of foreign-body giant cells was associated with Polyactive® than with any of the other three materials (Fig. 5). Such a high level of inflammatory activity appears to suppress osteogenesis. The negative influence of inflammation on osteogenic activity is mediated predominantly by cells of the monocyte/macrophage lineage, which align the polymer surface and are induced by the toxic chemical properties of the material itself or of its degradation products to undergo apoptosis. However, these cells escape death by fusing to form foreign-body giant cells [51, 52], which trigger a chronic inflammatory response.

Although the volumes of foreign-body giant cells that were associated with Ethisorb™ and PLGA were similar, the osteoinductive efficacy of BMP-2 was higher in association with the former than with the latter material (Fig. 9). This finding is probably accounted for by the higher surface-area density of Ethisorb™ (Fig. 3).

The data gleaned from the present study indicate that several factors contribute to the osteoinductive efficacy of BMP-2-functionalized polymeric scaffolds. However, the surface-area density of the material and its biocompatibility are important attributes. Furthermore, if the BMP-2 is incorporated into a calcium-phosphate coating rather than adsorbed directly onto the surface of the polymeric
material, which is the mode of delivery that is currently adopted in clinical settings, then the characteristically slower, cell-mediated liberation of the drug is associated with a significant attenuation of the inflammatory response and a consequent augmentation of osteogenic activity.

CONCLUSION

Bone-defect-filling polymeric materials can be rendered osteoinductive by functionalizing them with a BMP-2-bearing calcium-phosphate coating. This mode of BMP-2 carriage is more efficacious in inducing and sustaining bone formation at an ectopic site in rats than is a directly-adsorbed depot of the agent. It is also associated with a greater attenuation of the inflammatory response that is triggered by the native polymer, with the consequence that bone-formation activity is correspondingly augmented. Furthermore, during the initial 2-3 weeks of implantation that precede its degradation, the coating protects the host from the pro-inflammatory effects of the products of polymer degradation, thereby permitting bone-formation activity to gain a firm foothold. In a clinical setting, BMP-2-functionalized calcium-phosphate coatings would thus afford a means of curbing inflammatory reactivity and of dramatically lowering the pharmacological dose of the osteogenic agent to a safe level.

ACKNOWLEDGEMENTS
This study was supported by a grant from the Swiss National Science Foundation [to TI and EBH (no. 320000-107639/1)].
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Chapter 4

Cell-Mediated BMP-2 Liberation Promotes Bone Formation in a Mechanically Unstable Implant Environment

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doi:10.1016/j.bone.2010.02.010
Published online 11 Feb 2010.
ABSTRACT

The flexible alloplastic materials that are used in bone-reconstruction surgery lack the mechanical stability that is necessary for sustained bone formation, even if this process is promoted by the application of an osteogenic agent, such as BMP-2. We hypothesize that if BMP-2 is delivered gradually, in a cell-mediated manner, to the surgical site, then the scaffolding material’s lack of mechanical stability becomes a matter of indifference. Flexible discs of Ethisorb™ were functionalized with BMP-2, which was either adsorbed directly onto the material (rapid release kinetics) or incorporated into a calcium-phosphate coating (slow release kinetics). Unstabilized and titanium-plate-stabilized samples were implanted subcutaneously in rats and retrieved up to 14 days later for a histomorphometric analysis of bone and cartilage volumes. On day 14, the bone volume associated with titanium-plate-stabilized discs bearing an adsorbed depot of BMP-2 was 10-fold higher than that associated with their mechanically unstabilized counterparts. The bone volume associated with discs bearing a coating-incorporated depot of BMP-2 was similar in the mechanically unstabilized and titanium-plate-stabilized groups, and comparable to that associated with the titanium-plate-stabilized discs bearing an adsorbed depot of BMP-2. Hence, if an osteogenic agent is delivered in a cell-mediated manner (via coating degradation), ossification can be promoted even within a mechanically unstable environment.

Keywords: mechanical stability, implant, direct bone formation, mechanism, BMP-2-release

INTRODUCTION

In the fields of orthopaedic, maxillofacial and dental implantology, mechanical stability of the implant itself and of the implant environment are deemed to be necessary for sustained bone formation [1, 2] and osseointegration. Currently, investigators are faced with the challenge of expediting bone formation and implant osseointegration, with a view to rendering the materials fully functional at an earlier date. However, even an osseous environment can be mechanically unstable, for example, under osteoporotic conditions and at certain anatomic sites,
such as in the maxillofacial region. Moreover, in some locations, such as the latter, a very rigid (metallic) implant is not even desirable, the flexibility, mouldability and biodegradability of an alloplastic material being considered preferable. Although these alloplastic materials are biodegradable and biocompatible, eliciting limited inflammatory responses [3-6], they are not highly osteoconductive. In clinical studies, they have been shown to be replaced not by bone but by soft collagenous scar tissue [7].

Many attempts have been made to improve the osteoconductivity of both rigid and alloplastic implant materials [8-11]. However, the degree to which bone formation can be augmented and implant integration expedited by this means is limited. Further improvements in these processes are likely to be achieved only by conferring the materials with the property of osteoinductivity, which is accomplished by functionalizing their surfaces with an osteogenic growth factor. One such agent is bone morphogenetic protein 2 (BMP-2), which promotes the repair of bony defects in laboratory animals [12-14]. Albeit so, bone-formation activity is still compromised if either the implant itself or the implant environment is mechanically unstable.

We postulate that if an alloplastic material is rendered osteoinductive by functionalizing its surface with an osteogenic agent in such a manner as to ensure its gradual, sustained and cell-mediated release [15], then the material's lack of mechanical stability becomes a matter of indifference, even if the osseous environment itself is mechanically stable or unstable.

**MATERIALS AND METHODS**

*Experimental Set-Up*

Ethisorb™ is a co-polymer of glactin and p-dioxanone, which forms a compact, non-woven fibrous meshwork. It has a surface-area density of 100 (± 9.4) mm⁻¹ and a porosity (volume density of the internal space) of 42.2 (±3.8)%. (Wu et al.: submitted for publication). The material is soft and flexible in the native state, and these properties are preserved after coating with a homogenous, 3-µm-thick layer of calcium phosphate, which adheres well to the underlying polymer. (Wu et al.: submitted for publication).
Ten-millimeter-diameter, 0.6-mm-thick discs of Ethisorb™ (Ethicon, Norderstedt, Germany) were either left unsupported or mechanically stabilized by affixation to 10-mm-diameter and 1-mm-thick plates of titanium (Ti Grade 4 medical, Fredec AG, Murten, Switzerland). The circular plates of titanium were perforated peripherally with two 0.5-mm-diameter holes, one at each pole. The Ethisorb™ discs were sutured firmly, but without compression to the titanium plates via these holes using Vicryl 5-0 thread (Ethicon). Prior to this procedure, the Ethisorb™ discs were functionalized with BMP-2. Two modes of carriage were tested: direct adsorption and incorporation into a calcium-phosphate coating. The adsorbed depot of BMP-2 was produced by applying one 5-µl-drop of a stock solution (1 mg of BMP-2/ml of phosphate-buffered saline, pH 7.4) to each surface of the Ethisorb™ disc in turn, allowing for evaporation of the solvent on the first side (15 minutes at 37°C under sterile conditions) before applying a 5-µl-drop to the reverse side. In the case of titanium-plate-stabilized Ethisorb™ discs, a 10-µl-drop of the same stock solution was applied to the free (unattached) surface. The co-precipitated layer of BMP-2 and calcium phosphate was deposited on the Ethisorb™ discs according to a standard biomimetic procedure [16], which is described below (see “Coating of Discs”). The amount of BMP-2 that was incorporated into each coating was quantified by the ELISA technique [17], and was found to be 5.8µg per disc (± 0.26µg ; n = 3). The amount of BMP-2 that was deposited directly upon the surfaces of the Ethisorb™ discs was predetermined at 10 µg per sample.

Eight groups (including the appropriate controls) were established (see Table 1). The discs (n = 6 per group and per time-point) were implanted at a subcutaneous site in a total of 72 rats (see “Surgical Procedure”), and were retrieved after 4, 7 or 14 days for a histomorphometric evaluation of cartilage and bone volumes (see “Histomorphometric Evaluation”).

Coating of Discs

Ethisorb™ discs were coated first with a fine amorphous layer of calcium phosphate and then with a more substantial (3-µm-thick) crystalline one of the same inorganic salts. The amorphous layer of calcium phosphate, which served as a seeding substratum for the growth of the crystalline one, was produced by immersing the Ethisorb™ discs in concentrated simulated body fluid (733.5mM NaCl, 12.5mM CaCl₂, 2H₂O, 5nM Na₂HPO₄.2H₂O, 21nMNaHCO₃) containing 7.5mM
MgCl₂·6H₂O to promote nucleation and to inhibit crystal growth. The medium was prepared at ambient temperature as follows: Initially, each of the inorganic ingredients except for sodium hydrogen phosphate and sodium bicarbonate were dissolved in demineralized water. The resulting solution was then acidified to pH 4.0 by bubbling gaseous carbon dioxide through it. The remaining salts were then added. After allowing a few minutes for equilibration, during which time the pH of the solution rose to 6.2, the Ethisorb™ discs were introduced. They were immersed in the solution for 24 hours at 37°C. By the end of this incubation period, the pH of the solution had risen to 8.0.

The crystalline layer of calcium phosphate was produced by immersing the coated Ethisorb™ discs in a supersaturated solution of calcium phosphate (136mM NaCl, 4mM CaCl₂·2H₂O, 2mM Na₂HPO₄·2H₂O), which was buffered with 50mM TRIS (pH 7.4). The solution was prepared at ambient temperature as follows: Initially, 41ml of 1M HCl were added to 800ml of demineralized water. The sodium hydrogen phosphate was then introduced and allowed to dissolve before adding first the calcium chloride, then the sodium chloride, and finally the TRIS buffer. The pH was adjusted to pH 7.4, and the volume was made up to one litre. After passing the solution through a Steritop® filter-unit, the Ethisorb™ discs were introduced. They were incubated for 48 hours at 37°C. When BMP-2 was to be co-precipitated with the crystalline inorganic layer, then the drug was applied to the supersaturated solution of calcium phosphate at a final concentration of 10µg/ml.

The entire coating procedure (deposition of both the amorphous and the crystalline layers of calcium phosphate) was conducted under sterile conditions using sterilized solutions and sterilized Ethisorb™ discs.

**Surgical Procedure**

The study was approved by the local Animal Research Commission and was conducted in accordance with its regulations.

Seventy-two 6-week-old adult male wistar rats (weighing 185-250 g) were used for the study. They were fed a standard diet and had unlimited access to water. A total of 144 Ethisorb™ discs were distributed amongst the 8 experimental groups (Table 1), with n = 6 discs per group and per time-point (4, 7 and 14 days). General anaesthesia was induced and maintained as previously described [18]. The left and the right dorsal regions were shaved and disinfected, and the skin was then incised. One implant was inserted subcutaneously on each side of each
animal. Ethisorb™ discs that were mechanically stabilized with a titanium plate were placed with the latter surface facing uppermost. The implants were distributed between the two dorsal sites and amongst the different animals according to a systematic random protocol, with the sole restriction that any given animal carried exclusively BMP-2-bearing or BMP-2-lacking discs. The surgical incision was closed by suturing with Vicryl 5-0 thread. The discs were retrieved 4, 7 and 14 days after implantation.

<table>
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<tr>
<th>Group</th>
<th>Absence (-) or presence (+) of a calcium-phosphate coating</th>
<th>Mechanical stabilization of Ethisorb™ disc with a titanium plate</th>
<th>Mode of BMP-2 carriage</th>
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<td>Absence (-) or presence (+) of a calcium-phosphate coating</td>
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**Histomorphometric Evaluation**

The Ethisorb™ discs were excised together with a portion of the surrounding soft tissue, and were chemically fixed in 4% formaldehyde solution (buffered with 0.1 M sodium cacodylate, and containing 1% calcium chloride to prevent decalcification) for 3 days at ambient temperature. They were then dehydrated in ethanol and embedded in methylmethacrylate, which was polymerized at ambient temperature. Using a Leica diamond saw, the embedded material was cut into twelve 500-μm-thick slices according to a systematic random-sampling protocol [19]. All slices were glued to plastic specimen holders and ground down to a thickness of 80-100μm. They were then surface-polished and surface-stained with McNeal’s Tetrachrome, basic Fuchsine, and Toluidine Blue 0 according to a published protocol [20]. The sections were photographed at a final magnification...
of x200 in a Nikon Eclipse-1000 light microscope, likewise according to a systematic random-field-sampling protocol [19]. Using the photographic prints, the volume densities of bone (unmineralized and mineralized) and cartilage that were associated with each implant were determined by point counting [21]. The total net volumes of bone and cartilage were determined by applying stereological principles using Cavalieri’s method [19].

Statistical Analysis
All data are presented as mean values together with the standard error of the mean (SEM). Data pertaining to each group were compared using a two-way analysis of variance (ANOVA). The level of significance was set at $p<0.05$. SPSS statistical software (version 16 for Mac os x) was used for this evaluation. Post-hoc comparisons were made using Bonferroni corrections.

RESULTS

General Histological Findings
On the 4th day after implantation, a mild inflammatory response involving small numbers of lymphocytes, granulocytes and macrophages was observed around each implant. This reactivity had decreased by the 7th day and had ceased altogether by the 14th day. All implants were surrounded by a capsule of vascularized connective tissue. Cartilage and bone, which became apparent for the first time on day 4 and day 7, respectively, were associated, both at these junctures and subsequently, exclusively with implants that bore an adsorbed or a calcium-phosphate-coating-incorporated depot of BMP-2. Neither cartilage nor bone was deposited on or around either uncoated Ethisorb™ discs or coated ones that bore no depot of the drug, irrespective of whether they were mechanically unstabilized or stabilized with a titanium plate. On day 4, the volume of cartilage was too small to be quantified histomorphometrically. And, as aforementioned, no osseous tissue had been deposited at this stage. Cartilaginous tissue predominated over osseous tissue on day 7, and vice versa on day 14. Bone that had been deposited on and around mechanically unstabilized Ethisorb™ discs bearing an adsorbed depot of BMP-2 had been formed via an endochondral route (Fig. 1), whereas that associated with titanium-plate-stabilized Ethisorb™ discs bearing an adsorbed depot of BMP-2 had been formed mostly directly, viz., intramembranously. Bone that had been deposited on and around mechanically
unstabilized (Fig. 3) and titanium-plate-stabilized (Fig. 2) Ethisorb® discs bearing a coating-incorporated depot of BMP-2 had been formed mostly directly, viz., intramembranously, in both cases.

Fig. 1, A & B: Unstabilized Ethisorb™ Disc Bearing Adsorbed BMP-2. Low- (A) and high-magnification (B) light micrographs of a vertical section through a mechanically unstabilized Ethisorb™ disc (E) that bore an adsorbed depot of BMP-2, 14 days after implantation. In (B), the cartilage tissue (C) that serves for the endochondral formation of bone (B) is revealed at higher resolution.

Fig. 2, A & B: Stabilized Ethisorb™ Disc Bearing Coating-Incorporated BMP-2. Low- (A) and high-magnification (B) light micrographs of a vertical section through an Ethisorb® disc (E) that bore a layer of calcium phosphate into which BMP-2 was incorporated, and which was mechanically stabilized with a titanium plate (Ti), 14 days after implantation. The Ethisorb™ disc and the titanium plate are surrounded by a capsule of vascularized connective tissue (Cap.). Osseous tissue (B) has been deposited on the co-precipitated layer of calcium phosphate and BMP-2 that coats the “free” Ethisorb™ surface. V = Vicryl suturing thread.
Fig. 3, A & B: Unstabilized Ethisorb™ Disc Bearing Coating-Incorporated BMP-2. Low-
(A) and high-magnification (B) light micrographs of a vertical section through an
unsupported (mechanically unstable) Ethisorb™ disc (E) that bore a coating-incorporated
depot of BMP-2, 14 days after implantation. In (B), the osseous tissue (B) is seen to have
condensed directly (intramembranously) from the surrounding connective tissue, not via
an endochondral mechanism (as in Fig. 1).

Histomorphometric Analysis 7 Days after Implantation
On the 7th day after implantation, cartilage predominated over bone in each of the
4 groups whose Ethisorb™ discs bore a depot of BMP-2 (Fig. 4A). The volumes
of cartilage were higher in groups with an adsorbed depot of BMP-2 than in
those with a coating-incorporated one. In the former category, the volume of
cartilage was higher in association with mechanically unstabilized than with
titanium-plate-stabilized Ethisorb™ discs. The volumes of bone at this juncture
were very small in all groups, but tended to be higher (although not significantly)
in those whose Ethisorb™ discs bore an adsorbed depot of BMP-2.
Fig. 4 A: Total Volumes of Bone and Cartilage on Day 7
Total volumes of bone and cartilage associated with mechanically unstabilized (unst.) and titanium-plate-stabilized (stab.) Ethisorb® discs (Eth.) that bore either an adsorbed (ads.) or a coating-incorporated (inc.) depot of BMP-2, 7 days after implantation. Mean values are represented together with the SEM (n = 6 per group).
Fig. 4B: Total Volumes of Bone and Cartilage on Day 14. Total volumes of bone and cartilage associated with mechanically unstabilized (unst.) and titanium-plate-stabilized (stab.) Ethisorb™ discs (Eth.) that bore either an adsorbed (ads.) or a coating-incorporated (inc.) depot of BMP-2, 14 days after implantation. Mean values are represented together with the SEM. *p=0.034; **p=0.000; ***p=0.004.

**Histomorphometric Analysis 14 Days after Implantation**

On the 14th day after implantation, the volumes of cartilage were similar in each of the 4 groups whose Ethisorb™ discs bore a depot of BMP-2 (Fig. 4B). Compared to the values on day 7, the volume of cartilage had decreased significantly only for mechanically unstabilized Ethisorb™ discs that bore an
adsorbed depot of BMP-2 (from 0.32 mm$^3$ to 0.13 mm$^3$). In the other 3 groups, the volume of cartilage had either remained the same (titanium-plate-stabilized Ethisorb™ discs bearing either an adsorbed or a coating-incorporated depot of BMP-2) or increased slightly (mechanically unstabilized Ethisorb™ discs bearing a coating-incorporated depot of BMP-2). Moreover, the first-mentioned group (mechanically unstabilized Ethisorb™ discs bearing an adsorbed depot of BMP-2) was the only one in which the volume of bone had not increased dramatically during the intervening period. In the other 3 groups, the volume of bone had increased to a similar degree and by about 10-fold. Consequently, the ratio of cartilage to bone on day 14 was highest for mechanically unstabilized Ethisorb™ discs that bore an adsorbed depot of BMP-2; in the other 3 groups, it was between 5- and 7-fold lower (Fig. 5).
DISCUSSION

That BMP-2 is a potent inducer of bone formation has been long known [22, 23]. But it has only recently become apparent that the efficacy of this agent depends upon its mode of delivery. Using a well-established ectopic ossification model in rats, we have shown the osteoinductive efficacy of BMP-2 to be highest when the
drug is liberated gradually, via the mediation of local cells, from a bone-matrix-like, calcium-phosphate carrier, viz., in a physiological-like manner [18]. In this previous investigation, rigid titanium implants were used. In the present investigation, we wished to evaluate the osteoinductivity of BMP-2 that was borne by a soft, flexible, alloplastic material. For this purpose we used Ethisorb™, which is a co-polymer of glycolate and 1-lactate (90:10). In addition to testing two different BMP-2-delivery modes (adsorption versus incorporation into a calcium-phosphate coating), we also wished to assess the influence of mechanical stability on the osteoinductive response. To this end, the discs of Ethisorb™ were either left unsupported (mechanically unstabilized) or stabilized with a titanium plate prior to implantation at a mechanically unstable (subcutaneous) ectopic ossification site in rats, viz., between the skin muscle and the body muscle.

On the 7th day after implantation, the volumes of cartilage were higher in association with Ethisorb™ discs that bore an adsorbed depot of BMP-2 than with those that bore a coating-incorporated depot of the drug. We speculate that this finding can be accounted for by the differing kinetics of BMP-2 release. An adsorbed depot of BMP-2 is liberated rapidly in a single, high-dose burst of no more than a few hours’ duration, whereas a coating-incorporated depot of the drug is released gradually, in a cell-mediated manner [15], at a much lower dose, and for a longer period of time (some weeks). Hence, although on the 7th day the volumes of cartilage associated with Ethisorb™ discs bearing an adsorbed depot of BMP-2 were the highest, by the 14th day, the drug depot had been long exhausted, with the following consequences. Since the bone associated with unsupported (mechanically unstable) Ethisorb™ discs that bore an adsorbed depot of BMP-2 was formed via an endochondral route, and since the depot of BMP-2 had been exhausted by the 7th day, the volume of bone that could be subsequently laid down (by the 14th day) was limited by the volume of cartilage that had been deposited by day 7. Consequently, by day 14, the volume of cartilage had decreased at the expense of bone formation. This was the only group in which the volume of cartilage decreased between the 7th and the 14th days. On the other hand, the bone associated with titanium-plate-stabilized Ethisorb™ discs that bore an adsorbed depot of BMP-2 was formed directly, not via an endochondral route. Consequently, between the 7th and the 14th days, the volume of bone increased, but not at the expense of cartilaginous tissue, the volume of which remained
almost unchanged. The presence and persistence of this very small volume of cartilage could be attributable to the mechanically destabilizing influence of residual shear forces that may be still acting (or be less dampened) close to the skin muscles and at some distance from the titanium-plate-stabilized Ethisorb™ discs. Interestingly, the direct formation of bone, unlike its formation via the endochondral route, was sustained even though the entire adsorbed depot of BMP-2 had been liberated. Conceivably, the liberated drug was locally sequestered and subsequently made available for osteoinduction only to osteoblasts that were harbouring the soft connective tissue (from which bone condenses directly according to the intramembranous route), not to those that were more intimately associated with the calcified cartilage. The findings of greatest interest relate to the Ethisorb™ discs that bore a coating-incorporated depot of BMP-2. Irrespective of whether they were mechanically unstabilized or stabilized with a titanium plate, the osseous tissue deposited on and around these implants was laid down directly, viz., intramembranously, not via an endochondral route. On day 14, the volume of bone deposited in each of these 2 groups was similar, and comparable to that associated with the mechanically stabilized Ethisorb™ discs that bore an adsorbed depot of BMP-2 at the same juncture. Hitherto, a mechanically stable environment has been deemed necessary for sustained bone formation via the direct (intramembranous) route. Indeed, the formation of bone via an endochondral route at an ectopic site generally indicates that the environment is mechanically unstable. This tenet is supported by the findings for unstabilized Ethisorb™ discs that bore an adsorbed depot of BMP-2. However, the findings for mechanically unstabilized Ethisorb™ discs that bore a coating-incorporated depot of BMP-2 indicate that, if the osteogenic agent is delivered in a physiological-like manner, viz., via the cell-mediated degradation of a bone-matrix-like material (calcium-phosphate coating) into which it is incorporated, then the absence of a mechanically stable environment (unstabilized Ethisorb™ discs) becomes a matter of indifference. This revelation is of importance in the fields of dental, orthopaedic and maxillofacial surgery. It implies that the placement of an implant within an osseous environment that has been mechanically destabilized by pathological agencies (e.g., osteoporosis) or trauma can be rendered a safer, less risky and more reproducibly successful undertaking than heretofore in terms of the desired result, namely, speedy osseointegration.
CONCLUSION

In many clinical situations, the flexibility, mouldability and biodegradability of alloplastic materials are more desirable than the rigidity of permanent fixtures. However, neither type of implant is intrinsically osteoinductive, and alloplastic materials suffer from the additional disadvantage of lacking mechanical stability, which has been deemed necessary for direct bone formation. This mechanism of ossification is the preferred one in that bone is thereby produced more rapidly than when an endochondral route is pursued. Moreover, endochondral ossification yields a type of bone that does not persist for any great length of time. However, irrespective of the mechanism, bone formation must first be induced by introducing an osteogenic agent, such as BMP-2, the efficacy of which will depend upon its mode of delivery. The findings of our study indicate that when BMP-2 is incorporated into a bone-matrix-like layer of calcium phosphate that is deposited upon an alloplastic material (Ethisorb™), then the kinetics of its subsequent release at a mechanically highly unstable ectopic (subcutaneous) implantation site in rats are such as can overcome the impediment to direct bone formation that is presented by the material’s lack of mechanical stability.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Swiss National Science Foundation (to Y. Liu) and by the University of Bern. The authors would like to thank Dr. Lukas Enggist for his help with the statistical analysis, Wyeth (Cambridge, MA, USA) for its generous gift of human recombinant BMP-2, and Tatjana Arpagaus for her technical assistance.
REFERENCES

Chapter 5

BMP-2-incorporated Calcium Phosphate Coating Converts the Pro-inflammatory Activity within an Orbital-defect-filling-polymer, Ethisorb™ into a pro-osteogenic one

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In preparation
ABSTRACT

Objective:
The objective of this study was to evaluate whether BMP-2-coprecipitated calcium-phosphate (CaP) coating could convert the pro-inflammatory activity within a resorbable orbital-defect-filling-polymer, Ethisorb™ into a pro-osteogenic one in a mechanically unstable and non-osteogenic milieu.

Summary background data: Current treatments for large-area orbital bony defects such as autografts, allografts and non-resorbable artificial materials are associated with various limitations. In the non-osteogenic and mechanically unstable milieu of large-area orbital defects, resorbable materials such as Ethisorb™ are prone to a fibrous healing, which can be overcome by rendering them osteogenic.

Methods: The non-osteogenic and mechanically unstable milieu of large-area orbital bony defects was simulated in subcutaneous environment of rat back. Mechanically unstable milieu was shielded by fixing half of the samples with titanium discs. 7 or 14 days after implantation, the osteogenicity, foreign-body reaction and vascularization within the Ethisorb™ with a BMP-2-coprecipitated CaP-coating were histomorphometrically estimated using stereological principles with the Ethisorb™ with either a CaP-coating, or an adsorbed depot of BMP-2 (BMP-2-Ads.), or no treatment as controls.

Results: On the 14th day, Ethisorb™ with a BMP-2-coprecipitated CaP-coating exhibited significantly higher volume densities of total osteogenic tissues (bone and cartilage) than the others, which was attributed to a characteristic BMP-2-coprecipitated CaP-coating-originated intramembranous ossification. Foreign-body reaction was suppressed by CaP-coating irrespective of BMP-2.

Conclusion: The BMP-2-coprecipitated CaP-coating drastically converts the pro-inflammatory activity within Ethisorb™ into a pro-osteogenic one in a mechanically unstable and non-osteogenic milieu. These properties confer the modified Ethisorb™ a promising potential for large-area laminar bone defects.

Key words: Bone morphogenetic protein-2; Biomimetic coating; Calcium phosphate; Pro-osteogenic; Fibrous polymer
INTRODUCTION

Large-area defects of orbital laminar bone wall can cause enophthalmos and oculomotor disorders through the sagging of orbital contents e.g. the eyeball, extraocular muscles, and orbital fat into the paranasal sinus and thus incarceration of them [1-3]. Inappropriate bringing of the defect will leave a series of post-operational or late sequelae such as esthetic asymmetry and disorder of eye-globe movement, diplopia, enophthalmos, and exophthalmos, etc [4]. The treatment essential is to bridge the defect for the reposition of orbital content and to restore the attachment of ocular muscles for movement function [5].

Current grafts for the repair of orbital laminar bone defects, however, are associated with various limitations because of both their own disadvantages and the complicated environment for orbital bone regeneration [6, 7]. Autologous bone grafts were associated with the pain and morbidity of donor site [6], limited availability, the variable resorption after implantation [8], and the difficulty to reproduce the complicated curvature of orbital wall [9, 10]. Autografting periosteum is too weak to support the orbital content in the early period. Allogeneic grafts [11] have been discarded for its potential transmission of virus and infectious diseases like Creutzfeldt-jakob disease [12]. As alternative, non-biodegradable artificial materials such as titanium and silicone have been used tentatively [13, 14]. However, they are nonresorbable foreign bodies, and many complications especially in the late period (6months after operation) due to infections, foreign-body reactions, and post-graft displacement have been reported [15, 16]. Resorbable materials such as polydioxanone plates, polyglactin (e.g. Ethisorb™) are thus recommended to avoid the late complication [5, 17]. However the material is not osteogenic so that they can be replaced not by bone tissue but by dense collagenous scar tissue [5, 18]. The mechanical property of such scar tissue is insufficient to support the eyeball, thereby resulting in persistent enophthalmus or diplopia [5, 19].

The dilemma of resorbable materials can be overcome by rendering them osteoinductive using an osteogenic agent, such as bone morphogenetic protein-2 (BMP-2) which is a member of the superfamily of transforming growth factors [20]. BMP-2 has been used extensively to enhance bone formation both in experimental animals and in clinical trials [21-23]. Attempts have been made to
adsorb BMP-2 to polymeric bone-defect-filling materials [24]. Although bone formation can be thereby triggered, the pharmacological dose that must be applied to induce ossification is exceedingly high: the adsorbed depot of watersoluble BMP-2 diffuses away too rapidly to be effective [25]. Moreover, in addition to the unnecessary cost of such wastage, the transiently very high concentrations of this growth factor can trigger undesirable side-effects such as bone formation at unintended sites, and an augmentation of local neurophathy [26, 27].

The efficacy of BMP-2 can be drastically potentiated and the aforementioned side-effects can be avoided when they are delivered in a controlled and sustained manner. Our technique “BMP-2-incorporated biomimetic calcium-phosphate coatings [28]” can be a promising strategy for this purpose. The biomimetic calcium phosphate coating can be applied to a broad spectrum of biomaterials such as titanium implants, synthetic polymeric materials, deproteinized bovine bone natural-derived collagen. Furthermore, it can incorporate osteogenic agents such as BMP-2 through co-precipitation and controlled-release them slowly and sustainably [21, 29]. To provide a viable therapy for reconstruction of orbital bone defect, this technique has been applied onto four market-available and biodegradable polymeric membranes and has been shown to induce and sustain bone formation at a subcutaneous ectopic site after 5-week implantation using physiologically low loading doses [30]. Surprisingly, the volume densities of bone tissue induced by coating-incorporated BMP-2 at 5 weeks highly proportionally correlated to the surface-area density of polymers at time 0. Furthermore, the higher volume density of bone was associated with the lower foreign-body reaction. Among the four selected polymeric membranes, the functionalized Ethisorb™ that bore an as high surface-area density as 100 mm$^{-1}$ was superior in bone formation, degradation and osteoinductive efficacy.

This study was designed to further evaluate the functionalized Ethisorb™ in the early stage (within 2 weeks after implantation) of bone induction so as to unveil the mechanism whereby pro-fibrogenic microenvironment was turned into a pro-osteogenic one. Moreover, to predict the performance of functionalized Ethisorb™ in mechanically instable microenvironment caused by surrounding mobile tissues in orbital defect, either mechanically stable or unstable environments were created at a subcutaneous site by the presence or absence of fixing Ethisorb™ to titanium discs. Several biological events to functionalized
Ethisorb™ were monitored: tissue ingrowth rate, osteogenesis mode, foreign-body reaction, vascular permeation, and volume maintenance.

**MATERIALS AND METHODS**

*Material preparation*

Ethisorb™ (a co-polymer of glactin and -dioxanone, Johnson & Johnson) is a compact, non-woven fibrous meshwork with a surface-area density of 100 (±9.4) mm⁻¹ and a porosity (volume density of the internal space) of 42.2 (±3.8)% [31]. The material is soft and flexible in the native state, and these properties are preserved after coating with a homogenous, 3-µm-thick layer of calcium phosphate, which adheres well to the underlying polymer [31]. 0.6-mm-thick patches of Ethisorb™ were punched into discs with 10mm in diameter.

*Biomimetic coating deposition on Ethisorb™ discs and BMP-2 incorporation*

Ethisorb™ discs were biomimetically coated with a layer of calcium phosphate using our well-established protocol [21, 28, 29]. BMP-2 was incorporated by coprecipitated with the calcium phosphate coating. The coating procedure and BMP-2 incorporation were performed as described previously [32].

*Quantification of coating-incorporated depot of BMP-2 and adsorption of BMP-2*

The amount of BMP-2 that was incorporated into each coating was quantified by the technique of enzyme-linked immunosorbant assay (ELISA) [33] and was found to be 5.8mg per disc (± 0.26mg ; n = 3). [31, 32] The amount of BMP-2 that was deposited directly upon the surfaces of the Ethisorb™ discs was predetermined at 10-µg per sample.

*Creation of in vivo mechanically stable or unstable microenvironments*

The study was approved by the local Animal Research Commission and was conducted in accordance with its regulations. Seventy-two 6-week-old adult male wistar rats (weighing 185-250 g) were used for the study. Mechanically unstable microenvironment is surgically created for samples by inserting them between the dorsal skin and the underlying muscle of rats. The mechanical forces that were caused by the movement of skin and back muscles were imposed on the inserted samples. This was designed to mimic the mechanical forces that were caused by movement of orbital muscles surrounding orbital defect. On the other hand, to create mechanically stable microenvironments, the mechanical forces...
were blocked by fixing the other half of discs to 10-mm-diameter and 1-mm-thick plates of rigid titanium (Ti Grade 4 medical, Fredec AG, Murten, Switzerland). The surgical procedure and the fixation was performed as previously described [32].

Experimental set-ups
Eight groups were set up with n = 6 discs per group and per time-point (4, 7 and 14 days): 1) Mechanically unstable, uncoated, non-BMP-2; 2) Mechanically stable, uncoated, non-BMP-2; 3) Mechanically unstable, uncoated, adsorbed BMP-2; 4) Mechanically stable, uncoated, adsorbed BMP-2; 5) Mechanically unstable, coated, non-BMP-2; 6) Mechanically stable, coated, non-BMP-2; 7) Mechanically unstable, BMP-2-incorporating CaP coating functionalized; 8) Mechanically stable, BMP-2-incorporating CaP coating functionalized.

Histological processing
Samples were retrieved at 4 days, 7 days and 14 days after surgery. Histological processes such as fixation, dehydration, embedding, and sectioning were performed as previously described [32]. All slices were glued to plastic specimen holders and ground down to a thickness of 80-100mm. They were then surface-polished and surface-stained with McNeal’s Tetrachrome, basic Fuchsine and Toluidine Blue 0 according to a published protocol [34]. The sections were photographed at a final magnification of x20 (to estimate space occupying volume) or x380 (to estimate biological parameters within Ethisorb™) in a Nikon Eclipse-1000 light microscope, likewise according to a systematic random-field sampling protocol [35].

Histomorphometry

Space occupying volume
Space occupying volume of Ethisorb™ of any variant referred to the actual physical space occupied by Ethisorb™ and its related tissues such as newly formed tissues. A stable space occupying volume is an important criterion for ideal biomaterials for orbital reconstruction to precisely restore the orbital volume and thus to avoid enophthalmos or exophthalmos. To evaluate the characters in volume maintainance of Ethisorb™ either untreated or with other treatments, the space occupying volume at each time points were estimated determined by the point-counting technique using Cavalieri's methodology [36] on the photographs with a magnification of x20.

Rates of tissue ingrowth
The rate of tissue ingrowth indicated the progress of fibrovascular permeation within Ethisorb™. On the photographs with a magnification of x380, volume fraction of the space with tissue ingrowth to total space within Ethisorb™ was estimated using stereologically from its area density on tissue sections by the point-counting technique [35].

*Volume densities of bone, cartilage, vascular vessel and foreign body giant cells in tissue ingrowth within Ethisorb™*

Interactions of osteogenic activities, vascularization and foreign body reaction within in tissue ingrowth within Ethisorb™ were exhibited using bone, cartilage, vascular vessels and foreign-body giant cells as indicators respectively. The volume densities of bone, cartilage, and vascular vessels in tissue ingrowth within Ethisorb™ were directly estimated by point-counting system using point grid. [37]. The volume density of foreign-body giant cells in tissue ingrowth within Ethisorb™ was determined by subtracting the number of TRAP-positive cells (i.e., osteoclasts) from the total number of multinucleated cells (estimated using conventionally stained sections) as previously described [29].

*Statistical Analysis*

All data are presented as mean values together with the standard deviation of the mean (SD). Data pertaining to each group were compared using a two-way analysis of variance (ANOVA). The level of significance was set at \( p<0.05 \). SPSS statistical software (version 15.0 for Windows) was used for this evaluation. Post-hoc comparisons were made using Bonferroni corrections.

**RESULTS**

*Space-occupying volume*

The average space-occupying volume of Ethisorb™ varied from 47mm³ to 65mm³ and maintained in this level with time, no significant difference was found among Ethisorb™ with different variants at any selected time points (4, 7, 14 days after implantation).

*Rate of tissue ingrowth within Ethisorb™*

After 4-day implantation, no tissue ingrowth was found within the Ethisorb™ of any variants in either mechanically unstable or stable environment. After 7-day implantation, rates of tissue ingrowth within Ethisorb™ varied from 10% to 30%. Within Ethisorb™ of any variant, no significant difference in rates of tissue
ingrowth was found between under mechanically instable and stable microenvironments. In mechanically unstable microenvironments, rates of tissue ingrowth within either Ethisorb™ alone or Ethisorb™ with adsorbed BMP-2 were significantly higher than that of the Ethisorb™ with CaP coating but not than that of Ethisorb™ with coating incorporated BMP-2. In mechanical stable environments, no significant differences in rates of tissue ingrowth were found within Ethisorb™ with different variants. On 14 days, average rates of tissue ingrowth within Ethisorb™ varied from 75%-95%. Rate of tissue ingrowth within Ethisorb™ with coating incorporated BMP-2 under a mechanically unstable microenvironment was significantly higher than those within Ethisorb™ with any other variants in either mechanical microenvironment, among which no significant differences were found.

*Mode and quantity of osteogenesis within Ethisorb™*

No osteogenic activities could be detected either within Ethisorb™ after 4-day implantation. Osteogenic activities were associated only with Ethisorb™ with either coating-incorporated or adsorbed BMP-2 after 7-day implantation. In contrast, no osteogenic activities occurred to Ethisorb™ either with CaP coating or untreated on the three selected time points.

Tiny amount of osteogenic tissues including bone and cartilage could be ingrowth within the Ethisorb™ with either coating-incorporated BMP-2 or adsorbed BMP-2 after 7-day implantation. However, significant differences in volume densities of neither total osteogenic tissue nor bone nor cartilage were found Ethisorb™ with either coating-incorporated or adsorbed BMP-2 under either mechanically unstable or stable microenvironments.

After 14-day implantation, irrespective of the mechanical environments, the volume densities of total osteogenic tissues within the Ethisorb™ with coating-incorporated BMP-2 were significantly higher than those within Ethisorb™ with adsorbed BMP-2 (Fig. 1).
Both cartilage and bone could be detected within Ethisorb™s with either delivery mode of BMP-2. Within Ethisorb™ with coating-incorporated BMP-2, according to the relationship between of ossification site and Ethisorb™ fibers/coating, the bone regeneration could be classified into two types: 1) BMP-2-coating-originated; 2) connective tissue-localized. In the former type, ossification—a thin layer (0.3-0.6μm) of fuchsinophilic calcified osteoid started directly: formed “ossification rings” directly on the coatings that was deposited on Ethisorb™ fibers (fig. 2A); meanwhile, within the interval space nearby the ossification rings, neither uncalcified nor calcified osteoid were found. With the progress of BMP-2-coating-originated ossification, some fuchsinophilic reddish osteoids with scattered scarlet calcification points were detected surrounding the thin layer of calcified tissue, and in the nearer area to the original ossification rings the more calcification points were found (fig. 2B). The ossification rings thickened with the enrichment of calcification points (Fig. 2B). In this manner, the calcification area spread from the original ossification ring and formed larger and larger ossification area (Fig. 2C). The percentages of BMP-2-coating-originated intramembraneous ossification to total osteogenic activities within the Ethisorb™ with coating-incorporated BMP-2 were 82.9±13% under stable...
environments and were 87.3±16.0% under unstable environments. This BMP-2-coating originated ossification was not found within Ethisorb™ with adsorbed BMP-2. In contrast, in the connective tissue-localized ossification, the calcification area only occurred not directly on the surfaces of Ethisorb™ fibers but in the interval space among them, although the calcification area could be also surrounding the Ethisorb™ fibers (Fig. 2D). No significance in volume densities of connective tissue originated intramembraneous ossification was found between the Ethisorb™ with coating-incorporated BMP-2 and the Ethisorb™ with adsorbed BMP-2 (Fig. 2). Cartilage tissue occurred to all the Ethisorb™ with BMP-2 and no significant difference in volume density of cartilage was found among Ethisorb™ with either coating-incorporated or adsorbed BMP-2 under either mechanically unstable or stable microenvironments.

Fig. 2 Light micrographs of sections through the Ethisorb™ discs with either calcium phosphate coating-incorporated BMP-2 (A, B, C, D and E), or adsorbed BMP-2 (F) after 14-day implantation in subcutaneous ectopic bone induction model of rat.

Foreign-body reaction
With the tissue ingrowth after 7-day implantation, foreign body giant cells formed within Ethisorb™ with any variant (fig. 3). Average volume density of FBGCs (fig. 5A) within Ethisorb™ with adsorbed BMP-2 was relatively lower than that within Ethisorb™ untreated. Similarly, average volume density of
FBGCs within Ethisorb™ with coating-incorporated BMP-2 was relatively lower than that within Ethisorb™ with CaP coating in either mechanical microenvironment. The lowest value occurred to Ethisorb™ with coating-incorporated BMP-2 in either mechanical microenvironment. No significant difference in volume density of FBGCs was found for Ethisorb™ with any variant between under mechanically stable and unstable environments.

At the 14th day, average volume density of FBGCs within Ethisorb™ with coating-incorporated BMP-2 was still relatively lower than that within Ethisorb™ with CaP coating in either mechanical microenvironment. However, average volume density of FBGCs within Ethisorb™ with adsorbed BMP-2 was relatively higher than that within Ethisorb™ untreated. Furthermore, volume densities of FBGCs within Ethisorb™ with CaP coating and Ethisorb™ with coating-incorporated BMP-2 were significantly lower than the Ethisorb™ untreated or with adsorbed BMP-2 irrespective of the presence of BMP-2 or mechanical environments. No significant differences were found among the
coated or uncoated Ethisorb™ irrespective of the presence of BMP-2 and mechanical environments.

**Vascular permeation**

On both 7th and 14th day, the volume density of vascular vessels (fig. 5B) varied between 0.05 and 0.15 mm³/mm³ (fig. 4). No significant differences of volume densities of vascular vessels were found among Ethisorb™ of different variants at each time point.

Fig. 4 Graph depicting the volume densities of micro-blood vessels in the tissue influx within the Ethisorb™ discs either untreated, with adsorbed BMP-2 (Ads.BMP-2), with calcium phosphate coating (CaP coating) or with calcium phosphate coating-incorporated BMP-2 (CaP coating Inc.BMP-2) in either mechanical stable (M. stable) or instable (M. unstable) microenvironment after 7- or 14-day implantation in subcutaneous ectopic bone induction model of rat.
Fig. 5 Graph depicting the volume densities of foreign body giant cells in the tissue influx within the Ethisorb™ discs either untreated, with adsorbed BMP-2 (Ads.BMP-2), with calcium phosphate coating (CaP coating) or calcium phosphate coating-incorporated BMP-2 (CaP coating Inc.BMP-2) in either mechanical stable or instable microenvironment after 7- or 14-day implantation in subcutaneous ectopic bone induction model of rat.

DISCUSSION

Treatment of orbital bone defect has long been a formidable challenge to maxillofacial surgeons and the selection of materials remains an ongoing debate [7]. An ideal one should restore the defect with bone tissue which can integrate into surrounding original orbital bone wall and can restore the anatomic curvature and function of orbital wall without severe post-operative and late complications. However, to date, no single material has been universally successful in meeting every one of these criteria [7]. Therefore, this study aimed to seek a viable option for reconstruction of orbital wall.

Biodegradable materials possess a distinct advantage over the life-long risk of complications characteristic of non-degradable alloplasts. The importance of biodegradability also lies in that it enabled complete osseous restoration. In current concept of bone tissue engineering, the 3-dimensional geometry of the bone substitutes can be optimized to enhance their osteoconductivity for rapid bone ingrowth. A design standard like interconnectively porous structure with a pore size above 300μm and a porosity below 90% was recommended due to enhanced new bone formation and the formation of capillaries [38]. However, most biodegradable synthetic materials have been shown to present a fibrous encapsulation instead of bony restoration [5, 39]. This is due to the violation of osteogenic capacity in bone defects. In severe fractures, the disruption of the periosteum compromised the bone regeneration of orbital bone defects [40]. Under this condition, fracturing edges of orbital wall would be the only resource of osteogenic tissue to grow into the substitute. Anatomically, the weakest part of orbital wall, where the fractures mostly occurred, could be too thin (0.27mm [41]) to provide enough osteogenic elements to penetrate into the substitute. Meanwhile, the surrounding connective tissues, which are advantageous in proliferation and penetration over bone tissues [42], penetrate into and take over the whole substitute through the much larger upper and lower surface of the substitute. The microenvironment of large-area orbital wall defect is, thus, pro-
fibrogenic as an ectopic site instead of pro-osteogenic. Consequently, to achieve osseous restoration, the microenvironment of orbital defect has to be changed from pro-fibrogenic to pro-osteogenic by osteogenic materials. Although autografted periosteum could be used to confer osteogenecity to polymeric material [9] during surgery, a new methodology to confer osteoinductivity preclinically is still needed to avoid second surgical site and longer surgical period. Osteoinductivity can also be conferred using an osteoinductive agent BMP-2. However, current delivering mode of BMP-2 by simple adsorption was not recommended because the non-immobilized osteoinductive agents may diffuse rapidly and cause a serious of potential complications.

The dilemma of BMP-2 application can be overcome by our technique of BMP-2 incorporated biomimetic coating in which BMP-2 was co-precipitated with calcium phosphate and immobilized into the lattice work of the coatings. Four polymeric materials were adopted to test the technique for delivery of bioactive proteins. Consistent results were found for all the materials that a slow release of proteins could sustain over 5 weeks in vitro in contrast to the rapid depletion of the adsorbed ones within 3-5 days [31]. The in vivo evaluation has shown that the osteoinductive efficacy of coating-incorporated BMP-2 were higher or significantly higher than that of adsorbed BMP-2 for the four polymeric membranes after 5 week implantation [30]. In this study, on 14 days, results also confirmed that the volume of newly osteogenic tissues within Ethisorb™ with coating-incorporated BMP-2 was significantly higher than those within Ethisorb™ with adsorbed BMP-2.

One of the most striking discoveries was that, apart from connective tissue-localized ossification, a characteristic ossification type—“BMP-2 incorporated coating-originated ossification” could be found within the Ethisorb™ with coating-incorporated BMP-2. In this ossification type, the ossification originated directly on the surface of BMP-2-incorporating coating which was deposited polymers and formed an “ossification ring”. In the primary stage of this type of ossification, no ossification could be found in the surrounding connective tissue (fig. 2A). This suggested that, apart from the burst release, the BMP-2 incorporated into the coatings could be immobilized in coatings and did not diffuse into the surrounding connective tissue. The coating-immobilized BMP-2 could also exert the osteoinductive effect and enrich osteoblasts directly on the interface between coating and surrounding tissues. After the formation of
“ossification rings”, osteotoid formed surrounding the ossification rings and scattered calcified points. The more calcified points could be found in the nearer area to the original ossification rings (fig. 2B). The calcified points eventually joined and formed a calcified woven bone surrounding the original ossification rings (fig. 2C). These spatial characteristics suggested that this type of ossification was initiated and motivated by the coating-incorporated BMP-2. The “BMP-2 incorporated coating originated intramembranous ossification” was the main type of bone formation and the unique mechanism for the advantages of functionalized Ethisorb™ with coating-incorporated BMP-2 in bone regeneration over the Ethisorb™ discs with adsorbed BMP-2 (fig. 1). In contrast, the connective tissue-localized ossification lacked of the BMP-2-coating-centered spatial characteristics. Neither did the cartilage. The latter two ossification type didn’t contribute to the advantages of the functionalized Ethisorb™ discs in bone regeneration since no significant difference in either connective tissue-originated intramembranous ossification or endochondral ossification was found between Ethisorb™ discs with coating-incorporated BMP-2 and with adsorbed BMP-2 (fig. 1).

The spatial characteristics of “BMP-2-incorporated coating originated intramembranous ossification” could be extrapolated to a situation that the more interfaces between coating and connective tissues the polymer could provide, the more original ossification rings and thus the more bone formed. This hypothesis was supported by our previous findings that the bone volume densities within the four BMP-2-incorporated coating functionalized polymers after 5-week subcutaneous implantation positively correlated to the surface-area densities of polymers at time 0 in linear proportion. The findings suggested a novel concept of polymer design that emphasizes surface-area density in comparison to the aforementioned conventional concept for bone regeneration.

One concern on the use of alloplastic materials is the host rejection. FBGCs that are fused from depressed macrophages are signs of chronic host foreign body reaction and account for the failures of implants [43]. Relatively small increases in the amount of host reaction to a given material directly affect the permanence of a biomaterial for orbital defects [7]. On 7th day, the presence of BMP-2, irrespective of delivering mode, can reduce the volume density of FBGCs although the reduction was not significant. This may be due to the transient high amount of BMP-2 that were resulted from rapid release of both adsorbed and
coating-incorporated BMP-2 once they were exposed to physiological environments [31]. Coating-incorporated BMP-2 resulted in the lowest level of FBGCs within Ethisorb™ even from the early stage in either mechanical environment. Volume densities of FBGCs, thereafter, showed an equal or decreasing trend within Ethisorb™ with coating-incorporated BMP-2 but showed an increasing trend within Ethisorb™ with adsorbed BMP-2. On 14th day, biomimetic coatings overrode other factors and resulted in significantly lower volume densities of FBGCs than those of uncoated Ethisorb™ (either untreated or with adsorbed BMP-2). The trends of changes in FBGCs within Ethisorb™ were consistent with those on the titanium surfaces after 7- and 14-day subcutaneous implantation [29]. Our previous studies also showed that after 5 weeks, after the complete degradation of coatings, the volume densities of FBGCs within Ethisorb™ with coating-incorporated BMP-2 were still significantly lower than those of Ethisorb™ discs with adsorbed BMP-2 as well as Ethisorb™ either untreated or with CaP coatings [30, 44]. The tremendous bone formation induced by coating-incorporated BMP-2 accounted for this phenomenon. All these findings indicated that Ethisorb™ with coating-incorporated BMP-2 were superior in foreign-body reaction to Ethisorb™ with adsorbed BMP-2. Coating incorporated BMP-2 and biomimetic coating cooperatively suppressed the foreign body reaction to implants.

Fibrovascular permeation of the implant by host tissue is beneficial to locally active immune defense and implant fixation [45]. However, most of resorbable biomaterials were isolated by a thick fibrous capsule that creates an avascular interface between host and implant. Furthermore, the encapsulating sheath can thicken and outstrip its blood supply, leading to the degenerated vascularization [46]. Bacterial seeding of the peri-implant space is not accessible by the host immune defenses and can lead to abscess formation [47], which ultimately will lead to failure of implants. Optimal soft-tissue compatibility is characterized by or mesenchymal ingrowth with minimal macrophage activity and thin fibrous encapsulation [47].

On day7, 10% to 30% of spaces within Ethisorb™ with any variants were permeated but host tissue. Rates of tissue ingrowth within Ethisorb™ with CaP coating was lower than Ethisorb™ untreated or with adsorbed BMP-2, which was rescued by the incorporation of BMP-2. On day14, 85-95% of permeation was obtained and no significant difference in rates of tissue ingrowth among
Ethisorb™ with any variants. The highest rate of tissue ingrowth occurred to Ethisorb™ with CaP coating-incorporated BMP-2 in a mechanical unstable microenvironment, which enable it the most rapid integration into host tissues.

With the process of tissue ingrowth, the volume densities of vascular vessels maintained in a stable level within Ethisorb™ with any variant and no difference was found among them at the same time point or the same group at the two time points. The result suggested that the risk of vascular degeneration and abscess formation would not be increased during the monitoring span. Furthermore, the stable development of microvasculature ensured the osteogenic activities within Ethisorb™.

Accordingly, after 7- and 14-day implantation, the space occupying volume also maintained in a level 47-65mm³ that was similar to that after 35-day implantation. This finding that the compacted fibrous Ethisorb™ maintained its space occupying volume irrespective of any modification or mechanical microenvironment suggested a promising application potential for precise restoration of orbital volume during the monitoring period. After the complete degradation of the material, for Ethisorb™ alone, the rigidity of the fibrous tissue that ever encapsulated Ethisorb™ was not high enough to support orbital content and thus resulted in a second sagging [48]. However, when Ethisorb™ was functionalized with CaP coating-incorporated BMP-2, according to our results, it is not unreasonable to predict that the tremendously regenerated bone wall will integrate into the surrounding bone and support the orbital content.

The influence of mechanical microenvironments was not significant for all the monitored biological events including bone formation, osteogenesis mode, tissue ingrowth and foreign body reaction within Ethisorb™ of any variants. This may be due to relatively stable 3-dimensional environment provided by the compacted fibrous structures of Ethisorb™ [49]. In contrast, the osteogenic activities outside Ethisorb™ with adsorbed BMP-2 could be significantly suppressed in a mechanically unstable microenvironment. However, this was not the case for the osteogenic activities outside Ethisorb™ with coating-incorporated BMP-2. This finding suggested that the mechanical forces from muscular movement did influence the osteogenic activities outside the fibrous Ethisorb™ membrane. And the influence of mechanical forces could be resisted by coating-incorporated BMP-2 [32].
Our findings showed that the technique of biomimetic CaP coating incorporated BMP-2 synergistically changed the profibrogenic microenvironment of within and surrounding Ethisorb™ and thus conferred it a very promising treatment option for large-area orbital bone defect.
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Chapter 6

Functionalization of Deproteinized Bovine Bone with Bone Morphogenetic Protein 2- incorporated Calcium-Phosphate Coating Confers Efficient Osteoinductivity and Improves its Biocompatibility

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Submitted to European Cells and Materials

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ABSTRACT:
The repair of critical-sized bone defect remains a challenge in the field of implantology, maxillofacial surgery and orthopedics. The gold-standard treatment — autologous bone grafts — have various disadvantages, which limits their clinical application.
As an alternative, deproteinized bovine bone (DBB) is widely used in clinic for the repair of osseous defects. Albeit so, the material is not intrinsically osteoinductive. In the present study, this property was conferred by coating DBB with a layer of calcium phosphate into which bone morphogenetic protein 2 (BMP-2) was incorporated. Granules of BMP-2-functionalized, coated DBB — together with the appropriate controls (non-functionalized, coated DBB; DBB bearing adsorbed BMP-2; DBB alone) — were implanted subcutaneously in rats. Five weeks later, the implants were withdrawn for a histomorphometric analysis of the volume densities of (i) bone, (ii) bone marrow, (iii) foreign-body giant cells and (iv) fibrous capsular tissue. The volume densities of newly regenerated bone and bone marrow surrounding the BMP-2-functionalized, coated DBB were significantly higher than DBB with any other variants. The volume density of foreign-body giant cells surrounding the BMP-2-functionalized, coated DBB were significantly lower than both the non-functionalized, coated DBB and the DBB bearing adsorbed BMP-2. The volume density of fibrous capsular tissue surrounding the BMP-2-functionalized, coated DBB were significantly lower than the DBB with any other variants. Hence, this process of functionalization not only confers DBB with highly efficient osteoinductivity but also improves its biocompatibility — thus dually enhancing its clinical application potential in the repair of critical-sized bone defect.

Key words: Biomimetic coating, Deproteinized bovine bone, Bone morphogenetic protein-2, Calcium phosphate, Bone defect

INTRODUCTION:
For the repair of bony defects, osseous autografts are the gold standard. However, the supply of this tissue is not unlimited, and its removal is associated with donor-site morbidity and pain [1]. Consequently, a need has arisen for suitable bony substitutes that would be available on a large scale. One such material that
is widely used in clinical dentistry is deproteinized bovine bone (DBB). In terms of its inorganic chemical composition and three-dimensional geometry, DBB is akin to natural bone [2], and is available in unlimited quantities. However, the material intrinsically lacks the property of osteoinductivity, viz., it is not in itself capable of inducing the osteogenic differentiation of pluripotent mesenchymal stem cells and bone formation at an ectopic site. Consequently, it needs to be combined with ground autologous bone [3] — which supplies the necessary osteogenic elements — for the repair particularly of critical-sized defects. Attempts have been made to render DBB osteoinductive by the superficial adsorption of an osteogenic agent [4], such as bone morphogenetic protein 2 (BMP-2), which has been shown to induce bone formation in both animal models and human patients [5-8]. However, this mode of BMP-2 carriage and delivery is far from satisfactory, in so far as an adsorbed depot is released too rapidly (in a single high-dose burst) [9, 10] to induce a sustained osteogenic response at the site of implantation [5]. The difficulty cannot be satisfactorily overcome by increasing the loading dose of BMP-2, owing to the triggering of undesirable side-effects, which include an over-stimulation of local bone resorption and the induction of bone formation at unintended sites [11, 12].

We have refined a technique whereby implant materials can be biomimetically coated with an osteoconductive layer of calcium phosphate, which can be rendered osteoinductive by the concomitant incorporation of BMP-2 into the inorganic crystalline latticework. Physiological doses of incorporated BMP-2 are released slowly and steadily as the coating undergoes degradation at the site of implantation, and these low doses are effective in inducing sustained bone-formation activity at both ectopic [5] and orthotopic sites [13, 14] in animal models.

In the present study, this functionalized-coating technique was applied to DBB with a view to evaluating the osteogenic response at an ectopic (subcutaneous) site in rats.

**MATERIALS AND METHODS**

*Coating of DBB with a layer of calcium phosphate and the incorporation of bovine serum albumin or BMP-2*
Granules of DBB (0.25-1mm in diameter, Bio-Oss®, Geistlich, Switzerland) were biomimetically coated with a layer of calcium phosphate in the absence or presence of a protein according to a well-established protocol [5, 13, 15]. Briefly, 0.15-g samples of DBB granules were immersed in 300ml of five-fold-concentrated simulated body fluid (684mM NaCl; 12.5mM CaCl2·2H2O; 21mM NaHCO3; 5mM Na2HPO4·2H2O) for 24 hours at 37°C under high-nucleation conditions, viz., in the presence of 7.5mM MgCl2·2H2O to inhibit crystal growth. The fine, dense layer of amorphous calcium phosphate thereby formed serves as a seeding substratum for the deposition of a crystalline layer. The crystalline layer was produced by immersing the samples in 45ml of a supersaturated solution of calcium phosphate [40mM HCl; 2mM Na2HPO4·2H2O; 4mM CaCl2·2H2O; 50mM TRIS base (pH 7.4)] for 48 hours at 37°C.

Initially, we wished to confirm that the incorporated protein was homogeneously distributed throughout the crystalline latticework of the coating and to monitor its rate of release in vitro. For these evaluations, the coating was labelled with rhodamine, and the model protein bovine serum albumin (BSA, Sigma) — which was used as an inexpensive alternative to BMP-2 — was tagged with the fluorescent label fluorescein isothiocyanate (FITC) (Sigma). For the in-vivo investigation, tritiated BMP-2 was introduced into the coating medium.

Quantification of the amount of coating-incorporated BMP-2
The amount of coating-incorporated BMP-2 was determined by liquid scintillation counting [16]. 300µg of BMP-2 were labelled with tritium according to the reductive methylation procedure [17]. BMP-2 is thereby exposed to formaldehyde and sodium boro-[3H]-hydride (Perkin Elmer, Switzerland), which specifically labels the ε-amino groups of NH2-terminal residues and the ε-amino groups of lysyl residues. The reaction mixture was loaded onto a Sephadex G-25 PD-10 column (GE Healthcare, Otelfingen Switzerland) to separate the labelled from the unlabelled BMP-2. The concentration of BMP-2 within the purified preparation was determined using the bicinchoninic acid protein assay. BMP-2 containing 2.2% (w/w) of the tritiated species was introduced into the supersaturated solution of calcium phosphate at a final concentration of 10µg/ml, as aforementioned (previous section). The total uptake of tritiated BMP-2 by the coating was quantified by liquid scintillation counting. For this purpose, 60mg of coated DBB granules were dissolved in 1ml of 1M hydrochloric acid. 400-µl aliquots of the suspension were then added to scintillation vials containing 90µl
of 5M NaOH, 100µl of 4.5% saline (buffered with 50mM TRIS) and 3ml of scintillation fluid (Ultima Gold™). Radioactivity was measured in a liquid scintillation counter (TriCarb 2200CA, Packard, Downers Grove, IL, USA).

**Adsorption of BMP-2 onto DBB granules**

In current clinic situations, DBB can be functionalized by directly adsorbing BMP-2 onto its surface. Hence, we adopted the uncoated DBB bearing an adsorbed depot of BMP-2 as a control. According to the radiolabelling technique, 13.5±0.32 µg (mean±SD) of BMP-2 were incorporated into each coating. Hence, 13.5µg of the agent were likewise adsorbed onto 0.15g of DBB granules. The loading process was achieved by introducing a 75-µl aliquot of a stock solution (0.18 mg of BMP-2/ml) into 1-ml Eppendorf tubes containing 0.15g of DBB granules. The tubes were then centrifuged at 4500 rpm for 2 seconds, and finally vortexed to ensure that the DBB granules were homogeneously mixed and wetted. Thereafter, the samples were freeze-dried for 24 days. The entire procedure was conducted under sterile conditions.

**In-vitro characterization**

**Surface characterization of DBB and of the coatings**

The surface characteristics of DBB granules and of their calcium-phosphate coatings were assessed in a scanning electron microscope (XL 30, Philips, the Netherlands). For this purpose, samples of the material were mounted on aluminium stubs and sputtered with gold particles to a thickness of 10-15nm.

**Confirmation of the homogeneous distribution of a coating-incorporated depot of FITC-BSA by confocal laser-scanning dual-channel-fluorescence microscopy**

To confirm that the incorporated protein was homogeneously distributed throughout the crystalline latticework of the coating, BMP-2 was substituted with the less pricy alternative BSA, which was tagged with fluorescein isothiocyanate (FITC). FITC-BSA was introduced into the supersaturated solution of calcium phosphate at a final concentration of 10µg/ml. Rhodamine B was introduced into the same medium (at a final concentration of 0.1mg/ml) to label the crystalline layer of the coating. After thorough rinsing and freeze-drying, the coated samples were embedded in methylmethacrylate. 600-µm-thick sections were prepared and affixed to Plexiglas holders. These sections were then ground down to a thickness of 80 µm for inspection in a confocal laser-scanning microscope, which was equipped for fluorescence imaging (Zeiss LSM 510 META with LSM 510...
Acquisitions software and Images 3D software). Since the two fluorescent markers emit signals at different wavelengths, we were able to track separately the distribution of the crystalline coating (red signal) and that of the coating-incorporated depot of FITC-BSA (green signal).

**In-Vitro monitoring of the release kinetics of a coating-incorporated depot of FITC-BSA**

To monitor the release kinetics of a coating-incorporated depot of protein, FITC-BSA (10µg/ml) was introduced into the supersaturated solution of calcium phosphate. Six samples were used to determine the total amount of incorporated FITC-BSA. These samples were immersed in 1ml of 0.5% EDTA (pH 8.0) and vortexed twice for 5 minutes. The supernatants were withdrawn for analysis. Six samples of DBB bearing a coating-incorporated depot of FITC-BSA and six samples of DBB bearing an equivalent amount of adsorbed FITC-BSA (included for the purpose of comparison) were incubated in sealed 10-ml glass tubes containing 2ml of phosphate-buffered 0.9% saline (pH 7.4). The tubes were incubated for up to 35 days in a shaking waterbath (60 agitations/min), which was maintained at 37°C. Triplicate 200-µl aliquots of the medium (containing released FITC-BSA) were withdrawn for analysis after 3 hours, 6 hours, 9 hours, 1 day, 2 days, 3 days, 5 days, 7 days, 10 days, 14 days, 18 days, 23 days, 28 days and 35 days. Fluorescence density was measured in a spectrophotometer (excitation wavelength: 485nm; emission wavelength: 519nm). Fluorescence readings were converted to amounts of protein using a standard curve, which was generated by preparing a dilution series of FITC-BSA in 5ml of phosphate-buffered 0.9% saline. The temporal release of FITC-BSA was expressed as a percentage of the total amount that had been incorporated into the crystalline layer of the calcium-phosphate coating or that had been adsorbed directly onto the DBB granules.

**Assessment of the bioaffinity of coated and uncoated DBB granules by monitoring the attachment thereto of murine primary osteoblasts in-vitro**

Primary osteoblasts were isolated from the calvariae of 1- to 2-day-old mice by digestion with collagenase [18]. The cells were grown to 90% confluence on a plastic substratum, which was bathed with α-minimal essential medium (α-MEM) containing 10% fetal bovine serum (FBS), 1% pencillin and 1% streptomycin. The medium was exchanged every 3 days. 30mm³ of coated or uncoated DBB granules were then introduced into the chambers of 96-well plates and incubated
overnight with 50µl of α-MEM containing 20% FBS. After washing three times with phosphate-buffered 0.9% saline, triplicate 87.5-µl aliquots of the suspended osteoblasts (2×10⁴/ml) were introduced into the wells. After an incubation period of 24 hours at 37°C, the cells were rinsed three times, and their attachment to the DBB granules was then monitored using the proliferation agent WST-1 (Roche, Switzerland). The WST-1 reagent was mixed in a volume:volume ratio of 1:10 with Phenol-Red-free α-MEM containing 10% FBS. Tetrazolium salts in the reagent are converted by active cellular enzymes into intracellular deposits of formazan; the coloured signal thereby produced is directly proportional to the number of viable cells. The absorbance was measured at a wavelength of 490 nm (against a reference wavelength of 620 nm) using a microplate reader (Tex, Hercules, CA, USA).

**In-vivo investigation**

As an experimental animal model, we used adult male Sprague-Dawley rats (200-220g). One experimental and three control groups were established (n=6 samples per group): (1) BMP-2-functionalized coated DBB (experimental group); (2) non-functionalized, coated DBB; (3) uncoated DBB bearing an adsorbed depot of BMP-2; and (4) DBB alone (non-functionalized, uncoated). Each sample consisted of 0.15g of DBB granules. Two samples per rat were surgically implanted within dorsal subcutaneous pockets (one on the left side and one on the right), and were trapped therein by suturing the incision site. Five weeks after surgery, the samples were retrieved, chemically fixed and embedded as previously reported [5, 13]. Applying a systematic random-sampling strategy [19], 10-12 slices, each 600 µm in thickness and 1mm apart, were prepared from each sample. Odd- or even-numbered slices of each sample were separately mounted on Plexiglas holders and polished. The odd-numbered slices were surface-stained with McNeal’s Tetrachrome, basic Fuchsin and Toluidine Blue O [20] for the histomorphometric analysis of various parameters (see below), including the volume density of the entire population of multinucleated cells. The even-numbered slices were subjected to the tartrate-resistant acid phosphatase (TRAP) reaction [5, 21] for the histomorphometric estimation of the volume density of multinucleated osteoclasts. Applying a two-step systematic random-sampling strategy, 25-30 images at a final magnification of ×320 were recorded.
in a Nikon-Eclipse light microscope and printed in colour for the histomorphometric analysis. The spatial distribution of DBB at the implantation site was estimated by measuring the surface-area density of the granules with the aid of a cycloid test system [22] and by measuring their volume density using the point-counting technique [23]. The volume densities of bone, bone marrow, multinucleated cells and fibrous capsular tissue were likewise estimated using the point-counting technique [23]. The volume density of foreign-body giant cells was estimated by subtracting the volume density of TRAP-positive osteoclasts from the volume density of the entire population of multinucleated cells [5].

Statistical analysis

All data are presented as mean values together with the standard deviation (SD). Data were compared using a one-way analysis of variance (ANOVA), the significance level being set at p<0.05. Post-hoc comparisons were made using Bonferroni’s corrections.

RESULTS

Characterization of DBB and of the Calcium-phosphate Coatings

In the scanning electron microscope, the surface relief of DBB granules was characterized by parallel arrays of ridges and troughs (Fig.1A: A1); the calcium-phosphate coatings were characterized by an interlacing network of needle-like crystals (Fig.1A: A2). Confocal laser-scanning dual-channel-fluorescence microscopy revealed the crystalline layer of the coatings (red signal) to be homogeneously deposited along the surfaces of the DBB granules (Fig.1B: B1/B1a), and the coating-incorporated depot of FITC-BSA (green signal) to be evenly distributed throughout the crystalline layer (Fig.1B: B2/B2a & B3/B3a). As anticipated, the DBB-adsorbed depot of FITC-BSA was released rapidly, being completely exhausted after 13 days (Fig.1C). On the other hand, the coating-incorporated depot of FITC-BSA was released gradually and at a steady rate after the 3rd day until the 35th day, at which juncture the initial depot had been depleted by no more than 52% (Fig.1C). The attachment of murine primary osteoblasts to coated DBB granules was 1.34±0.02 (mean±SD)-fold higher than
to uncoated ones ($p<0.05$). The total amount of BMP-2 that had been incorporated into the coatings was $13.5\pm0.5\mu g$ (mean$\pm$SD) per 0.15g of DBB.

**Fig. 1** Characterization of uncoated, coated and BMP-2-functionalized DBB granules. **A:** Scanning electron micrographs of native DBB granules (A1) and of DBB granules bearing a calcium-phosphate coating (A2). Bars=5$\mu$m.
B: Confocal laser-scanning fluorescence micrographs illustrating the homogeneous deposition of the rhodamine-labelled crystalline layer of a calcium-phosphate coating (red signal) along the surfaces of DBB granules (B1/B1a), and the even distribution of a depot of FITC-BSA (green signal) that was co-precipitated with it (B2/B2a). The images in B1/B1a and B2/B2a are merged in B3/B3a, which reveal the crystalline layer of the coating and the BSA to be co-localized (orange signal). Bars=100µm (B1, B2, B3) and 10µm (B1a, B2a, B3a).

C: Graph depicting the release kinetics of a directly-adsorbed and a coating-incorporated depot of FITC-BSA from DBB granules in vitro. The directly-adsorbed protein depot was released rapidly, in a single high-dose burst. More than 80% of the initial loading dose had been liberated by the 1st day, and the depot had been completely exhausted by the 13th day. The temporal release profile of the coating-incorporated protein depot was characterized by an initial rapid phase, spanning 3 days, and a subsequent slower one, which was protracted until the end of the monitoring period (35 days). During the initial 3 days, 34.6% of the initial protein load was released at a rate of 11.5% per day. During the subsequent phase, the protein was released at a steady rate of 1.5% per day; by the 35th day, the initial depot had been depleted by no more than 52%.

In-vivo Investigation

Five weeks after their subcutaneous implantation in rats, the surface-area densities [9-10 mm²/mm³ (Fig. 2A)] and the volume densities [0.46-0.48 mm³/mm³ (Fig. 2B)] of DBB granules were similar in each of the four groups, there being no significant differences (p>0.05) between the mean values. Bone and bone marrow were associated only with BMP-2-functionalized DBB, viz., with granules that bore either a coating-incorporated (Fig.3A) or a directly-adsorbed (Fig. 3B) depot of the agent. In the two unfuctionalized groups [non-functionalized coated DBB (Fig.3C) and nonfunctionalized uncoated DBB (Fig.3D)], neither bone nor bone marrow was deposited. Osseous tissue was deposited directly upon the surfaces of coated or uncoated BMP-2 functionalized DBB granules, without intervening connective tissue. Bone marrow was deposited abundantly within the spaces between the DBB granules that bore a coating-incorporated depot of BMP-2 (Fig.3A) but only sporadically within those between DBB granules that bore a directly-adsorbed depot of the agent (Fig.3B).

The volume densities of bone (Fig.4A) and bone marrow (Fig.4B) were substantially higher in association within DBB granules that bore a coating-incorporated depot of BMP-2 than in association within those that bore a directly-adsorbed depot of the agent (p<0.05 for bone and p<0.01 for bone marrow).
Fig. 2 Graph depicting the surface-area densities (A) of and volume densities (B) of DBB within the subcapsular space (reference volume) for the four groups, 5 weeks after subcutaneous implantation in rats. Mean values (n= 6 animals per group) are represented together with the standard deviation.
Fig. 3 Light micrographs of cross-sections through DBB granules in the four groups, 5 weeks after subcutaneous implantation in rats. 
A: DBB granules bearing a coating-incorporated depot of BMP-2. Bone (black arrows) has been deposited directly upon the surfaces of the DBB granules (white arrowheads). The spaces between the granules are abundantly filled with bone marrow (asterisks).
B: DBB granules bearing a directly-adsorbed depot of BMP-2. Also in this group, bone (black arrow) was deposited directly upon the surfaces of DBB granules (white arrowheads), albeit in smaller quantities than in (A). Bone marrow was observed only sporadically within the spaces between the granules.
C: Non-functionalized, coated DBB. Neither bone nor bone marrow has been deposited on or around the DBB granules (white arrowheads).

D: Non-functionalized, uncoated DBB. Likewise in this group, neither bone nor bone marrow was deposited on or around the DBB granules (white arrowheads).

E: Higher-magnification view of a region represented in (A), illustrating osseous tissue in direct contact with the DBB granules (white arrowheads) and osteoid (black arrows) aligning the bone-marrow spaces (asterisks).

F: Higher-magnification view of a region in (D), illustrating the profiles of two foreign-body giant cells (white arrows) along the surface of a DBB granule (white arrowheads).

The presence of foreign-body giant cells (Fig.3F) is a sign of acute inflammatory reactivity, whereas the degree to which an implanted material is encapsulated with dense fibrous connective tissue (Fig.3G) is a gauge of chronic inflammatory reactivity. The volume density of foreign-body giant cells (Fig.4C) and fibrous capsular tissue (Fig.4D) were lower in an association with DBB granules that bore a coating-incorporated depot of BMP-2 than with those in any of the other three groups.
Fig. 4 Graphs depicting the volume densities of bone (A), bone marrow (B), foreign-body giant cells (C) and dense fibrous capsular tissue (D) that were associated with DBB granules within the subcapsular space (reference volume) for the four groups, 5 weeks after subcutaneous implantation in rats. Mean values (n=6 animals per group) are represented together with the standard deviation. a: \( p < 0.05 \); b: \( p < 0.01 \); c: \( p < 0.001 \).

DISCUSSION

To be optimally efficacious as osteogenic agents, BMPs need to be delivered to the target site gradually, at a low (physiological) level and a steady rate, not in a single high-dose burst [1, 24]. For such a mode of BMP-delivery, our
biomimetically-prepared calcium-phosphate coatings are an ideal vehicle [5, 13, 14]. In the present study, we have shown that DBB can be unproblematically coated with a co-precipitated layer of calcium phosphate and BMP-2. Substituting BMP-2 with FITC-BSA, the release kinetics of a coating-incorporated protein depot were revealed to follow the desired course (slow, sustained rate of delivery), whereas those of a protein depot that was adsorbed directly onto DBB were characterized by a single, rapid, high-dose burst. A protein depot that is adsorbed upon a coated carrier has been likewise shown to be characterized by burst-release kinetics [25]. The in-vivo investigation revealed a coating-incorporated depot of BMP-2 to be more efficacious than a directly-adsorbed one in inducing sustained osteogenic activity. This finding is consistent with existing data relating to diverse polymeric carriers [26], and affords further evidence that the functionalized-coating technique can be applied to a range of scaffolds with a view to augmenting bone formation at the site of implantation. Interestingly, larger quantities of bone marrow were associated with a coating-incorporated than with a directly-adsorbed depot of BMP-2. Given the high immunological competence of this tissue, its direct contact with BMP-2-functionalized coatings bears witness to their being well tolerated by the host tissues. Moreover, since bone marrow is an important source of nutrients for osseous tissue and of pluripotent progenitor cells to sustain its turnover, its abundance bodes well for the health and the endurance of the bone.

The three-dimensional geometric configuration of a bone-defect-filling material is known to influence its capacity to support bony ingrowth in vivo, and we have ourselves shown that the surface-area density of a polymeric scaffold can impact osteogenesis [26]. However, in the present study, the surface-area and volume densities of DBB in the four different groups were similar. Hence, a possible influence of differences in these parameters can be excluded.

Although a bone-defect-filling material should ideally provoke no inflammatory reactivity at the host site, this is seldom the case. An inflammatory response can be manifested both acutely and chronically. Acute inflammatory reactivity is epitomized by the accumulation of foreign-body giant cells, which are formed by the fusion of macrophages whose capacity for the phagocytosis (“isolation”) of the implanted material undergoing degradation has been overburdened [27]. Chronic inflammatory reactivity is indicated by an encapsulation of the implanted material with dense fibrous connective tissue [28]. The
biocompatibility of DBB is a controversial issue, albeit that its tolerance by host tissues appears to be related to the absence [29] or presence [30] of residual proteins. In the present study, substantial numbers of foreign-body giant cells were associated with native DBB granules, which were also surrounded by a robust fibrous capsule. These findings indicate that the material is not well tolerated within the subcutaneous space of rats. However, the volume densities of foreign-body giant cells and fibrous capsular tissue were significantly lower in association with DBB granules that bore a coating-incorporated depot of BMP-2 (Fig. 4, C&D). Non-functionalized coatings likewise suppressed chronic inflammatory reactivity – as demonstrated also by Anderson et al. [27] – but to a lesser degree.

On the other hand, the acute inflammatory response was not significantly influenced by the absence or presence of a coating-incorporated depot of BMP-2. Taken together, these findings indicate that at the early post-implantation stage, before BMP-2-stimulated bone-formation activity has gained a firm foothold, a slow, steady release of the agent from the coating as it undergoes degradation neither hinders nor potentiates the formation and accumulation of foreign-body giant cells. But once bone-formation activity is well underway, the presence of the osseous tissue suppresses the fibrous encapsulation of the DBB granules. The volume density of foreign-body giant cells was significantly higher in association with DBB granules that bore a directly-absorbed depot of BMP-2 than with those that bore a coating-incorporated depot of the agent. This finding indicates that the transiently high local concentration of BMP-2 that is generated by its burst release from an absorbed depot enhances the formation and accumulation of foreign-body giant cells. Since bone-formation activity cannot be sustained when BMP-2 is liberated in a single high-dose burst, the volume density of osseous tissue that is laid down is low (Fig. 4A) and insufficient to hinder the process of fibrous encapsulation (Fig. 4D).

The impact of a coating-incorporated depot of BMP-2 on the volume density of foreign-body giant cells appears to be influenced by the nature of the underlying carrier, which will become gradually exposed during the course of coating degradation. Using diverse organic polymers instead of DBB as carrier materials, we have previously shown the volume density of foreign-body giant cells to be significantly lower in the presence than in the absence of a coating-incorporated depot of BMP-2 [26]. However, since in this previous as well as in the present
study the volume density of foreign-body giant cells that was associated with non-functionalized coatings and with the native carrier did not differ significantly from each other, the discrepancy cannot be accounted for by differences in the pro-inflammatory effects of the carrier or its breakdown products per se. It is probably attributable to differences in the impact of BMP-2-stimulated local activities on the strength of the pro-inflammatory response that is provoked by the different carriers and their breakdown products.

The effectiveness of the BMP-2-functionalized coating in lowering the level of chronic inflammation could be accounted for in several ways. In the first instance, the coating itself could enhance the adhesion of osteoprogenitor cells – as indeed was found to be the case in the in-vitro culturing experiments – thus promoting the BMP-2-stimulated formation of bone, which would shield the host tissues from residual surface antigens on the DBB granules. Moreover, BMP-2-induced osteoinductive activity is known to enhance vascularization [31], which could suppress fibrotic activity [32] and the formation of fibrous capsular tissue. This effect might be partially mediated by a local increase in the level of fibronectin [33]. Furthermore, mesenchymal stem cells within the bone marrow — which was abundant between the granules of DBB that bore a coating-incorporated depot of BMP-2 — are also capable of exerting an immunosuppressive effect [34].

In summary, our findings demonstrate that the functionalization of DBB with a co-precipitated layer of calcium phosphate and BMP-2 not only promotes bone-formation activity but also dampens the inflammatory response that is provoked by the native material. The acquirement of these attributes would greatly enhance the clinical potential of DBB as a bone-defect-filling material.

ACKNOWLEDGEMENTS:

The authors would like to thank Geistlich AG (Switzerland) for their generous gift of DBB (Bio-Oss®).
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Chapter 7

RhBMP2/7 Heterodimer: an Osteoblastogenesis-inducer of Not Higher-potency But Lower-effective-concentration Compared to RhBMP2 and RhBMP7 Homodimers

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Published Online 30 January 2010
ABSTRACT
Heterodimeric bone morphogenetic proteins (BMPs) were exhibited to be more potent than and thus potential substitutes for homodimeric BMPs whose clinical application is limited for the drawbacks resulted from their higher effective doses. This study aims to delineate the bio-functional characteristics of rhBMP2/7 heterodimer in inducing osteoblastogenesis of MC3T3-E1 through in vitro time-course and dose-response studies. RhBMP2/7 heterodimer induced cell migration with a significantly lower optimal concentration and higher peak effect than the respective homodimers. RhBMP2/7 heterodimer induced cell differentiation with significantly lower threshold concentrations but similar maximum effects. On day28, area of calcium depositions induced by 50ng/ml rhBMP2/7 was 12- or 38-fold more than that of 50ng/ml rhBMP2 or 50ng/ml rhBMP7 respectively. The results indicated that rhBMP2/7 heterodimer was an osteoblastogenesis-inducer of not higher-potency but lower-effective-concentration compared to rhBMP2 and rhBMP7 homodimers.

Keywords: Bone morphogenetic protein; Heterodimer; Homodimer; Osteoblastogenesis

INTRODUCTION
The osseous restoration of voluminous bone defect remains a challenge in orthopedics, maxillofacial surgery and implantology.1,2 Bone tissue engineering including gene-, cell- and cytokine-therapies has been reported to substitute autografting and allografting whose clinical applications are hampered for their varieties of limitations.3,4 Among the three therapies, cytokine therapy is advantageous in safety, feasibility and potential for nearest clinical application over the other two.1,5
Bone morphogenetic proteins (BMPs), a group of dimeric disulfide-linked polypeptide growth factors under transforming growth factors-β superfamily, are one of the paramount cytokines in promoting bone regeneration.6 However, the clinical effective doses of the homodimeric BMPs to induce bone formation are extremely high (e.g. up to milligrams),7,8 which results in not only a substantial economic burden to patients but also a series of potential side effects, such as overstimulation of osteoclastic activity and ectopic bone formation in unintended area.9,10
One alternative approach to solve the problem is to adopt more potent forms of BMPs. Previous studies showed that BMP heterodimers achieved several- or dozens-fold more effects than the respective homodimers in inducing alkaline phosphatase (ALP) activity and bone formation.\textsuperscript{11-14} These findings suggested that BMP heterodimers were feasible and promising substitutes for homodimers. However, the bio-functional characteristics and mechanisms of BMP heterodimers on osteogenesis remain ambiguous. How BMP heterodimers effect on the sequential osteoblastogenetic events such as chemotactic migration, proliferation, differentiation and mineralization, hereto, remains uncovered. Earlier studies confined the parameters to ALP activity, which is inadequate to reveal the characteristics of their bio-functional activities.\textsuperscript{11,12} Recently, some conclusions were based on the conditioned medium of gene-transfected BMP producers instead of purified BMP heterodimers, which could not exclude the influence of other factors in the medium on cellular activities.\textsuperscript{13,14} These studies mostly focused on the \textit{in vivo} gene therapy which could only suggest their clinical efficacy instead of the functional characteristics of BMP heterodimers. The parameters such as cell migration index, DNA content, ALP activity as early marker of differentiation, osteocalcin (OCN) as terminal marker of differentiation, and calcium deposition of cell culture were adopted to evaluate the biological activities of BMPs in previous studies.\textsuperscript{13,15-17} Murine calvaria-derived MC3T3-E1 Cell, a precursor of functionalized osteoblast, was also shown to be able to examine the potency of osteoinductive agents.\textsuperscript{13,17} In this paper, we wished to unveil functional characteristics of BMP heterodimers by comparing osteoblastogenesis-inducing effects of rhBMP2/7 heterodimer with rhBMP2 and rhBMP7 homodimers.

**MATERIALS AND METHODS**

**Cell culture**

Preosteoblasts, MC3T3-E1 cell line (ATCC, Chinese Academy of Sciences), were cultured in α-MEM containing 10%FBS (Gibco\textsuperscript{TM} Invitrogen Corp. USA). The medium was changed every three days. Exponentially growing cells were plated at 1.5×10\textsuperscript{4} cells/well in 48-well plates for cell proliferation, at 4×10\textsuperscript{4} cells/cm\textsuperscript{2} in 6-well plates for ALP activity assay, in 24-well plates for OCN detection or in 48-well plates for alizarin red staining. After 24h incubation, cells
were subjected to a low serum medium (2%FBS) for another 24h and then followed by rhBMPs (R&D Systems, USA) treatment.

*RhBMPs concentration selection test*

To identify the concentration range for examining the biological activities of rhBMP2/7 heterodimer, a dose-response test of ALP assay was performed the same way as described in the section of "ALP activity assay". The results showed that the ALP activity induced by rhBMP2/7 reached the maximum at 150-200ng/ml and significantly decreased at 250ng/ml. Besides, ALP activity induced by rhBMP2 or rhBMP7 reached a plateau stage at 200-400ng/ml respectively with similar highest level. Consequently, the maximum concentration in this study was determined at 200ng/ml.

*Real-time cell migration monitoring*

Cell migration of MC3T3-E1 induced by rhBMPs was continuously monitored using Real Time Cell Electronic Sensing/Cell Invasion/Migration System (RT-CES/CIM™ System, ACEA Biosciences, Inc. USA)\(^{18,19}\) according to the manufacturer’s instructions. Briefly, the bottoms of the upper chambers, the both sides, were coated with 0.01% bovine plasma fibronectin (Invitrogen Corp. USA), and 20µl of 0.1%BSA-α-MEM and 150µl of 0.1%BSA-serum free medium containing various amounts of rhBMP2/7, rhBMP2, rhBMP7 or their 1:1 mixture (0 as negative control, 0.01, 0.1, 1, 10ng/ml)\(^{15,16}\) were added into the upper and lower chambers respectively. Confluent MC3T3-E1 cells were subjected to serum-free starvation for 4h and then suspended in 0.1%BSA-α-MEM at a density of 12.5×10^4 cells/ml followed by adding 80µl of cells suspension into the RT-CIM upper chambers. Cell migration was continuously monitored for a period of 0–8h, and data presented as cell index (CI) were collected every 15min, chiefly indicating the numbers of cells that migrated to the lower side of upper chambers.

*Cell proliferation assay*

To investigate cell proliferation of MC3T3-E1 cells in response to various concentrations (0 as control, 5, 10, 50, 100, 150, and 200ng/ml) of different rhBMPs, the number of cells was determined by the fluorometric quantification of amount of cellular DNA after the stimulation for 1 day, 4 days and 7 days using Quant-iT™ PicoGreen dsDNA Reagent and kits (Invitrogen Corp. USA) as previously described.\(^{20}\) The fluorescent intensity of the cell lysate and dye mixed solution was measured using a fluorescence spectrometer (SPECTRAmax M2, Ex 480nm/Em 520nm).
ALP activity assay
To determine the early differentiation of preosteoblasts stimulated by rhBMPs at different concentrations (0 as control, 5, 10, 50, 100, 150, and 200ng/ml), ALP activity and protein content were measured after rhBMPs treatment on day2, day4 and day7. ALP activity in the cell lysates (Sigma-Aldrich, USA), was determined using LabAssay™ ALP colorimetric assay kit (Wako Pure Chemicals, Ind., Ltd., Osaka, Japan). The cell number was estimated by determining total protein content which was measured at 570nm using a commercial BCA Protein Assay kit (Beyotime, Inst., China). The values that presented ALP activity were expressed as μmol p-NP/mg total protein.

OCN expression assay
To assess the terminal differentiation of preosteoblasts stimulated by rhBMPs (concentrations set at 0 as control, 5, 10, 50, 100, 150, and 200ng/ml), OCN secreted into the cell culture medium was determined. The cell supernatants were collected on day4 and day7 and were centrifuged (10000 rpm, 4°C, 5 minutes) before detection. The OCN concentrations of the supernatants were determined by ELISA using a mouse osteocalcin elisa kit (Biomedical Technologies Inc., USA).

Alizarin red staining
According to the results of above cellular events, we compared the mineralization possibility of MC3T3-E1 cells stimulated by 50ng/ml rhBMPs. Quadruplicate cell cultures were prepared in the same way as described previously and then treated with mineralizing medium (10%FBS, 50µg/ml L-Ascorbic acid and 5mM β-glycerophosphate, Sigma-Aldrich, USA) containing 50ng/ml rhBMPs. The medium was replaced every three days. After 3 weeks and 4 weeks, mineralized nodules were determined by alizarin red staining (Sigma-Aldrich, USA). Culture plates were photographed by NIS-Elements F2.20 (Nikon Eclipse 80i) and the calcified area was quantified using Image-Pro Plus 6.0 analysis.

Statistical analysis
Statistical comparisons between results obtained with the various concentrations of rhBMPs and different kinds of rhBMPs were made by one-way analysis of variance (ANOVA). Post Hoc comparisons were made using Bonferroni corrections. The level of significance was set at p<0.05. SPSS software (version 15) for a Windows computer system was employed for the statistical analysis.
RESULTS

Chemotactic migration
CI of cell chemotactic migration for each rhBMP was in the same pattern which contained two stages: the initial steady-increase stage in the first 4-5 hours and the following plateau stage (Fig. 1A, 1B, 1C, 1D). Cell started to migrate immediately after being installed in the system regardless of the presence of rhBMPs. The speeds of cell migration were respectively enhanced to higher levels by different rhBMPs of certain concentrations than negative controls especially after 1-2 hours. The chemotactic effects of rhBMPs lasted for 5 hours after which relative cell migrations tapered. Despite the same pattern of CI, the optimal concentration (the lowest concentration to get the maximum effect) and the maximum value of chemotactic effects differed according to rhBMP types. The chemotactic effect of rhBMP2/7 showed a bell-shape dose-dependent curve. The optimal concentration was 0.1ng/ml, followed by 1ng/ml (Fig. 1A). The effect dramatically decreased when the concentration decreased to 0.01ng/ml or increased to 10ng/ml. The maximum values of rhBMP2 and rhBMP7 were obtained around 1ng/ml (Fig. 1B, C). Their decreasing trends were demonstrated when the concentration went either higher or lower. The chemotactic effect of 1:1 mixture of rhBMP2 and rhBMP7 increased slowly in the selected concentration range (Fig. 1D). The maximum fold of CI (CI normalized by CI of negative control group) of rhBMP2/7 (1.93, at 0.1ng/ml) was significantly higher than that of rhBMP2 (1.48, at 1ng/ml), rhBMP7 (1.31 at 1ng/ml) or their 1:1 mixture (1.43, at 10ng/ml) (Fig. 1E).
Fig. 1 (A-D) Comparison of cell migration under different rhBMPs treatment. Real-time cell migration was continuously monitored using RT-CES/CIM™ System in a period of 8h. The data presented as cell index (CI) were collected every 15min, chiefly indicating the numbers of cells that migrated. (E) Fold of cell index at 5h normalized by CI of control group (**p<0.01, *p<0.05, indicated significant increase vs. Control group,
Cell proliferation

After stimulation for 1 day, there is no significant difference in cell proliferation among different BMPs of the selected concentrations. On day 4 (Fig. 2), compared with the control group, the DNA contents increased significantly under the stimulation of rhBMP2/7 and rhBMP2 at the concentrations of 5-50ng/ml or 200ng/ml. In contrast, rhBMP7 showed a stimulative effect on the proliferation of preosteoblasts in a broader range of concentration, from 5 to 200ng/ml. The highest values of DNA content were obtained respectively at 5ng/ml for rhBMP2 or rhBMP2/7, at 200ng/ml for rhBMP7 and at 50ng/ml for the mixture of rhBMP2 and rhBMP7.

The DNA contents in all rhBMPs groups further increased on day 7. Compared to the respective control groups, DNA contents were significantly increased by rhBMP2/7 at the concentration of 5-10ng/ml. For rhBMP2, the effective concentration range was 5-50ng/ml. In accordance with the result on day 4, the DNA contents were increased by rhBMP7 in the selected concentration range from 5 to 200ng/ml with the peak value occurring at 100ng/ml. The highest values of stimulative effects on cell proliferation were obtained respectively at 5ng/ml for rhBMP2 or rhBMP2/7, at 100ng/ml for rhBMP7 and at 50ng/ml for the mixture of rhBMP2 and rhBMP7. The mixture of rhBMP2 and rhBMP7 showed no synergistic effect. Besides, no significance was found among the highest values of DNA contents in different rhBMP groups on day 4 or on day 7 ($p>0.05$).
Fig. 2 RhBMPs showed different optimal concentration with similar peak values on stimulating MC3T3-E1 proliferation. The cellular DNA contents were measured on day1, day4 and day7. Values shown are means±SD for quadruplicate cultures. (*p<0.05 indicated significant increase vs. control group).

**ALP activity**

The minimum concentration for rhBMP2/7 heterodimer to exhibit a significant promoting effect on ALP was as low as 5ng/ml, at which either rhBMP2, rhBMP7 or their mixture remained ineffective (Fig. 3). The rhBMP homodimers started to exert their promotive effect on ALP activities at the concentration of 50ng/ml, at which the effect of rhBMP2/7 on stimulating the ALP activity has already reached a plateau (Fig. 3A-C). Therefore the threshold concentration of rhBMP2/7 heterodimer was almost 1/10 of that of homodimers. In addition, the levels of ALP activity induced by rhBMP2/7 on day2 were about 1.4-2.4 fold of that of rhBMP2 or/and rhBMP7 at all the selected concentration (Fig. 3A). After 4-day stimulation, the ALP activities further increased under the stimulation of rhBMP2/7 heterodimer at the concentrations of 5 and 10ng/ml at which rhBMP2 and rhBMP7, whereas, remained ineffective (Fig. 3B). At the concentration of 50ng/ml, the ALP activity stimulated by rhBMP2/7 was still significantly higher than rhBMP2, rhBMP7 and their 1:1 mixture. The ALP activities induced by rhBMP2/7 maintained stable from the concentrations of 50-200ng/ml at which the effects of rhBMP2, rhBMP7 and their 1:1 mixture showed a dose-dependent increasing curves. The advantages of rhBMP2/7 over the homodimers, thus, tapered with the concentration increased. At the concentration of 100-200ng/ml, rhBMP2/7 was only advantageous over rhBMP7. On day7, the ALP levels induced by all rhBMPs were about 1.5-fold more than those of day4 (Fig. 3C).
The effect pattern of rhBMPs at the concentrations of 5 and 10ng/ml was similar to that of day4. The effect of rhBMP2 reached the same level of rhBMP2/7 at the concentrations of 50-200ng/ml. In contrast, rhBMP7 showed a weaker effect on inducing ALP activity of preosteoblast than rhBMP2/7 or rhBMP2 in the span of the given concentrations. The effect of the mixtures of rhBMP2 and rhBMP7 lied between the two homodimers.

Fig. 3

![Graph showing ALP activities of MC3T3-E1 under different rhBMPs treatment](image)

Fig. 3 ALP activities of MC3T3-E1 under different rhBMPs treatment were determined by colorimetric assay on day2 (A), day4 (B) and day7 (C). Values shown are means±SD for triplicate wells in duplicate experiments normalized by total cellular protein (*p<0.05 indicated significant increase vs. control group; **,***p<0.05 indicated significant increase vs. rhBMP2, rhBMP7, or their 1:1 mixture group respectively).

**OCN expression**

In accordance with its effect on ALP activity, the threshold dose of rhBMP2/7 heterodimer to promote OCN expression (5ng/ml) was also significantly lower than those of rhBMP homodimers (50ng/ml) (Fig. 4).

After 4-day culture, the OCN expression level induced by rhBMP2/7 (5ng/ml) increased with concentration until 50ng/ml after which its effect maintained stable (Fig. 4A). OCN expression showed a dose-dependent increasing curve for rhBMP2 and rhBMP7 from 50ng/ml at which the effect of rhBMP2/7 was 6-fold higher than the respective homodimers. The advantage of rhBMP2/7 decreased with concentration increase but maintained significant over the homodimers until at the concentration of 200ng/ml at which there was no significant difference between rhBMP2/7 heterodimer and the respective homodimers (p>0.05).
After culturing with rhBMPs for 7 days, the OCN expression increased to much higher levels, about 2-fold higher than those measured on day 4 (Fig. 4B). The effect of rhBMP2/7 on stimulating OCN was still significantly advantageous over rhBMP2 or rhBMP7 at the concentration of 5-150 ng/ml. rhBMP2 increased the OCN expression significantly from 50 ng/ml and reached a plateau at the concentrations of 100-200 ng/ml. At 200 ng/ml, no significant difference could be found among the OCN levels induced by different rhBMPs.

**Cell matrix mineralization**

After 21-day culture, matrix mineralization of MC3T3-E1 was only found under the stimulation of 50 ng/ml rhBMP2/7 (Fig. 5). On day 28, calcium depositions were detected in all groups, with the significantly maximal level occurring in rhBMP2/7 group, which was 12-fold higher than that of rhBMP2, 38-fold higher than that of rhBMP7, or 26-fold higher than that of the mixture group. Besides, comparing the two time points, the area of calcified nodules was over 3 times at day 28 than that at day 21 for rhBMP2/7 group (Fig. 5B).
Fig. 5 (A) The calcium depositions of cell culture were stained using alizarin red staining. (B) Quantitative calculation of calcified area in each rhBMP group. Values shown are means±SD for quadruplicate wells (**p<0.01, *p<0.05 indicated significant increase vs. control group; a,b,c p<0.05 indicated significant increase vs. rhBMP2, rhBMP7, or their 1:1 mixture group respectively).

DISCUSSION

BMPs signals play pivotal roles, from the earliest embryonic patterning events to organ development. The actions and activities of BMPs are targeting cell-specific and BMP producer-dependent. In this study, we compared the actions of the recombinant human heterodimer and homodimer of homologous producer (E. coli) on a series of cellular events of committed osteogenic cell type-MC3T3-E1 during osteoblastogenesis. Infiltration of bone forming cells is an important event during the restoration of bone defect. Exogenous BMPs may be one of the dominant modulators on cell migration when BMP are applied in an unphysiologically high amount for bone regeneration. Different BMP homodimers differed in their chemotactic effect to different cell types. BMP heterodimers may be even distinct from homodimers since they could exclusively assemble heteromeric type I receptors and thus pattern the dorsoventral axis of drosophila embryo while the homodimers could not. It is, therefore, worthwhile to monitor the chemotactic effect of BMP heterodimers. Our results showed that the optimal concentration of rhBMP2/7 for chemotactic effect was significantly lower (1/10) and the
maximum effect of the heterodimer was significantly higher than those of the respective homodimers. This result suggested that rhBMP2/7 heterodimer might play a more important role than homodimers in enhancing chemotactic migration and patterning the spatial interrelationship of osteogenic cells.

In consistent with previous findings, we found that the threshold and the optimal concentration of rhBM2/7 heterodimer for each cellular event were significantly lower than the respective homodimers. However, our results showed that the maximum effects of rhBMP2/7 heterodimer on stimulating proliferation, differentiation were similar to instead of higher than those of rhBMP2 or rhBMP7 homodimers. In addition, gene expression results of ALP and OCN on day1, day4 and day7 determined by Real-time PT-PCR also indicated that the final maximum levels stimulated by rhBMP2/7 heterodimer were similar to that by rhBMP2 homodimer (Supplementary Fig. S1). Although it was not in conflict with the previous definition of higher potency to rhBMP2/7 heterodimer than the respective homodimers in the low concentration range (0-50ng/ml), the advantage of heterodimer in osteogenic potency tapered in the high concentration range (50-200ng/ml). BMP2/7 heterodimer produced by transfected CHO cells was shown to be more potent than BMP2 homodimer in stimulating osteogenic differentiation.25 The use of mammalian producer, conditioned medium instead of purified protein and the single selected concentration may account for the inconsistency. Moreover, our results also exhibited that, for stimulating cell migration (Fig. 1) and proliferation (Fig. 2), dose-dependent effects of rhBMPs, no matter heterodimer or homodimer, unanimously showed a bell-shape curve in which rhBMPs at concentrations lower or higher than the optimal one would down-regulate their effects. In the concentration selection test, the results also partially indicated that the ALP activities also analogously performed a bell-shape pattern. These findings strongly suggested that specificity of rhBMP2/7 heterodimer as an osteoblastogenesis-inducer was lower effective-concentration instead of higher potency in comparison with the homodimers. In fact, the phenomenon that different BMPs take effect in different concentration range (usually shown as different potencies) may generally occur to different heterodimers and homodimers.14,26

By now, the molecular mechanism accounting for the specificities of BMP remained unclear. One mechanism would be the various receptor complex
created by the combinations of three type I and three type II receptors. On the other hand, this mechanism is challenged by the phenomenon that several BMPs can use one receptor and the absence of one receptor does not totally block signaling activated by specific BMPs. A recent study which revealed different reliance of BMPs on different receptors, whereas, made the mechanism more complete and reasonable. Another study provided a new explanation that the specificity of BMP heterodimer lies in a higher combined affinity for both the type I and type II receptors, thus facilitating formation of active signaling complexes endowed by the presence of both BMP2 and BMP7 moieties in the same ligand. Another mechanism may be the complicated BMP antagonism network. BMP2/7 heterodimer was shown to induce a significant lower level Noggin, a BMP-induced competitive antagonist, and lower affinity to Noggin than the respective homodimers. Another antagonist Cas-interacting zinc finger protein (CIZ) behaves similarly on BMP2/7 heterodimers to Noggin. Similarly, the biological significance of the low-effective-concentration specificity of heterodimer also remains unclear. While the co-expression of two different BMP genes have been detected in developing limb and during fracture healing, heterodimer might be a pivotal effector on the site of less BMP producers which could only provide a lower concentration of BMPs, such as the beginning phase of fracture healing and embryonic development. For this hypothesis, our results provided several clues that the significantly higher maximum level of rhBMP2/7 for chemotactic effect (the initial step of osteogenesis), the significantly lower threshold of rhBMP2/7 for promoting proliferation and differentiation than the respective homodimers. Besides, it is also important to note that the ALP activities induced by rhBMP2/7 heterodimer were significantly higher than those of homodimers under concentrations of 100-200ng/ml on day2 (Fig. 3A) and the advantages tapered with time. On day7, rhBMP2/7 was only advantageous over rhBMP7 in inducing ALP activities. The differences of OCN expression between rhBMP2/7 and the respective homodimers at concentrations of 50-150ng/ml were more significant on day4 than on day7. Mineralization induced by rhBMP2/7 was obviously observed on day21 but not by the homodimers. These findings that rhBMP2/7 heterodimer effected more rapidly, accelerated the process of differentiation and mineralization suggested a pivotal role of BMP heterodimer in the beginning stage of osteogenic activities.
As we showed, although rhBMP2/7 heterodimer was a low-concentration effector, its action also showed a concentration-dependent bell-shape curve for the cellular events which sequentially occurred with concentration increase. One event always booms with the former one tapering although there were broad overlaps. Only the specifically appropriate concentration of rhBMP2/7 heterodimer could exert an optimal promoting effect on certain cellular event. This finding is significant for designing the *in vivo* application pattern of rhBMPs that the appropriate level of BMPs should fit a certain stage of osteoblastogenesis. Overdose of BMPs in the initial stage may lead to the down-regulation of cell migration, thus suppress the accumulation of osteogenic cells possibly as well as their proliferation. The delivering mode of BMPs was shown to influence the osteoconductivity of implant surfaces during the early phase of osseointegration. Slow-released and low level of BMPs (low local concentration of BMP in initial stage of bone regeneration) is superior to burstly-released ones in recruiting cells. We also observed fewer osteogenic activities in the initial stage when applying a higher amount of BMPs in an *in vivo* bony site (unpublished data). Although the over-stimulation of osteoclastic activity was also believed to be one of the mechanisms for the less bone volume, our findings strongly suggested that the suppression of osteoblastic activities by the locally transient high concentration of BMPs might also account for the attenuated bone formation. Therefore, caution must be especially taken to the application pattern of rhBMP2/7. However low its effective concentration is, the BMP heterodimer should also be delivered in a physiological-like manner. The principle was suitable not only for protein therapy but also for gene therapy. Controlling the level and duration of heterodimeric BMPs by using the lower titer or reduced numbers of transduced cells or regulated expression system was also recommended to avoid bone-overgrowth or immune-overreaction in combinatorial gene therapy with BMP2/7.

Significantly lower effective dose of BMP heterodimers was also expected to bring fewer side effects than the homodimers. However, since the maximum capability of rhBMP2/7 in inducing osteogenic activities, despite being obtained in a relatively lower effective concentration range, was similar to the homodimers, the capability of the heterodimer in inducing the side effects might be also similar to that of the homodimers in the same range. Consequently,
further study should clarify this point before clinical application of BMP heterodimers.

One limitation in this study is that we only adopted one kind of osteogenic cell line, which might behave differently from the others such as bone marrow stromal cells. Moreover, a broader concentration range could be further studied to completely exhibit the functional characteristics of both heterodimer and homodimers. The rhBMPs used in this study were generated from *E. coli*, the bio-activities of the rhBMPs might be different from the ones generated by mammalian cells through gene-transfection.

The Specificity of more rapidness and lower-effective-concentration range conferred rhBMP2/7 heterodimer a promising clinical application potential over its respective homodimers. However, further studies must be performed on the potential side effects and *in vivo* application of rhBMP2/7 heterodimer.

ACKNOWLEDGEMENTS

We thank Y KE for providing RT-CES/CIM system biosensor and also H FU for technical assistance in Laboratory of cell signaling and molecular genetics, Zhejiang University. We also want to thank Laboratory of Pathology for supplying ABI PRISM 7900HT Sequence Detection System and K Song for technical assistance. This work was supported by International Team of Implantology (ITI) research foundation (No. 547-2007).
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