Bone growth response with porous hydroxyapatite granules in a critical sized lapine tibial-defect model

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Abstract. This study evaluated the tissue reaction to porous hydroxyapatite (HA) granules in a critical sized tibial-defect of New Zealand white rabbits for a period of 2, 6, 12 and 24 weeks. Physicochemical characterizations of the granules were done using transmission electron microscopy, scanning electron microscopy, X-ray diffraction, and fourier transform infrared spectroscopy to analyse the microstructure, composition, phase purity crystallinity and functional groups of HA. Prior to *in vivo* testing, the HA granules had proved to be biocompatible and cytocompatible *en route in vitro* studies using L929 mouse fibroblast cells. In the histologic evaluation, as early as 2 weeks, bone ingrowth was observed in the pores and interstices of the granules forming a network of bony trabeculae and over 6, 12 and 24 weeks, it was seen that the granules assisted in bone formation. Fluorochrome multilabels of yellow, red and orange lines showed active sites of bone mineralization in progress in the pores and periphery of the granules. Good osteointegration of the granules with the host bone was observed. There was neither inflammation nor fibrous tissue interposition while resorption of the material was in effect a slow process, since the HA granules still persisted after 24 weeks.

Keywords. Hydroxyapatite granules; new bone; fibroblast cells.

1. Introduction

In orthopaedic surgery, repair of large bone defects is a major reparative problem and it is one of the most challenging areas of skeletal reconstruction. Despite bone's considerable ability to regenerate, healing can be delayed or impaired due to multifunctional causes. So there is a need for bone grafts and preferably used are autografts or allografts (Damien and Parsons 1992). Nevertheless, problems such as donor site morbidity, limited donor bone supply, difficulty of contouring the bone graft and immunological responses are the disadvantages of transplantation (Glowacki and Mullikan 1985; Buck *et al* 1989; Binderman and Fin 1990). Over the past three decades, various biomaterials have come into widespread use in clinical practice both for the repair of osseous defects and augmentation procedures. So the concomitant need for grafts, especially bioactive ceramics is given considerable attention (de Groot *et al* 1988; Le Geros 1988; Passuti *et al* 1990; Ricci *et al* 1992; Schepers *et al* 1993) to restore or replace lost, diseased or congenitally missing bone. The use of ceramics as implantation materials originated from research in the early 1970s, which lead to the introduction of a variety of bioceramics. Calcium phosphate ceramics have received attention because much effort has been directed to the development of bone

implant materials because of their close chemical and crystal resemblance of these materials to the bone mineral. The exceptional biocompatibility is attributed to their being primarily composed of calcium and phosphorous ions, which are the most common constituents of vertebrate hard tissue systems (de Groot 1980, 1983; Jarcho 1981). Therefore, it is clearly understood that materials whose physicochemical characteristics similar to tissues and organs are highly desirable for bone regeneration. Furthermore, HA in the form of porous granules (Roth *et al* 1984) is of considerable interest as possible bone graft substitutes or as carriers of antibiotics (Wahlig and Dingeldein 1986) or osteogenic substances (Urist *et al* 1984) or even for any other drugs.

This preliminary study characterizes hydroxyapatite (HA) granules, synthesized in-house to evaluate their biocompatibility *in vitro* and compare their respective osseous regeneration in the healing of the defect *in vivo* in a lapine (rabbit) tibial-defect model.

2. Experimental

2.1 Materials

Porous hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) granules were used for the study.

2.1a Preparation of hydroxyapatite granules for *in vitro* and *in vivo* studies: HA powder was synthesized by a

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wet chemical method involving calcium nitrate and ammonium dihydrogen phosphate (Varma and Sivakumar 1996). The precipitated HA powder was freeze dried to generate free flowing powder which was compacted with a pore-former, sintered and granulated to get HA porous granules.

2.1b HA dense disc for in vitro study: The HA powder was compressed to form discs of 10 × 3 mm size using a uniaxial die. The discs were sintered at 1200°C for 2 h to get dense discs having density > 98%.

The separated granules and discs were first washed with distilled water and later subjected to ultrasonic cleaning for the complete removal of the fine powders adhered over the surface. The materials were then sterilized prior to *in vitro* and *in vivo* experiments.

2.2 Characterization of HA

The following characterizations were performed to analyse the microstructure, composition, phase purity and crystallinity of HA.

2.2a X-ray diffraction: Sintered HA granules were powdered and XRD spectrum was recorded using Siemens D-5005, at 40 kV and 30 mA, using a step size of 0.02°, scan rate of 2° per minute and a scan range between 20° and 40° 2 Theta in flat plate geometry with K- α 1 radiation.

2.2b Fourier transform infrared spectroscopy: Sintered HA granules were powdered and mixed with KBr powder in a weight ratio of 1 : 100 and pressed into pellets (10,000 psi). The FTIR spectrum was recorded using a Nicolet Impact 410 FTIR spectroscopy in the absorption mode at a resolution of 4 cm⁻¹ wave numbers, operating from 400 to 4000 cm⁻¹.

2.2c Chemical composition: Chemical composition of HA powder was analysed for calcium and phosphorous content. Estimation of calcium was carried out by a substitution titration method using EDTA solution as described by Vogel (1980). The phosphorous content was analysed by a gravimetric method (Vogel 1980). Here, the phosphate was precipitated as ammonium phosphomolybdate by adding excess ammonium molybdate to the hot and acidic solution of phosphate ions. Calcium and phosphorous ratio was calculated.

2.2d Transmission electron microscopy: HA powder was dispersed in acetone and a drop of the suspension was placed on copper support film (formvar/carbon) grids of 300 mesh type and viewed under TEM (H-600) at an accelerating voltage of 75 kV.

2.2e Scanning electron microscopy: The microstructure (pore shape, size and interconnectivity) of the granules, after gold coating in an ion sputter (E101-Hitachi) was examined using SEM (S2400-Hitachi).

2.3 In vitro studies

Direct toxicity was evaluated by morphological examination using phase contrast microscopy (PCM) and SEM. Since the geometry of the system studied was three dimensionally complex and the substrates for the cells were opaque, this work necessitated the use of SEM. L929 mouse fibroblast cell line (National Centre for Cell Sciences, Pune, India) was used for the *in vitro* experiments. Schematic representation of the cell culture procedure is outlined separately (figure 1).

2.3a Cytotoxicity—cells in direct contact with HA granules: Cells (1×10^3 cells/well) were seeded in 24 well tissue culture polystyrene plates enriched with Dulbecco's Minimum Essential Medium (DMEM) with Earl's salt (High media, Pune, India), supplemented with 10% calf serum and containing 100 U/100 µg/ml of penicillin and streptomycin, respectively and incubated at 37°C in humid atmosphere and 5% CO₂ in air for 24 h. When the cells attained confluency, the cleaned and sterilized granules were placed in direct contact with the cells in the wells and left for 24 h under the same condition (figure 1a). Thereafter, the cells were observed under PCM for any change in morphology or any other cytopathic effect, compared with negative (high density polyethylene-HDPE) and positive (copper) controls. Photographs were taken.

2.3b Cytotoxicity—cells in indirect contact with HA granules: To prepare the HA extract, known weight of the granules were placed in DMEM with Earl's salt for 24 h, after which the extract was taken.

Fibroblast L929 cells (1×10^3 cells) were seeded in small plastic vials and cultured to confluency in DMEM with Earl's salt supplemented with 10% calf serum and antibiotics and incubated at 37°C in humid atmosphere and 5% CO₂ in air for 24 h. To study the effect of the extract on the ultrastructure of the cells, the confluent cells were then kept in contact with the HA extract for 24 h. Thereafter, the cells were fixed in 1% glutaraldehyde, post-fixed in 1% osmium tetroxide, dehydrated in graded series of alcohol and embedded in spur resin and polymerized. Ultrathin sections (60–80 nm) were cut in an Ultramicrotome (LKB V) using a diamond knife and taken on copper grids (300 mesh type). They were stained with uranyl acetate and lead citrate, and viewed under TEM at an accelerating voltage of 75 kV and photographed.

2.3c Cytocompatibility: To visualize cell adhesion on the surface of the material, L929 fibroblast cells (1×10^3 cells) were seeded on the surface of conditioned (pre-incubated for 24 h in the medium) hydroxyapatite disc (3 cm diameter and 1.5 cm thickness) stuck on a glass coverslip and were maintained in DMEM with Earl's salt supplemented with 10% calf serum and antibiotics and incubated at 37°C in humid atmosphere and 5% CO₂ in air for 48 h (figure 1b). Thereafter, the disc with the cells were fixed in 1% glutaraldehyde, post-fixed in 1% osmium tetroxide, dehydrated in acetone, critical point dried (HCP2-Hitachi), gold coated in an ion sputter (E101-Hitachi) and viewed using SEM (S2400 Hitachi) and photographed.

2.3d MTT assay—cell viability: A decrease in metabolic activity offers an earlier indication of cell death. One of the most common assays for testing cellular viability is the MTT assay based on the reductive cleavage of yellow tetrazolium salt (3–4, 5-dimethylthiazol-2yl)-2,5-diphenyl-tetrazolium bromide) to a purple formazon compound by the succinic dehydrogenase present in intact mitochondria. Consequently, this conversion only occurs in living cells.

Fibroblast cells (1×10^2 cells) were seeded in 96-well tissue culture plates in the culture medium and the cells were kept in contact with HA extract for 24 h. The multi-well plate was washed and allowed to react with MTT dye solution. The plate was incubated at 37°C for 4 h. MTT solution was removed and propanol was added to all wells. The plates were mixed for 10 min to ensure crystal dissolution and absorbance was measured using a

Multiplate Reader (Biotek, USA) at 570 nm. The percentage of live cells after contact with the HA extract was determined by comparing their respective absorbances with cells having no material extract contact.

2.4 In vivo studies

2.4a Animal experiments: Eight New Zealand white male rabbits weighing 2–2.5 kg, were randomly assigned to two groups of four rabbits each. The rabbits were pre-conditioned with atropine sulphate (0.2 mg/kg body weight) and diazepam (4 mg/kg body weight) and then anesthetized with xylazine (3 mg/kg body weight) and ketamine (50 mg/kg body weight) intramuscularly. ECG and body temperature were monitored during surgery and the animal was supplemented with 100% O₂. The surgical site was shaved, scrubbed with antiseptic (Savlon) and painted with betadine/povidone iodine and draped using window drapes. The skin over the proximo-medial aspect of the tibia was incised and the skin and the periosteum were retracted. Implantation site was chosen about 4 cm away from the tibia-femur articular joint and a 3 cm gap between the proximal and the distal implantation site was maintained in all the experimental groups. A critical sized tibial cortical bone defect in rabbit measuring 2 mm in diameter (Greiff 1978 and American Society for Testing of Materials (ASTM), F981-91, West Conshohocken PA, standard) was drilled using a surgical dental drill unit (Satellac, France) to a depth of 1.5 mm, with simultaneous irrigation of cold physiological saline to remove all the bone particles and to avoid thermal necrosis. The HA

Figure 1. Schematic representation of the cell culture procedure using L929 mouse fibroblast cells (a) in direct contact with hydroxyapatite (HA) granules and (b) seeded on HA disc.

granules were press fit into the defects using a dental filler and the periosteum, muscle and skin were closed in layers. The wound was dressed with neosporin dust. On the 7th postoperative day sutures were removed. Care and management of experimental animals were followed by the guidelines issued by Indian National Science Academy (2000), approved by the Institutional animal ethics committee (IAEC) and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The animals were sacrificed at consecutive periods of 2, 6, 12 and 24 weeks for assessment of the tissue/material interaction.

2.4b Fluorescence labelling in bone: To label active zones of bone mineralization, one animal of the 3 months period group was injected intramuscularly with three types of fluorochromes viz. tetracycline (25–50 mg/kg), xylene orange (90 mg/kg) and alizarin complexone (30 mg/kg) at 2 weeks interval, consecutively prior to sacrifice.

2.4c Angiography: To study the vasculature status of the implanted site at 3 months post-implantation, contrast dye method was performed. Diatrizoate meglumine and diatrizoate sodium injection USP Urovideo® 60% (Merck) with a bolus of 5 ml of dye at a time was used for angiogram (Siemens-Siremobil 4 K).

2.5 Evaluation methods

2.5a Radiography: Radiographic assessment of the HA granules in the tibia bone was done after the implantation and before sacrifice using the dental X-ray unit (N Villa Sistemi Medicali-model 83603). The area of the defect was analysed for the percentage of radioopacity of the same density as the surrounding host bone.

2.5b Histology and histomorphometric analysis: Animals were euthanized with high dosage of sodium thiopentone and the disarticulated tibia was immediately fixed in 10% buffered formalin. The bone was carefully cleaned of all attached muscles and cut perpendicular to the implant with a diamond blade in an Isomet (Buehler) into 5 mm thick blocks which encloses the ceramic implant. After gross examination, the blocks were immediately immersed in 10% phosphate-buffered formaldehyde while fluorescence-labelled bone samples were fixed in 70% ethyl alcohol. Thereafter, both the labelled and unlabelled samples were dehydrated in graded acetone series and embedded in polymethylmethacrylate (PMMA) and sectioned to a thickness of 100–150 µm using the Isomet. Undecalcified sections stained with Stevenal's blue and counter stained with van Gieson picrofuschin (Gurr 1973; de Groot 1980) were observed by light microscopy and histomorphometric analysis was done to determine

the percent area of new bone formed at the defect site, using image analysis software programme (Optimas 6.1 version). Images were captured using a CCD camera in a 24 bit RGB mode and suitably thresholded, calibrated and the percent area of new bone and the implant at 12 and 24 weeks were calculated based on the differential staining intensity. Statistical analysis was done using Student 't' tests, with the level of significance set-up at $P < 0.05$.

2.5c Fluorescence microscopy: Undecalcified and unstained fluorescence labelled PMMA polished sections of 100–150 µm thickness were used for fluorescence microscopy (NIKON E600 Epifluorescent Microscope) with the respective filters (table 1).

2.5d SEM: Undecalcified bone block (1 mm thick) with the implant fixed in 3% glutaraldehyde, post fixed in 1% OSO_4 , dehydrated in graded acetone series, dried in a CPD (critical point drier-HCP2-Hitachi) using CO_2 and gold coated in an ion sputter (E101-Hitachi) was observed by scanning electron microscopy (SEM-S2400, Hitachi) for the osteointegration of the granules in the host bone.

3. Results

3.1 Material characterization

3.1a TEM: At higher magnification under TEM, sub-micron crystallites (200–400 nm) of HA were seen confirming that the freeze-dried powders were agglomerates of submicron crystallites (figure 2a).

3.1b SEM: The HA particles showed a size distribution between 10 and 1200 µm (figure 2b). The size of the porous granules ranged from 0.5–1 mm and the pore size was between 200 and 300 µm. There were only few intra-granular porosities (figure 2c).

3.1c Chemical analysis: The Ca/P mole ratios of the freeze-dried powder as well as the porous granules is summarized in table 2.

Table 1. The fluorochromes with their respective excitation and emission wavelengths and colours in the bone labelling studies.

Fluorochromes	Filter excitation max. (nm)	Emission max. (nm)	Colour
Tetracycline	405–435	525	Green/yellow
Xylene orange	377	615	Orange
Alizarin complexone	580	625	Red

Figure 2. (a) Transmission electron micrograph of hydroxyapatite (HA) submicron crystallites of 200–400 nm size ($\times 40,000$), (b) scanning electron micrograph of HA particles of 10–1200 μm size ($\times 300$), and (c) SEM of HA porous granule of 0.5–1 mm size with pore size between 200 and 300 μm ($\times 60$).

3.1d XRD: The XRD pattern is similar to any other submicron sized crystalline HA materials. To confirm the structural integrity, the freeze-dried powder was heat treated at 1100°C for 2 h. The peaks of the diffractogram correspond to the standard HA (JCPDS9–432) and are highly crystalline (figure 3a).

3.1e FTIR: The IR spectrum of the synthetic HA showed the presence of the characteristic hydroxyl peaks of HA at 3570 and 630 cm^{-1} (OH^- str. vibration), and phosphate peaks of HA at 1089, 1046 and 960 cm^{-1} (ν_3 str.) and 600 and 568 cm^{-1} (ν_2 str.) (figure 3b).

3.2 *In vitro* studies

3.2a Cytotoxicity—cells in direct contact with HA granules: HA did not show any signs of toxicity embarrasment after 24 h in contact with L929 cells in culture. Cells spread centripetally from the granules to form a monolayer on the glass cover slip (figure 4a). They showed the same morphology in comparison with the negative control.

3.2b Cytotoxicity—cells in indirect contact with HA granules: The ultrastructure of the monolayer of fibroblast cells was observed as cells lying parallel in contact with each other by short pseudopodia. The cell membrane was ruffled and the presence of the cytoplasmic organelles like the endoplasmic reticulum and golgi bodies together indicated cellular activity. Side by side association of the elongated cells, cell-to-cell contact at the edges of adjacent cells with the formation of cell-to-cell junctions were the additional features observed (figure 4b). Even at the ultrastructural level no cytotoxic effect was observed in the cells after contact with the HA extract.

3.2c Cytocompatibility: SEM examination revealed good anchorage, attachment, adhesion, spreading and cell replication on HA disc. Cells were elongated in shape and actively colonized the surface either in singles or in rosettes. Neighbouring cells maintained physical contact with one another through multiple extensions. Division of cells for proliferation were apparent in single spreading cells (figure 4c).

Simultaneously, dissolution of HA was apparent, since crystallization was observed on the surface of the disc

Table 2. Calcium phosphate mole ratio of the freeze dried, heat treated and powdered hydroxyapatite.

Materials	Ca/P ratio
Freeze dried HA	1.62 $\pm$ 0.3
Heat treated HA	1.64 $\pm$ 0.2
Powdered granule	1.65 $\pm$ 0.3

due to the physicochemical interaction with the culture medium (figure 4d).

3.2d MTT assay—cell viability: With MTT assay 80% of the cells in contact with the HA extract showed good metabolic activity in comparison with the cells without any HA contact.

3.3 *In vivo studies*

3.3a Radiography: Radiograph of the implanted tibia at 3 months showed the material to be well integrated with the host bone (figures 5a–c).

3.3b Angiography: Angiography of the implanted site did not reveal any microcapillaries, however, the major vessels such as the femoral and periosteal vessels were visible (figure 5d).

3.3c Gross appearance: The implanted bone site did not show any inflammation, infection nor swelling at all periods of post-implantation.

3.3d SEM: SEM revealed the osteointegration of the granules within the new bone. By 24 weeks, no gaps were observed between the host bone and the newly formed bone (figure 6a).

3.3e Fluorescence microscopy: The different fluorescence colours (yellow → red → orange) corresponded to those zones, which at that time of administration of the fluorochromes showed active mineralization adjacent to the HA granules as well as away from the granules after 12 weeks (figure 6b).

3.3f Histological examination: 2 weeks: The newly formed bone was immature woven bone. The granules were found intact in the defect within the network of bony trabeculae without any intervening fibrous tissue encapsulation. Cellular infiltration was seen in the interstices of the granules. Active osteoblast cells were seen lining the newly formed trabeculae and microcapillaries in the vicinity of the granules (figure 7a).

6 weeks: The bony defect was gradually closed with new bone enclosing and infiltrating into the pores of the

Figure 3. (a) X-ray diffraction spectrum (* = apatite) and (b) Fourier transform infrared spectrum of hydroxyapatite (HA) powdered granules.

granules. Yet few gaps still persisted between the new bone and the granules. Morphologically the granules did not show any signs of degradation. Formation of the Haversian system and the maturation of woven bone were observed (figure 7b).

12 weeks: The defect was completely closed in the periosteal and endosteal areas without any gaps. The interface between host bone and new bone was intact. The granules showed good osteointegration but did not show any signs of degradation. In the cortical region, mature woven bone was undergoing remodeling into lamellar bone with well developed Haversian system (figure 7c).

24 weeks: Defect was completely healed showing good osteointegration with the granules. Mature woven bone was remodelled into lamellar bone but granules still persisted without degradation (figure 7d).

3.3g Quantitative histomorphometric analysis: A comparative study of the implant site at 3 and 6 months post-implantation did not reveal any significant change in the percentage of new bone formed to that of the implant granules (table 3).

4. Discussion

In the present study, HA is primarily evaluated as a possible substitute for cortical bone graft. In view of this application they should be bioactive, biodegradable and/or resorbable and favour bony ingrowth at least by conduction. Now again, these properties depend on the physical and chemical properties of the material as well as greatly on the structural parameters such as pore size, the width and integrity of the partition pore walls. Then to design an implant for osteoconduction it would seem logical to mimic the architecture of the bone. Since

Figure 4. (a) Photomicrography of monolayer of L929 mouse fibroblast cells in direct contact with hydroxyapatite (HA) granules on glass coverslip after 24 h ($\times 100$), (b) ultrastructure of monolayer of fibroblast cells stained with uranyl acetate and lead citrate, showing ruffled cell membrane in contact with each other by short pseudopodia after 24 h ($\times 17,000$), (c) scanning electron micrograph of fibroblast cells seeded on HA disc after 48 h, showing good anchorage, attachment, adhesion and spreading ($\times 500$), and (d) SEM of surface crystallization observed on HA disc in cell culture medium after 48 h ($\times 400$). (C = cell; G = granule).

osteons in the bone average 190–230 μm diameter and intercommunicate through Volkmann canals, an idealized bone graft substitute would mimic either an osteon evacuated cortical bone and have an interconnected porous system of channels of similar dimensions or an osteon evacuated cancellous bone and have a thin lattice interconnected by pores of 500–600 μm (Shors and Holmes 1993), for site specific clinical application. So the aim of this study was to observe the subsequent process of bone formation after implanting HA granules as the on lay graft in the lapine cortical tibial-defect.

Surface active cell adhesion comprises a sequence of events following cell attachment, cell spreading, organization of an actin cytoskeleton and formation of focal

adhesions (Lebaron and Athanasiou 2000). With cell adhesions on material surfaces the beneficial interactions with surfaces can be understood and in turn we are able to far see their behaviour to materials in an osseous site. HA mimics the extracellular matrix by providing a structural component only that will physically sustain tissue regeneration and repair. So the cells that adhered and proliferated on the HA disc is expected to organize the extracellular matrix (ECM) to convey mechanical and chemical stimuli, and other signals influencing cellular shape, actin cytoskeleton organization and transcription activity (Blaschke *et al* 1994; Chen *et al* 1997; Lelievre *et al* 1998) and subsequently to engineer a functional tissue. These cell behaviours may possibly promote inte-

Figure 5. Radiograph of hydroxyapatite granules implanted in rabbit tibia bone: (a) normal bone (control), (b) immediately after implantation (zero day), (c) 3 months after implantation and (d) angiogram showing the implanted site and the major blood vessels in the tibia bone.

gration of the material with the host tissue and potentially enhance tissue remodeling *in vivo*. Our HA granules proved to be non toxic and the fibroblast cells exhibited excellent cell phenomenology (adhesion, attachment, spreading etc.), when visualized in SEM and TEM. This was also supported by the fact that HA allows the proliferation of fibroblasts (Orly *et al* 1989). Cell-induced resorption or dissolution of the hydroxyapatite ceramic crystals is frequently observed *in vitro* and *in vivo* (Daculsi *et al* 1989; Orly *et al* 1989). It is reported that cells cause dissolution of the HA ceramic crystals intra-cellularly by phagocytosis or extracellularly by producing a microacidic environment (Baron *et al* 1985; Daculsi *et al* 1989; LeGeros *et al* 1991). The isolated rosette crystals on the HA surface in SEM could be probably dicalcium phosphate dihydrate (DCPD) which could demonstrate a high surface area and be a factor in promoting the osteoconductive process. The formation of carbonate-apatite crystals on HA surfaces is believed to be a dissolution-precipitation process of calcium and phosphate ions (LeGeros 1988; LeGeros and LeGeros 1993). Reactions on material surfaces, including the formation of carbonate-apatite may be important in establishing the strong 'bonding zone' at the bone-material interface unique to bioactive materials. HA is highly crystalline as revealed by the sharp peaks of the XRD spectrum with a Ca/P ratio of 1.65 ± 3 . Material stoichiometry is an important factor in the resorption of HA and amorphous or less crystalline materials have been found to be more resorbable and there is evidence to suggest that they may be more beneficial for early bone ingrowth

than HA with high crystallinity (Maxian *et al* 1993; de Bruijn *et al* 1994; Klein *et al* 1994).

Bone is a composite structure consisting of collagen fibres embedded in a calcium phosphate matrix. Bone forming cells first fabricate a highly organized collagen matrix, osteoid, which subsequently mineralizes. The geometric properties (3D shape, porosity, particle size etc) of materials have been proven to affect bone formation (Reddi and Huggins 1973; Sampath and Reddi 1984; Ripamonti *et al* 1992) although the mechanisms have not been clearly demonstrated. Studies have indicated that ceramic forms of HA are inert as implant materials and do not show signs of bioresorption (Klein *et al* 1983; Hoogendoorn *et al* 1984) whereas others have observed some degree of degradation of the implanted material (Holmes *et al* 1984; Shimazaki and Mooney 1985; van Blitterswijk *et al* 1985). This controversy may be due to differences adopted in ceramics preparation condition like sintering and that the presence of impurities may have also been partially responsible for this discrepancy. So degradability of HA is still under dispute (Winter *et al* 1981; Klein *et al* 1983; Hoogendoorn *et al* 1984). Resorption is believed to be necessary for bone formation and bonding and solubility of the material increases with decreasing pH, which accelerates dissolution of the low crystalline material (Maxian *et al* 1993; Klein *et al* 1994; Paschalis *et al* 1995). Crystallization has revealed that reactions such as dissolution of the ceramic, precipitation from the solution and ion exchange caused structural rearrangement at the ceramic tissue interface. This would lead to a biologically equivalent apatitic surface on the

Figure 6. (a) Scanning electron micrograph of the cross-section of undecalcified tibia bone implanted with hydroxyapatite (HA) granules showing osteointegration after 24 weeks ($\times 30$) and (b) fluorescence micrograph of undecalcified, unstained polymethylmethacrylate (PMMA) sections of HA granules in the tibial cortical defect showing multilabel fluorescence colours (tetracycline, yellow; xylenol orange, orange and alizarin complexone, red) indicating active mineralizing zones after 12 weeks (Bar = 500 μm). (g = granule; m* = mineralizing zones; NB = new bone; HB = host bone; BM = bone marrow).

implant material accompanied by adsorption and incorporation of the biological molecules, which would favour attachment of cells (Hyakuna *et al* 1989; El-Ghannam *et al* 1999). Resorption of HA without loss of bony anchorage of implant is a balance between the release of calcium and phosphate ions and bone formation (Ducheyne *et al* 1994). With our HA granules, there was neither histotoxicity nor foreign body reaction and was osteo-tolerated but the resorption of the material was in effect, rather a slow process, since the HA granules still persisted after 24 weeks. The absence of chronic inflammation may be due to the smooth, rounded particle shape as well as the bioactive chemical nature of the HA. It has been reported that HA is regarded as a material with extremely good affinity to bone tissues (Holmes *et al* 1987; Alper *et al* 1989; Grenga *et al* 1989).

Porosity and interconnectivity are the key determinants of the amount and type of tissue ingrowth. Micropores determine the rate of biodegradation independent of the chemical composition of the ceramic while macropores would just influence bony ingrowth and interconnectivity channels are important for bony ingrowth. A pore size of over 100 μm is thought to be necessary for bony invasion (Klawitter and Hulbert 1971) and pore interconnections are thought to facilitate the invasion of cells and blood vessels (Holmes 1979; Shimazaki and Mooney 1985). Flautre *et al* (2001) reported that the best osteoconduction was obtained in the centre of the ceramic by means of a 130 μm interconnection size and a 175–260 μm mean pore size in HA. High surface area may be a factor in the osteoconduction behaviour of this porous HA material. A mechanical obstructive effect due to pore size is not very

Figure 7. Photomicrographs of undecalcified polymethylmethacrylate (PMMA) sections stained with Stevenal's blue and counter stained with van Gieson acid picrofuschin after a. 2, b. 6, c. 12, and d. 24 weeks post implantation of HA granules in the tibial cortical defect (Bar = 500 μm). (g = granule; m = marrow cavity; NB = new bone).

likely for scaffolds with pore size ranging from 150–710 μm (Ishaug-Riley *et al* 1997, 1998). The feasibility for vascular invasion is one of the essential factors of a bone inducing cell substratum (Jin *et al* 2000) and a porous structure, which is feasible for vascularization rapidly, will allow differentiation and growth of osteoblasts leading to direct bone formation. The HA granules in our study, with a pore size of 200–300 μm promoted neo-vascularization in rabbit tibia-defect. Pioneering studies showed that pore sizes less than 15–150 μm result in fibrovascular ingrowth, pores of 50–150 μm encourage osteoid formation, and pore sizes > 150 μm encourage the ingrowth of mineralized bone (Hulbert *et al* 1970). It has been described that in highly porous and interconnected implants, fibrovascular tissue ingrowth starts by day 3 or 4. By 28 days, this fibrovascular tissue ingrowth is completed throughout the implant and apposition of bone against the pore walls began (Finn *et al* 1980; Piecuch *et al* 1983; Butts *et al* 1989). The irregular and unorganized orientation of the collagen fibres and distribution of osteocytes (immature woven bone) are subsequently replaced with mature parallel fibre lamellar bone, which will remodel throughout life.

In this study, as early as 2 weeks, a network of bony trabeculae covered with osteoid and lined with osteoblasts were observed in the interstices of the existing HA granules. Osteoblastic activity with initiation of bone and adjacent islands of bone showed incipient regeneration and the presence of active osteoblast cells in laying down new bone in apposition to the material in the tibial-defect, further justified that cells did not seem to distinguish between HA and bone surfaces. Thereby, this indicated a significant similarity in their surface chemistry. The stimulatory role of the biological apatite layer favours osteoblastic differentiation, which correlates with the bioactivity of the material (Ohgushi *et al* 1992, 1996). In short, the osteoconductive potential of the material reflects its bioactivity (Hench and Wilson 1993). Chemical bonding of synthetic HA to bone comprises a partial dissolution of the ceramic surface and the subsequent formation of carbonated apatite crystals together with biomolecules of the surrounding tissue fluid (Daculsi *et al* 1990; LeGeros *et al* 1991). Chemical bonding is related to the osteoconductivity (Ono *et al*

1990) of the materials and the osteogenic potency of the tissue at the implant site (Schmitz and Hollinger 1986). Though the mechanisms are not clear, it is speculated that the binding of biologically active molecules to the HA surface can activate the cell membrane receptors of cells resulting in osteoblastic differentiation and expression of its specific phenotype and cell coordination to bone formation (Hench 1991; Ohgushi *et al* 1993). This is an indication of the bioactivity of HA (Jarcho *et al* 1977). So the speed of formation of carbonate apatite or biological apatite on the surface of bioactive materials *in vivo* (Hench 1994) controls the bone ingrowth rate and also the new bone formation rate around the particles at a very early period (Oonishi *et al* 2000). It is now understood that the role of apatite crystals is critical for bone formation and inhibition of bone resorption and in addition, a novel catalytic role for matrix metalloproteinases (MMPs) that can degrade newly formed extracellular matrix (ECM) and osteoid (Nesbitt and Horton 1997; Kremer *et al* 1998). The physicochemical changes of the material surface and the biological behaviour of the bone tissue is the key issue to accomplish the bone bonding via formation of an apatite (Kokubo *et al* 1990). It is known that apatite formation on HA is a slow process and the functional groups such as the hydroxyl and phosphate ions observed in the FT-IR studies of HA granules would permit substitutions of ions for the carbonate apatite formation to encourage activation of osteoblastic differentiation.

After 6 weeks of postimplantation, osteoblastic activity seemed preponderant and the bone defect was almost closed, leaving only a few gaps. After 12 weeks, there was complete healing with no gaps at the interface and after 24 weeks, bone defect was closed with lamellar bone. Bone formation progressed from the margin of the cavity towards the granules and the amount of bone formed increased with time. Osteogenesis has occurred through an intramembraneous process and the endochondral ossification was not observed and the connectivity of a porous implant is believed to play an important role in encouraging tissue ingrowth (Holmes *et al* 1984; Eggli *et al* 1988). It is established that HA is a bioactive osteoconductive material (Hench 1994) in bone in contrast to Class A bioactive materials (bioactive glass)

Table 3. Quantitative histomorphometric analysis of the hydroxyapatite granules implanted in rabbit tibia bone, showing the percentage area of new bone to that of the implant, after 3 and 6 months ($n = 3$ sections).

Implanted material	Implant zone (area %)		New bone zone (area %)	
	3 Months	6 Months	3 Months	6 Months
Hydroxyapatite	38.62 $\pm$ 1.74	39.22 $\pm$ 2.56	54.31 $\pm$ 1.35	54.47 $\pm$ 6.83

Note: Implant zone = the whole defect area which encloses the new bone and implant granules; New bone zone = the area of new bone formation in the defect.

that accelerates bone proliferation by a process called osteoproduction (Wilson and Low 1992).

The position of the multilabels of fluorochromes around the implant indicated that apposition of bone began both on the implant and on the bone. In the periosteal and cortical regions, most of the bone deposited towards the ceramic and bone appeared to be laid down on several foci of the implant surface and pores. In the histological sections, the demarcation between host bone and newly formed bone was always clearly visible because of differences in mineralization, lamellar orientation and the occasional presence of small haversian canals situated where the new bone bordered the host bone. Degree of mineralization and number of lamellae in the new bone were seen to increase over the weeks, attaining maturity. The interface between host bone and implant could be identified because of differences in the degree of mineralization and perpendicular arrangement of the lamellae. The normal physiological behaviours of bone (maturation sequence and response to Wolff's law) within the pores of HA characterizes the implant composition and microstructure (pore shape, size and interconnectivity) as highly biocompatible. It is expected that an ideal matrix material would stimulate osteoprogenitor cell function and the expression of the osteoblast phenotype and they should also integrate with the tissue, gradually be degraded and fully replaced by bone tissue.

The measurement of new bone formation between an implant and bone is very useful in evaluating bone bonding which is also an indication of the osteoconductivity of the material and of the bone forming capability of the adjacent tissues. The computer imaging system can discern differences between morphologic features. New bone formation around the implant is similar to the biological process of fracture healing. There was no significant increase in bone formation between the 3 and 6 months indicating that much of the bone formed was at an early period and gradually slowed down in par with the slow resorption of HA. Bone regeneration within porous synthetic material has been documented by the concept of osteoconduction: the 3-D process in which sprouting capillaries, perivascular tissue, and osteoprogenitor cells grow from the recipient bone into the bone graft by the so called "creeping substitution" process (Urist 1980).

The tibial defects did not hamper the animals. The osteoconductive potential of a material reflects its bioactivity and different bioactive materials have different bioactive response to the cells. In certain species, an osteoconductive biomimetic biomaterial may progressively function as an osteoinductive substance by binding to endogenous bone morphogenetic proteins (BMPs) establishing a physiological-physicochemical continuum between HA and endogenous BMPs (Reddi 2000). The osteoconductive property of bioactive ceramics however

depends on their proximity to the host bone and on the viability of bone cells in the host bone. When these criteria are met satisfactorily, the successful union of ceramics to the host bed is ensued.

5. Conclusions

The combination of *in vitro* and *in vivo* studies with various evaluation parameters led to a better understanding of the effect of bioactive HA on cell function and bone formation to guide morphogenesis and regeneration. By and by, granules are easy to handle and can be molded into the desired shape while granules of large pores and high osteoconductive potential can be used for packing bone defects of non-load bearing areas. Since the diversity of reconstructive requirements for clinical defects of the skeleton is great, porous HA coating of metals or a bone hybrid of HA with cells and growth factors could be used to reconstruct defects. So fabrication of varied composites and combinations could help in the development of second generation bioactive ceramics for implantation.

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