The induction of bone formation by coral-derived calcium carbonate/hydroxyapatite constructs

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ABSTRACT

The spontaneous induction of bone formation in heterotopic rectus abdominis and orthotopic calvarial sites by coral-derived biomimetic matrices of different chemical compositions was investigated in a long-term study in the non-human primate Papio ursinus. Coral-derived calcium carbonate constructs were converted to hydroxyapatite by hydrothermal exchange. Limited conversion produced hydroxyapatite/calcium carbonate (HA/CC) constructs of 5% and 13% hydroxyapatite. Rods of 20 mm in length and 7 mm in diameter were implanted in heterotopic rectus abdominis sites; discs 25 mm in diameter were implanted in orthotopic calvarial defects of six adult non-human primates P. ursinus. Heterotopic samples also included fully converted hydroxyapatite replicas sintered at 1100 °C. To further enhance spontaneous osteoinductive activity, fully converted hydroxyapatite replicas were coated with the synthetic peptide P15 known to increase the adhesion of fibroblasts to anorganic bovine mineral. Bone induction was assessed at 60, 90 and 365 days by histological examination, alkaline phosphatase and osteocalcin expression, as well as by the expression of BMP-7, GDF-10 and collagen type IV mRNAs. Induction of bone occurred in the concavities of the matrices at all time points. At 365 days, bone marrow was evident in the P15-coated and uncoated implants. Resorption of partially converted calcium carbonate/hydroxyapatite was apparent, as well as remodeling of the newly formed bone. Northern blot analyses of samples from heterotopic specimens showed high levels of expression of BMP-7 and collagen type IV mRNA in all specimen types at 60 days, correlating with the induction of the osteoblastic phenotype in invading fibrovascular cells. Orthotopic specimens showed prominent bone formation across the different implanted constructs. The concavities of the matrices biomimetize the remodeling cycle of the osteonic primate cortico-cancellous bone and promote the ripple-like cascade of the induction of bone formation. This study demonstrates for the first time that partially converted HA/CC constructs also induce spontaneous differentiation of bone, albeit only seen one year post-implantation.

1. Introduction

The pursuit of understanding pattern formation, the attainment of tissue form and function or morphogenesis [1] has been vigorously pursued for decades and has been the source of significant advances and even greater frustration. It has dramatically carved the definition [2–7] and later the discovery of morphogenetic soluble signals or morphogens that are now at the very crux of tissue induction and morphogenesis [8–11]. Whether morphogen gradients cross threshold values at which genes are turned on or off, or influence each other triggering the spontaneous emergence of stable, long-range patterns of morphogen activity, only ‘Morpheus unbound’ can trigger the cascade of pattern formation resulting in tissue induction and morphogenesis [2,4,5].

The intrinsic or ‘spontaneous’ induction of bone formation by a variety of porous matrices is a very interesting phenomenon [10–12]. To the best of the authors’ knowledge, the first reported heterotopic induction of bone, cartilage and hemopoietic tissue by subcutaneously implanting tissue diaphragms in the subcutaneous space of the rat was published by Selye et al. in 1960 [13]. Heterotopic bone also formed after implantation of porous poly-hydroxyethyl-methacrylate in the sub-cutis of Large White pigs [14]. The morphogenesis of bone in porous bioceramics when implanted in heterotropic sites was first reported when implanting coral-derived hydroxyapatite specimens in the rectus abdominis muscle of adult non-human primates Papio ursinus [15]. Systematic experiments were then initiated to study the morphogenesis of bone when porous biomimetic matrices were implanted heterotopically in the ventral and dorsal musculature of P. ursinus [10,15–17]. The morphogenesis of bone following heterotropic implantation of coral-derived porous hydroxyapatites has been instrumental for
the development of sintered highly crystalline hydroxyapatites [18] culminating in the construction of smart biphasic hydroxyapatite/tricalcium phosphate constructs inducing significant amounts of bone in both heterotopic and orthotopic sites of P. urusinus [19].

This paper investigates the intrinsic morphogenesis of bone by coral-derived porous constructs with different chemistries, with and without coating of the P15 residue peptide long-term implanted in heterotopic and orthotopic sites of adult non-human primates P. urusinus. Previous studies have demonstrated that the collagen-derived P15 peptide enhances cell attachment to inorganic bone mineral, providing cell orienting signals enhancing the biominetic environment of substrata [20,21]. In previous experiments, partially converted hydroxyapatite/calcium carbonate constructs did not induce bone formation by induction, even when implanted after reconstitution with highly purified naturally derived bone morphogenetic/osteogenic proteins (BMPs/OPs) [22].

The aim of this long-term study was to ascertain whether the hydrothermal conversion alters the prerogative of the ‘spontaneous’ induction of bone formation by coral-derived porous constructs. The long-term study wished also to evaluate the remodeling and fate of the newly formed bone by induction in the various bioceramic constructs particularly in heterotopic sites where implanted specimens were subjected to limited mechanical forces.

2. Materials and methods

2.1. Description and specification of coral-derived hydroxyapatite implants

Six different porous hydroxyapatite replicas of the calcium carbonate exoskeletal microstructure of coral (genus: Goniopora) were prepared by hydrothermal chemical exchange with phosphate, to the protocol specification (Interpore International, Irvine, CA) [23]. Limited conversion to hydroxyapatite resulted in calcium carbonate constructs with 5% and 13% hydroxyapatite, respectively, designated 5% and 13% hydroxyapatite/calcium carbonate (5% and 13% HA/CC) constructs. Samples also included fully converted hydroxyapatite replicas sintered at 1100 °C (SHA). The fully converted implants were coated at two concentrations of P15 (Interpore International, Irvine, CA). The low dose was at 10 μg P15 per milliliter of phosphate buffered saline (PBS) (10 μg P15 SHA); the high dose of the peptide used to coat sintered hydroxyapatite specimens was at 500 μg P15 per milliliter of PBS (500 μg P15 SHA). Implants for heterotopic implantation consisted of rods of porous fully converted or partially converted calcium carbonate to hydroxyapatite as described above, 20 mm in length and 7 mm in diameter. The solid components of the framework average 130 μm in diameter, and their interconnections average 220 μm; the average porosity is 600 μm, and their interconnections average 260 μm in diameter [23,24].

2.2. Primate models for tissue induction

Six clinically healthy adult Chacma baboons P. urusinus, with a mean weight of 21.3 ± 3.5 kg, were selected from the primate colony of the University of the Witwatersrand, Johannesburg. Criteria for selection, housing conditions and diet were as described [15]. Research protocols were approved by the Animal Ethics Screening Committee of the University [AESC no. 96/95/5], and conducted according to the Guidelines for the Care and Use of Experimental Animals prepared by the University in compliance with the National Code for Animal Use in Research, Education and Diagnosis in South Africa [25].

The heterotopic rectus abdominis and orthotopic calvarial models of tissue induction and morphogenesis by osteoinductive biomimetic matrices have been described in detail [15–19]. A total of 18 fully converted or partially converted HA/CC rods were implanted bilaterally in 18 ventral intramuscular pouches created by sharp and blunt dissection in the rectus abdominis muscle of each animal. Rods were implanted in triplicate (complete conversion to hydroxyapatite, sintered hydroxyapatite and 5% and 13% conversion to hydroxyapatite) or duplicate (fully converted hydroxyapatite with P15 high and low doses and complete conversion to hydroxyapatite with P15 diluent vehicle, PBS, as control) (Fig. 1A).

Orthotopic implantation in calvarial defects was as previously described [17–19]. The implantation design and the specification of the various hydroxyapatite/calcium carbonate heterotopic and orthotopic implants are presented in Fig. 1B.

2.3. Tissue harvest, histology and histomorphometry

Anaesthetized animals were killed with an intravenous overdose of sodium pentobarbitone on days 60, 90 and 365, two animals per observation period as described [15,16,19,26]. Prior to carotid perfusion, heterotopic specimens were harvested and subjected to molecular, biochemical and histological analyses. Calvarial specimen blocks were cut along the sagittal one-third of the implanted defects, and further fixed in 10% neutral buffered formalin. Heterotopic and orthotopic calvarial defects were subjected to molecular analysis, in particular the matrix formation and mineralization:

![Fig. 1.](image-url)
orthotopic specimen blocks were cut into halves along the sagittal diameter of the implanted rods and discs, and one half of the specimen was immersed in phosphate buffered formalin for paraffin decalcified histology while the other half immersed in 70% alcohol for resin embedding. Histological preparations were as previously described [15,16]. Two ground sections from each specimen block, 300 μm apart, were stained with a modified Goldner’s trichrome, omitting the de-waxing procedure, and subjected to histomorphometric analyses as described [10,11,17–19]. Each source represented a field of 7.84 mm². Morphometry was performed on two decalcified sections per implant, analyzing 39.2 mm² per section for a total of 32 sections. Morphometry was also performed on two cross sections per heterotopic implants superimposing the Zeiss graticule over the centre of the sections. Newly formed and mineralized bone stained blue and the osteoid seams stained orange-red at the bone–bone marrow or fibrovascular space interface.

2.4. Bone specific alkaline phosphatase assay

The concentration of bone specific alkaline phosphatase was determined using an Alkaline Phosphatase kit (Metra Biosystems Inc., Mountain View, CA). Absorbance data was analyzed using EIA Smart software (Packard Instruments Co., Canberra, Australia).

2.5. Osteocalcin assay

The concentration of osteocalcin was determined using a Novocalcin™ kit (Metra Biosystems Inc., Mountain View, CA). Absorbance data was analyzed using EIA Smart software (Packard Instruments Co., Canberra, Australia).

2.6. Northern blot analyses

The expression of type IV collagen, osteogenic protein-1 (OP-1) or BMP-7, and growth and differentiation factor-10 (GDF-10) were measured by Northern blot analyses normalized to actin message. These markers were chosen for Northern blot analyses as early indicators of osteogenesis because they play such important roles in angiogenesis and bone cell differentiation.

Samples of 100–150 mg from quadruplicate specimens of harvested tissue specimens from the rectus abdominis muscle on days 60, 90 and 365 before bilateral rectus abdominis free flap transplantation were used for Northern blot analyses as early indicators of osteogenesis because they play such important roles in angiogenesis and bone cell differentiation.

Linearized vectors containing DNA probe inserts were radio labelled to high specific activity with 32P-dCTP by random prime labelling using a DNA Megaprime labelling kit (RPN 1606 Amersham, UK). Hybridisation was at 68 °C, and the membranes were washed twice for 15 min at room temperature with 150 ml of 0.1 SSC, 0.1% SDS followed by the stringency wash at 68 °C with 0.1× SSC, 0.1% SDS. The hybridised membranes were exposed to Kodak film (Biomax MS) with intensification screens for 15–75 h at ~70 °C. Signals were quantified relative to γ-actin blots by densitometric analysis (Gel Documentation and Analysis System, Syngene, U.K.) [28].

2.7. Statistical analysis

The data was analyzed using the GraphPad Prism™ Version 2.0 (GraphPad Software Inc., San Diego, CA). An F-test was used to analyze variances, and unpaired t-tests were used to detect any differences between the coralline-derived hydroxypatite/calcium carbonate specimens. For Northern blot analysis, a two-way analysis of variance test was used to determine the differences in expression of selected genes with respect to time and type of porous biomaterials used. The level of significance used was at p < 0.05.

3. Results

3.1. Bone formation by self-induction in heterotopic specimens

At harvest, the implants were firmly attached to the ventral fascia and the rectus abdominis muscle without fibrous encapsulation. On days 60 and 90, the porous spaces in all types of coral-derived biomaterials were infiltrated by a highly vascular connective tissue characterized by a cellular loose connective tissue matrix and a condensed mesenchymal tissue aligned parallel to the surface of the implanted scaffolds; this resulted in mesenchymal tissue condensations within the porous spaces aligned along the different coral-derived constructs (Fig. 2). The remarkable morphogenesis of mesenchymal tissue condensations, as a morphological phenomenon, predating the morphogenesis of bone, was as previously described reporting the induction of bone formation in fully converted coral-derived hydroxypatites implanted in the rectus abdominis muscle of adult baboons [15,16].

On day 60, all specimens showed complete mesenchymal tissue invasion across the porous spaces with vascular invasion and mesenchymal tissue condensations at the HA–soft tissue interface (Fig. 2A–C). Mesenchymal condensations at the HA interfaces with prominent vascular invasion characterized most of the implanted specimens on days 60 and 90 including SHA and 5% HA/CC specimens (Fig. 2D, F, G, and I). On day 60, specimens of fully converted HA replicas showed the induction of bone formation tightly attached to the HA substratum surrounding a central blood vessel replicating the osteogenic structure of cortical bone (Fig. 2E). SHA's showed extensive mesenchymal tissue condensations across the porous spaces and in direct contact with the hydroxypatite biomatrix (Fig. 2D). Mesenchymal condensations were also a prominent feature of 5% HA/CC specimens on day 60 (Figs. 2G), as well as P15 high dose specimens (Fig. 2F).

On day 90, there was further remodeling of mesenchymal condensations at the HA interfaces with the development of large compact bundles of connective tissue fibres aligned along the interfaces of the implanted matrices (Fig. 2I). Bone had formed by induction in P15 PBS control and P15 low dose specimens and vascular invasion and angiogenesis always characterized the induction of bone formation (Fig. 2H).

One year after implantation, bone had formed by induction in all the implanted specimens (Fig. 3). Significant amounts of bone had formed by induction in fully converted HA replicas (Fig. 3A and B). The highest amount of bone was found in P15 high dose specimens (29% bone volume) as calculated on decalcified sections (Table 1). 5% and 13% HA/CC constructs also initiated the induction of substantial bone formation in heterotopic sites (Fig. 3E, F, and G, H, respectively).

On day 365, the induced bone was organized and remodeled in direct apposition to the differently prepared coral-derived constructs (Fig. 3C–H). Substantial bone induction had formed and remodeled in fully converted hydroxypatite biomaterials (Fig. 3A). Specimens also showed the continuous induction of bone formation at the periphery of the implanted scaffolds and in concavities of the substratum (Fig. 3B). Low power view of decalcified sections showed that the initiation of bone formation always initiated within concavities and/or lacunae of the substratum as shown unequivocally in Fig. 3B. There was remodeling of the newly formed bone surrounding the porous spaces with the generation of bone marrow (Fig. 3C–G). Hydroxypatite constructs treated with low and high doses of P15 showed induction of bone within the porous spaces together with the morphogenesis of bone marrow (Fig. 3C and D). Resorption and dissolution of the implanted 5% and 13% HA/CC constructs together with prominent induction of bone formation were observed on day 365 after implantation of the partially converted coral-derived constructs (Fig. 3E–H). There was remodeling of the newly formed bone into lamellar bone directly attached to the implanted substrata with multinucleated giant cells resorbing the implanted partially converted matrices (Fig. 3H).

3.2. Morphology of tissue morphogenesis and calvarial implants’ incorporation

On days 60 and 90, calvarial specimens of 5% and 13% HA/CC constructs showed complete fibrovascular invasion across the porous spaces of the implanted matrices (Figs. 4 and 5). The
Fig. 2. Self-inducing porous biomimetic matrices and the induction of bone differentiation by smart coral-derived bioceramic constructs 60 and 90 days after implantation in the rectus abdominis muscle of adult baboons Papio ursinus without the addition of exogenously applied osteogenic soluble molecular signals. (A–C) Low power microphotographs of sintered fully converted hydroxyapatite (A), fully converted hydroxyapatite with 500 μg P15 (B), and 13% hydroxyapatite/calcium carbonate constructs harvested on day 60 after implantation in the rectus abdominis muscle. Complete fibrovascular invasion within the porous spaces with differentiation of mesenchymal condensation attached to the biomimetic matrices. (D) Detail of (A) showing fibrovascular invasion, angiogenesis (magenta arrows) and mesenchymal condensations at the bioceramic interfaces (blue arrows). (E) Induction of bone differentiation (blue arrow) in a specimen of fully converted coral-derived calcium carbonate construct organized around a central blood vessel (magenta arrow). Prominent mesenchymal condensations in specimens of fully converted hydroxyapatite pretreated with 500 μg P15 (F) and 5% hydroxyapatite/calcium carbonate constructs harvested on day 60 showing prominent condensations (blue arrows) assembled in the porous spaces (G). (H) ‘Spontaneous’ induction of bone differentiation (blue arrows) in a fully converted hydroxyapatite construct coated with phosphate buffered saline as control (PBS HA/CTRL) harvested on day 90. ‘Osteogenetic vessels’ (magenta arrows) surrounded by newly formed woven bone (blue arrows) within the porous spaces of the coral-derived biomimetic construct harvested on day 90. (I) Prominent mesenchymal condensations (blue arrows) self-assembled in a 5% hydroxyapatite/calcium carbonate construct harvested on day 90. Decalcified sections cut at 5 μm stained with Goldner’s trichrome. (A–C) Original magnification ×2.7; (D–I) original magnification ×75.
Fig. 3. Induction of bone formation by porous biomimetic matrices implanted in heterotopic sites of the rectus abdominis muscle of adult baboons and harvested on day 365 after implantation. (A, B) Induction of bone formation by fully converted coral-derived hydroxyapatite constructs. Bone formation by induction within the porous spaces of the biomimetic construct with remodeling of the newly formed bone attached to the biomimetic matrix. (B) Detail showing continuous osteogenesis (blue arrow) within concavities of the substratum up to one year after implantation. Magenta arrow points to multinucleated macrophage-like giant cells attached to a convexity of the coral-derived construct. (C, D) Low and high dose P15, respectively, showing the induction of bone formation within the porous spaces with bone marrow formation (magenta arrows). (E, F) 5% Hydroxyapatite/calcium carbonate constructs showing the induction of bone differentiation and bone marrow differentiation. (G, H) 13% Hydroxyapatite/calcium carbonate constructs showing the induction of bone differentiation throughout the porous spaces and bone marrow differentiation. (H) Remodeling of the newly formed bone with osteoclastic activity (blue arrow). Decalcified sections cut at 5 μm stained with Goldner's trichrome. Original magnification: (A) ×2.7; (B) ×125; (C) ×25; (D, E) ×47; (F) ×75; (G) ×25; (H) ×125.
induction of bone within the porous spaces was limited on day 60 at the interfacial regions, although islands of newly formed bone were also seen in the central and internal regions of both 5% and 13% HA/CC specimens (Fig. 5A, E, and F). On day 90, 13% HA/CC constructs showed central regions of the specimens as early as 60 days post-implantation (Fig. 5A, E, and F). On day 90, 13% HA/CC constructs showed central regions of the specimens as early as 60 days post-implantation (Fig. 5A, E, and F). The orientation of the corallites was evident on the pericranial aspects of the specimens. Several multinucleated macrophage/osteoclastic cells were found in close association with the newly formed bone formation attached to both 5% and 13% HA/CC calvarial constructs (Figs. 4B, F, and 5B).

On day 365, there was complete penetration of bone throughout the porous spaces (Figs. 4C, 5C and D) with remodeling of the newly formed bone into lamellar osteonic bone (Figs. 4I and 5I). Resorption and dissolution of the implanted scaffolds were particularly evident on the pericranial aspects of the specimens. Several multinucleated macrophage/osteoclastic cells were found in close association with fragments of matrix particles undergoing resorption.

3.3. Histomorphometry: heterotopic specimens

The results of the histomorphometric analysis on decalcified sections prepared from heterotopic specimens are presented in Table 1. The higher dose of peptide P15 significantly increased the amount of bone at 12 months (p < 0.0013) when compared to the low dose of the peptide (Table 1). The amount of bone in the 13% HA/CC specimens was significantly higher than in the 5% HA/CC specimens (p < 0.002). Fully converted HA and SHA specimens also induced substantial amounts of bone formation 365 days after heterotopic implantation.

3.4. Histomorphometry: orthotopic specimens

Volume fraction of tissue components of decalcified orthotopic specimens is presented in Table 2. There was no statistical difference between the amount of bone formed in the 5% vs. 13% HA/CC specimens at 2, 3 or 12 months. The amount of bone that formed at 12 month in both HA/CC specifications was significantly greater when compared to 2 and 3 months (p < 0.0001).

3.5. Northern blot analyses

The intensity of the expression of BMP-7, type IV collagen, and GDF-10 mRNAs was normalized as percentages against γ-actin intensity levels (in densitometric units). The relative densitometric units (RDUs) of the evaluated mRNAs are presented in Fig. 6. BMP-7 and type IV collagen mRNAs were highly expressed at 60 days in all the implanted biomimetic matrices (Fig. 6A) decreasing at 90 and 365 days (Fig. 6B and C) with the exclusion of type IV collagen mRNAs generated in fully converted HA, 13% HA/CC and SHA specimens remaining at relatively high levels at 90 days (Fig. 6B). At 90 days there was a several fold decrease as compared to 60 days in the expression of BMP-7 mRNA of fully converted HA specimens with the highest expression of collagen type IV at the same time period (Fig. 6A and B).

Collagen type IV mRNA expression was approximately two and fourfold higher in 13% HA/CC vs. 5% HA/CC and 90 and 365 days, respectively, even though levels were similar both at 60 days (Fig. 6B). The levels of GDF-10 mRNA expression were low relative to the mRNA levels of collagen type IV and BMP-7 at 60 days (Fig. 6A). The level of GDF-10 mRNA decreased at 3 months with further reduction at 12 months. SHA specimens expressed no detectable GDF-10 mRNA at 12 months (Fig. 6C). It was surprising that the GDF-10 mRNA levels at 12 months in 13% HA/CC implants were higher than expression levels at 3 months in the same implanted samples showing a fluctuating pattern also evident in GDF-10 mRNA expression in P15 treated samples (Fig. 6C).

3.6. Alkaline phosphatase and osteocalcin assays

The alkaline phosphatase activity expressed as units per milligram of protein is summarized in Fig. 7A. In the majority of specimens there was an increase in alkaline phosphatase activity from 2 to 12 months. The same trends are also seen when evaluating the bone specific alkaline phosphatase where the highest values were found to be at 12 months (Fig. 7B). Similarly, the results of the osteocalcin assay show a dramatic increase in the amount of osteocalcin detected over the 2–12 month period with the exclusion of the partially converted 5% and 13% HA/CC specimens (Fig. 7C).

4. Discussion

We have shown that 5% and 13% HA/CC constructs induced the remarkable induction of spontaneous bone differentiation albeit only 365 days after implantation in the rectus abdominis muscle. Additionally, the foregoing results demonstrate the efficacy of fully converted coral-derived hydroxyapatite constructs coated with and without a promoter of cell attachment, the P15 residue peptide [20,21] to induce the morphogenesis of bone in heterotopic rectus abdominis sites of adult P. ursinus. We have previously demonstrated that the specific configuration of the porous coral-derived...
Fig. 4. Calvarial incorporation of 5% hydroxyapatite/calcium carbonate constructs harvested on days 60 (A, D), 90 (B, E, F) and 365 (C, G, H and I) after implantation in orthotopic calvarial sites of adult non-human primates Papio ursinus. (A) Islands of bone formation throughout the calvarial implant as early as 60 days after calvarial implantation within the central and internal regions of the implanted construct (blue arrows). (D) Detail showing the induction of bone formation around invading ‘osteogenetic capillaries’. (B) Calvarial construct highlighting the geometric orientation of individual corallites of the original coral calcium carbonate exoskeleton. (E, F) Details of (B) and (F) showing the induction of bone formation according to the orientation of the corallites (blue arrows) with pronounced vascular invasion. (C, G, H and I) Substantial induction of bone formation within the porous spaces of 5% hydroxyapatite/calcium carbonate constructs harvested on day 365 after calvarial implantation with solid blocks of newly formed bone surrounding sprouting capillaries and ‘osteogenetic vessels’ (H and I). Decalcified sections cut at 5 μm stained with Goldner’s trichrome. Original magnification: (A, B, C) ×2.3; (D, E) ×75; (F, G, H) ×25; (I) ×75.
Hydroxyapatite substratum is conducive and inducive to cellular and extracellular matrix interactions leading to the unique pattern of membranous bone differentiation in extraskeletal sites of *P. ursinus* [16].

In previous experiments, partially converted coral-derived HA/CC substrata with a “coating” of 2 μm depth of hydroxyapatite on the porous resorbable framework of calcium carbonate failed to induce the intrinsic induction of bone morphogenesis as evaluated.
30 and 90 days after implantation in the rectus abdominis muscle of P. ursinus [22]. These partially converted HA/CC constructs did not induce bone formation even when reconstituted with highly purified naturally derived bone morphogenetic/osteogenic proteins (BMPs/OPs) [22]. Limited induction of bone formation was also seen in coral-derived constructs of different ratios of calcium phosphate to calcium carbonate with or without highly purified naturally derived BMPs/OPs implanted in non-healing calvarial defects of P. ursinus [31]. Interestingly thus, the long-term implantation of 5% and 13% partially converted HA/CC constructs in the rectus abdominis muscle resulted in the morphogenesis of bone when tissue specimens were harvested 365 days after implantation, indicating that post-implantation modifications of the substrata are critical for the differentiation of osteoblastic-like cells expressing, secreting and embedding osteogenic molecular signals into the biomimetic matrices.

There is no bone formation by induction without the osteogenic soluble molecular signals of the transforming growth factor-β (TGF-β) superfamily [10]. Indeed, the basic tissue engineering paradigm is tissue induction and morphogenesis by combinatorial molecular protocols whereby soluble molecular signals are combined with insoluble signals or substrata acting as three-dimensional scaffolds for the initiation of de novo tissue induction and morphogenesis [9–11]. The induction of bone formation, by combining soluble osteogenic molecular signals with different insoluble signals or substrata, is the essence of the tissue engineering paradigm. This and other studies on the intrinsic osteoinductivity of porous biomimetic matrices are changing the paradigm since the osteogenic signals of the TGF-β superfamily are not previously combined to the biomimetic matrices but rather self-expressed, secreted and embedded into specific geometric configurations of the matrices by differentiating osteoblastic-like cells which attach and differentiate onto the biomimetic substrata [10,11,19,26].

As previously shown using biphasic biomimetic matrices [26], expression of mRNA species is followed by secretion and embedding of the osteogenic gene products, i.e. BMP-7, onto the biomimetic matrices resulting in the induction of bone formation as a secondary response. Northern blot analyses have shown that the induction of bone formation is continuously supported by the expression of type IV collagen mRNA [26]. This reflects the induction of angiogenesis and capillary sprouting as shown by morphological analyses of the implanted specimens. Expression of type IV collagen mRNA can thus be seen as an ‘early event’ in mesenchymal cell differentiation predating the induction of bone formation.

Animal variation might have been responsible for the lack of bone formation on day 90 in fully converted coral-derived hydroxyapatites correlating with the several fold reduction of BMP-7 mRNA on day 90 vs. day 60 resulting in lack of bone differentiation. The ability of the porous biomimetic matrices to induce differentiation of invading mesenchymal stem cells could be investigated at earlier time points to study the population on invading cells’ ability to express pre-osteoblastic markers such as RunX2, D1x5 and Msx2 [32].

The intrinsic induction of bone formation is a relatively novel and cost effective bone tissue engineering strategy based on the expression and secretion of osteogenic soluble molecular signals as initiated by geometrically ‘smart’ self-inducing matrices [11,18], i.e. biomimetic biomaterial matrices that arouse and set into motion the mammalian body’s natural ability to heal [12,21] initiating the cascade of bone differentiation by induction.

The understanding of tissue induction and bone morphogenesis within the porous spaces of the coral-derived HA/CC constructs would not be possible without the knowledge of the binding and sequestration of both angiogenic and osteogenic proteins that provides the conceptual framework of the supramolecular assembly of the extracellular matrix of bone attached to the implanted matrices [10,11]. Angiogenic and osteogenic proteins bind to type IV collagen of the invading capillaries, and are presented in an immobilized form to responding mesenchymal cells to initiate osteogenesis in angiogenesis [10]. Basement membrane components by sequestering both initiators and promoters of angiogenesis and bone morphogenesis are thus modeling bone formation by induction in angiogenesis.

How do porous biomimetic matrices induce the differentiation of resident resting mesenchymal cells attached to the substratum into osteoblast-like cells, which are able to secrete selected osteogenic molecular signals of the TGF-β superfamily? Translating the morphogenesis of bone in coral-derived hydroxyapatites to sintered highly crystalline hydroxyapatites, we have learned that a specific geometric configuration carved in highly crystalline hydroxyapatites [18] or biphasic HA/TCP matrices [19,26] replicates the remodeling cycle of the primate cortical-cancellous bone, connecting osteoclastogenesis – the induction of concavities in the form of resorption pits and lacunae – with the induction of bone differentiation [10,11,19]. The remodeling of the skeleton, the formation of bone by osteoblasts and the resorption of bone by osteoclasts, is a closely integrated homeostatic system [33] . The sequential phases of the remodeling of the primate cortical-cancellous bone are quiescence, activation, resorption, reversal, formation/induction and quiescence again [33,34]. Of critical importance for the induction of bone, the resorption lacunae as formed by osteoclastogenesis are in the form of concavities. The concavities are thus regulators of bone initiation and deposition during the remodeling processes of the skeleton [10,11,19]. Biomimetic matrices of highly crystalline hydroxyapatites or biphasic HA/TCP bioeramics constructed with a series of repetitive concavities offer a geometric configuration which vividly reproduces and biomimetics the remodeling processes of the primate osteonic bone [19,33,34]. It was thus right, though much later understood, that the first description of the spontaneous induction of bone formation in porous bioeramics had occurred when studying coral-derived hydroxyapatite implants [15,16] mimicking the cortical-cancellous structure of primate osteonic bone.

Ultimately, which are the resident mesenchymal cells capable of transformation/differentiation into active secreting osteoblastic-like cells at the hydroxyapatite interface? It is obvious that the rectus abdominis muscle of adult baboons is endowed with a stem cell niche [35] which provides a large number of differentiating cells including osteogenic progenitors which attach and differentiate
onto the biomimetic matrices. The presence of a stem cell niche in adult rectus abdominis muscles is additionally supported by the recent identification of myogenic endothelial cells in human skeletal muscle [36]. Myogenic and endothelial cells may derive from a common somatic precursor, and cells co-expressing myogenic and endothelial cell markers residing in the interstitial spaces of skeletal muscle, i.e. myoendothelial cells, may contribute to postnatal tissue morphogenesis [36]. Importantly, in the context of the
spontaneous induction of bone formation in porous biomimetic matrices implanted in the rectus abdominis muscle, clonally expanded myoendothelial cells differentiate into myogenic, chondrogenic and osteogenic cells under appropriate culture conditions [36].

Multipotent myoendothelial cells residing in stem cell niches within the rectus abdominis muscle do respond to endothelial cell mitogens including angiogenic factors [36]; myoendothelial cells may also respond to bone morphogenetic proteins previously bound to collagen type IV and other extracellular matrix components further inducing the ripple-like cascade of the induction of bone formation within the porous spaces of the biomimetic matrices, mimicking the cortico-cancellous remodeling cycle of the primate bone [11,19].

The concept that biomimetic matrices may concentrate endogenously produced BMPs/OPs [12,18,37] is complicated by the fact that specific circulating BMPs/OPs are bound to protein carriers and attachment proteins which inhibit and/or reduce osteogenic activity. Expression of mRNA of osteogenic proteins of the TGF-β superfamily by resident differentiating osteoblastic-like cells attached to the biomimetic matrices has postulated that ‘inductive’ geometric configurations of biomimetic matrices via surface modifications are endowed with the striking prerogative of differentiating resident mesenchymal cells into osteoblastic-like cells able to express and secrete the osteogenic molecular signals into the implanted substrata [26]. Previous studies have shown that specific surfaces of porous hydroxyapatite ceramics do support osteoblastic cell differentiation and the expression of the osteoblastic phenotype [38].

The formation of a bone-like biological carbonated apatite layer forming on the surface of heterotopic implanted bioceramics has been suggested to be the physiochemical trigger for resident stem cells to differentiate into the osteogenic phenotype [12,37]. The role of molecularly designed matrices in regenerative medicine should be guided by the biomimetic of the extracellular matrix. Clinical trials in skeletal reconstruction have used doses of recombinant hBMPs/OPs which are several hundred times greater than the doses suggested by experiments in animal models including non-human primates [39]. Further development of ‘smart’ biomimetic matrices affecting and controlling the release of soluble osteogenic proteins is a viable alternative to enhance the osteogenic activity of hBMPs/OPs previously absorbed onto the carrier. The use of cell attachment promoting peptides such as P15 in this study not only promote the recruitment of specific cells to the matrix, but by playing a role in cell orientation, also provide additional signals that promote osteogenic differentiation [20,21]. ‘Smart’ biomimetic matrices deployed in clinical contexts will help to deliver optimal osteogenic activity of low doses of human recombinant osteogenic proteins [10,40].

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Appendix

Figures with essential colour discrimination. Most of the figures in this article are difficult to interpret in black and white. The full
colour images can be found in the online version, at doi:10.1016/j.biomaterials.2008.10.065.

References


