



HAL
open science

CELL-DEGRADATION OF CALCIUM PHOSPHATE CERAMICS

Patrick Frayssinet, Nicole Rouquet, Francis Tourenne, Jacques Fages, D.
Hardy, Gilbert Bonel

► **To cite this version:**

Patrick Frayssinet, Nicole Rouquet, Francis Tourenne, Jacques Fages, D. Hardy, et al.. CELL-DEGRADATION OF CALCIUM PHOSPHATE CERAMICS. CELLS AND MATERIALS, 1993, 3 (4), pp.383-394. hal-01508687

HAL Id: hal-01508687

<https://hal.science/hal-01508687v1>

Submitted on 18 Apr 2017

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

CELL-DEGRADATION OF CALCIUM PHOSPHATE CERAMICS

P. Frayssinet^{1,*}, N. Rouquet¹, F. Tourenne¹, J. Fages¹, D. Hardy², G. Bonel¹

¹Bioland, 132 Route d'Espagne, 31100 Toulouse, France

²Service de Chirurgie Orthopedique, Hopital St. Pierre, Bruxelles, Belgium

(Received for publication September 29, 1993, and in revised form December 27, 1993)

Abstract

Calcium phosphate ceramics are used in bone surgery under different forms: dense or porous ceramics as bone substitute, thin ceramic coatings on metallic implants as an osseointegration enhancer. Their degradation depends on their physico-chemical properties and particularly on their chemical composition. Natural calcium phosphates of bone are degraded by mononuclear or multinuclear cells and the extracellular matrix induces the differentiation of the degrading-cells. Hydroxyapatite, which is one of the most used calcium phosphates, is known as a low degradation material. However, the histological analysis of implanted HA-materials both in animals and in humans showed that a cellular degradation took place on the surface of the material.

Key Words: Calcium phosphate ceramics, hydroxyapatite, hydroxyapatite-coatings, macroporous ceramics, cell degradation, osteoclast, macrophage, remodelling, dissolution.

*Address for correspondence:

P. Frayssinet,
Bioland,
132 Route d'Espagne
31100 Toulouse, France

Telephone number: (33) 62200395
FAX number: (33) 62200346

Introduction

Hydroxyapatite (HA) ceramics are used daily in human and animal surgery under different forms [31, 36]. This material is classified as a surface-active biomaterial [33]. It means that an interfacial bond is formed between the material and the tissue in which it is implanted. Calcium phosphate materials, currently used in human surgery, are assumed by surgeons to be non- or slowly degradable materials. Depending on its use, a degradation may be necessary to improve osseointegration [20].

HA-materials such as HA-coatings and macroporous-HA can be considered to have a chemical composition which is close to bone mineral [42]. All other characteristics are radically different. The macroporous ceramic can be composed of a single calcium phosphate phase. HA-coatings can contain other calcium phosphate phases in their amorphous phase [61]. The size of the crystallites of bone is much smaller than the size of ceramic grains, and the structure and the texture of bone and ceramics are totally different [51]. Despite these differences, based on its close chemical composition to that of the bone mineral, HA is assumed to be a biodegradable material.

The purpose of this article is to discuss some observations made on HA ceramic degradation in a biological environment.

Calcium phosphate ceramics

Ceramic material can be defined as all solid materials which are neither metal nor organic. Ceramics are heterogeneous material constituted by grains which are assembled using a thermal process (sintering stage). They can be used in the form of bulk or porous material obtained by slip casting or machining. Moreover, thin coatings of calcium phosphate ceramic on metal alloys, polymers and composites can be obtained by plasma-spraying of HA-powder. The characteristics of the ceramic will have a great influence on the resorption of the material. Moreover, different calcium phosphates can be used to manufacture bioceramics. The HA and the β -tricalcium phosphate (β -TCP) are the most commonly used. The choice between these two compounds depends

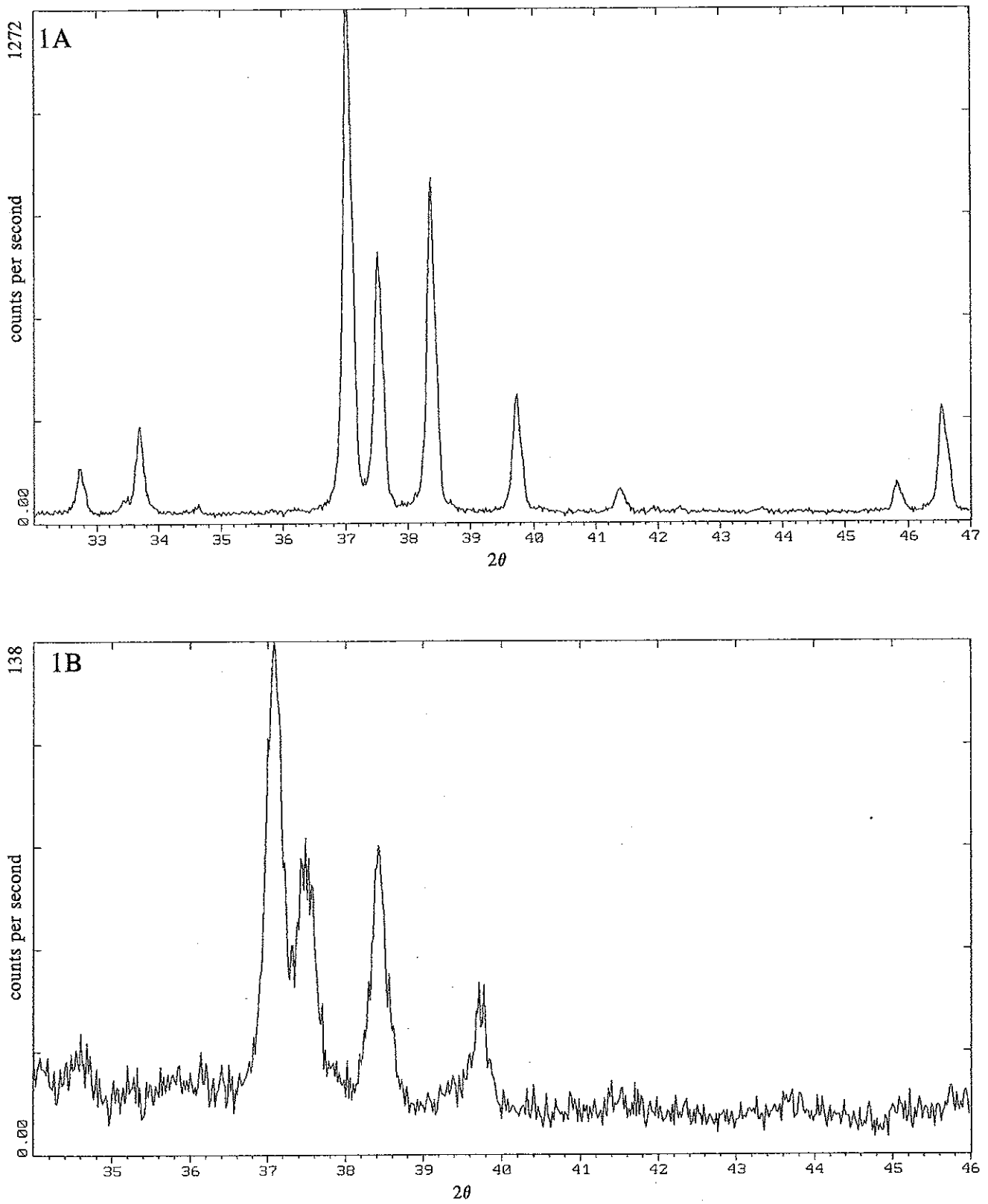


Figure 1. X-ray diffraction patterns of: HA-powder before plasma-spraying (A), and of the obtained HA-coating (B). The peaks are broader after the plasma-spraying indicating the presence of an amorphous phase after the spray.

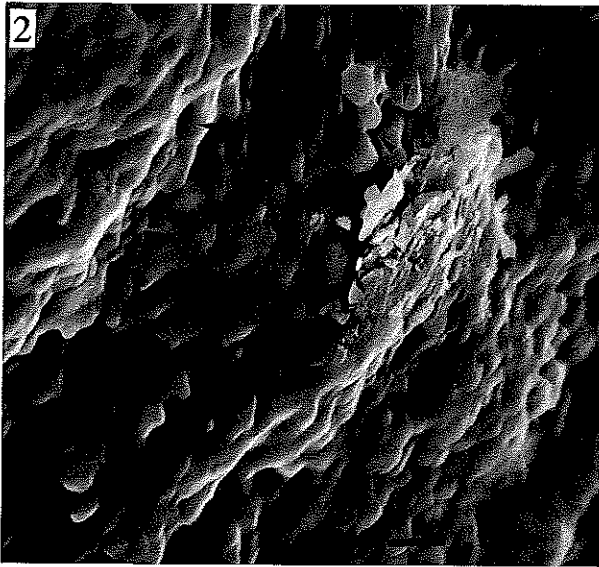


Figure 2. Scanning electron micrograph of an HA ceramic surface showing the grains constituting the ceramic. Small holes exist between the grains (arrowheads); they are randomly distributed and not interconnected, constituting the microporosity of the ceramic. Bar = 50 μm .

on the solubility required for the material. HA is considered to be quite insoluble and β -TCP soluble when tested *in vitro*.

Different phases can appear in the HA-ceramics according to the sintering protocol adopted and the purity of the powder used to manufacture the ceramic. β -tricalcium phosphate (β -TCP) can appear during the manufacturing process of HA-ceramics. An amorphous phase, which has a Ca/P ratio identical to the Ca/P ratio of HA but which cannot be identified as HA by X-ray diffraction, can also be found in HA-ceramic coatings. These phases do not have the same solubility as pure crystalline HA. The crystallinity of the ceramic is measured by X-ray diffraction (XRD). The broadening of the XRD pattern peaks after plasma-spraying is representative of the amount of amorphous phase in the ceramic. Amorphous phase which can contain other calcium phosphate phases, such as β -TCP or calcium oxide, is more soluble than the crystalline phase [61] (Fig. 1).

The porosity of the material is often divided into microporosity and macroporosity. The microporosity is due to the small spaces existing between the grains of the ceramic which were left by the shrinkage occurring during the material diffusion process at the grain boundaries (Fig. 2). They have a diameter of less than a few micrometers and are not interconnected. The macroporosity results from the shaping of the ceramic done in order to obtain a bone tissue ingrowth inside the ceramic. The macropores should be interconnected and have a diameter of from 100 μm up to several millimeters.

The porosity of the material determines the size of the surface which is in contact with the body fluid. The surface in contact with the tissue depends also on the interconnection of the material pores. It is generally admitted that a pore diameter of more than 100 μm is necessary for bone ingrowth [30] depending on the volume of the implanted ceramic. If the interconnection of the pores is poor, the size of the exposed surface will be smaller than if the pore connection is high.

The size and the shape of the grains obtained after the sintering stage or plasma-spraying can vary and have an influence on the amount of the surface of the crystalline phase exposed to the body fluids.

Degradation of bone calcium phosphate

Bone mineral of the adult is composed of an A and AB carbonated non-stoichiometric apatite [42]. Nucleation of carbonated apatite crystals within the bone extracellular matrix can be due to the removal of biological inhibitors, to interaction between Ca^{2+} and calcium-binding proteins, or to apatite precipitation out of locally supersaturated calcium phosphate solution made by body fluids inside some microdomains [4, 27, 45, 58]. Bone mineral crystals are first formed either inside "the holes" existing between the fibrils in the collagen bundle or inside extracellular matrix vesicles synthesized by osteoblasts. The bone mineral nucleation taking place inside these organic compartments is an heterogeneous secondary nucleation controlled by the molecules contained inside these compartments. Subsequently, the mineralization spreads out of the nucleation site throughout the extracellular matrix, this, making the bone an organo-mineral composite.

A high degradation rate of the organo-mineral composite constituting the bone tissue by bone cells is already known in several pathological and physiological processes. Osteoclasts [12, 44] are known to resorb bone tissue during remodelling phenomena which occur during the life of the bone. Bone tissue is resorbed by osteoclasts or giant cells, during Paget's disease. Some forms of osteoporosis, idiopathic osteolysis, and hyperparathyroidism are also characterized by the presence of many osteoclasts resorbing bone tissue. From the histological studies carried out in these pathologies, two distinct kinds of cells were shown to degrade bone tissue and calcium phosphate mineral: osteoclasts and mononuclear cells [13]. Histological analysis of bone allograft shows, in the same resorption sites, mononuclear cells, giant cells, and osteoclasts.

Osteoclasts: Osteoclasts are multinucleated (2 to 50 nuclei) giant cells (20 to 100 μm). Once in contact with the bone, the newly formed osteoclast begins to erode a cavity of characteristic shape and dimension. They have a central ruffled border with many cytoplasmic folds, surrounded by a clear zone where the cell is closely attached to the bone surface, forming a temporary seal around the site of bone destruction [17]. Cell attachment could be mediated by oligosaccharides [48]. The chemical processes mediating bone resorption are

numerous and complex. Dissolution of the mineral is the main task of the osteoclast which produces biochemical changes that includes release of acid, particularly lactic acid and citric acid [43], coupled with increased synthesis and release of lysosomal enzymes and other proteases [32]. Carbonic anhydrase, which hydrolyses carbonate into proton and carbon dioxide, plays a role in the acidification of the medium at the interface of bone and osteoclasts. The ruffled border membrane contains a protein identical to H⁺-pump found in the lysosomal membrane [7]. The decrease of the pH induced by the proton-pump in the extracellular compartment facing the ruffled border seems to be responsible for the bone mineral degradation. Lysosomal enzymes secreted in this compartment could help to degrade the organic matrix. Furthermore, collagen fibrils could be degraded by other cells containing large amounts of collagenase such as osteoblasts [32] although osteoclasts could resorb the organic and inorganic components of bone as reported by Blair *et al.* [9]. However, the digestion of the collagen coating, existing on bone surface, by osteoblasts and lining cells could be necessary for osteoclast digestion of bone [15]. Complement (C₃) adsorption on the exposed mineralized bone surfaces could mediate the recruitment of mononuclear preosteoclasts to this site [46]. Osteoclasts have been known for a long time to be involved in relation with osteoblasts in the normal remodelling of bone tissue. Osteoclast precursors may respond to specific humoral signals emanating from other bone cells such as active osteoblasts, lining cells, or immune cells synthesizing interleukin I α and β , tumoral necrosis factor α and β , and transforming growth factors α and β [5, 55].

Osteoclasts originate from hemopoietic tissue. They derive from mononuclear phagocytes as precursors. They arise from a derivative of the hemopoietic stem cell that can become committed specifically to osteoclast generation [14]. The separation between osteoclasts and macrophages or giant cells lineage could come early [11]. Alternatively, it remains possible that osteoclasts could develop from mature mononuclear phagocyte by induction of osteoclastic differentiation in the special microenvironment of bone surface. Osteoclast phenotype has been well characterized and shown to express many of the antigens of the monocyte-macrophage lineage [5]. Mononuclear cells identified in histological sections of bone, applied to the bone surface, show a similar immunophenotype to that of the multinucleated osteoclasts [6].

Marrow-derived giant cells express osteoclast-specific cell surface antigens when cultured with calvariae. In the mesenchymal cell containing chorioallantoic membrane, intact bone matrix induced the formation of giant cells that express osteoclast-specific antigens [54]. Demineralized matrix and matrix components such as type I collagen, osteocalcin, osteopontin, osteonectin, bone-derived growth factors, cartilage proteoglycan, hyaluronic acid, laminin, and fibronectin do not induce the formation of giant cells expressing osteoclast markers

(antigen 121 F) on their membrane [54]. In the same experiment, a culture medium, conditioned by chick hatched calvariae, induced the formation of giant cells having osteoclast markers from the culture of a monocyte or marrow mononuclear cell population. It showed that the terminal stages of osteoclast differentiation were influenced by soluble factors found in the bone environment. Glowacki *et al.* [28] showed that osteocalcin-deficient bone particles were not associated with osteoclasts when implanted subcutaneously in rats. However, in the same experiment, it was shown that synthetic microcrystalline HA recruited multinucleated cells lacking the osteoclastic features [28].

Thus, osteoclast recruitment at the bone contact depends on specific or non-specific humoral signals and factors emanating from bone extra cellular matrix (ECM). However, ECM calcium phosphate materials can have an influence on osteoclast recruitment mediated by non-specific humoral signals such as C₃ adsorption on the naked calcium phosphate surface.

Other cells: It is not clear whether macrophages contribute directly to bone remodelling *in vivo*. Evidence has been presented that cells of the mononuclear phagocytic system are capable of bone resorption in various culture system. Cells of the mononuclear phagocytic system were shown to resorb implanted isogenic bone matrix on animal. These cells are also present at the point of contact of bone tissue allograft in humans. Mononuclear cells could be responsible for the digestion of the bone collagen [32] and a part of the erosion cavity dug by the osteoclast [21]. It is unclear if these cells are precursor cells having failed to fuse or resorbing monocytes [52] helping osteoclasts by the release of cytokines.

Furthermore, macrophages are interrelated with osteoblasts and synthesize cytokines which are able to regulate their activity [34, 56].

Degradation of ectopic natural HA-crystals

Ectopic calcium phosphate crystals found in soft tissues induce a cellular response involving phagocytic cells. Calcium oxalate and dicalcium pyrophosphate deposits in soft tissues are surrounded by histiocytes and giant cells [10]. HA-crystals, identical to the HA-crystals found in the human joint disease, having a diameter of between 0.1 and 5 μ m, induce prostaglandin E₂ release by macrophages [2]. Apatite-containing particles are frequently found in osteoarthritic joints. Hydroxyapatite has been reported to induce acute inflammatory reaction in human skin [18] and synovitis after injection into dog's knee joints [60]. Articular chondrocytes were shown to secrete collagenase, protease, and prostaglandins E₂ and F₂ after phagocytosis of HA-particles.

Cheung *et al.* [16] showed that the phagocytosis of HA-crystal found in osteoarthritic joint induced the multiplication of synovial cells. These results were attributed to the increase of cytoplasmic Ca²⁺ suggested by a degradation of the crystals once phagocytosed.

Degradation of hydroxyapatite-ceramics

Chemical, biochemical and biological evidence of Hydroxyapatite-solubility: Hydroxyapatite is one of the less soluble calcium-phosphates used as bone substitute material. The solubility phase diagram of the most frequently used calcium phosphates as biomaterials shows that although HA is the most stable phase under many conditions, it becomes less stable than dicalcium phosphate dihydrate (DCPD) if the solution is sufficiently acidic. Such ranges of acid pH are commonly found in some cell compartments and particularly in cells responsible for the bone degradation. It is considered that both the endocytic and exocytic vacuolar apparatus in cells are maintained at low pH [3]. Endocytic vesicles, lysosomes, portions of the trans-Golgi apparatus, certain acid-producing secretory vesicles, and the "resorption chamber" of the osteoclast are low pH cell compartments. Using fluorescein-labelled dextran, pH values of 4.7-4.8 were measured in macrophage lysosomes [57]. Similar pH values were measured by Baron *et al.* [7] in the extracellular resorption region of osteoclasts. A recent report showed that cultured fibroblasts phagocytosed HA-particles inside or in close proximity to their acidic intracellular compartment identified by neutral red uptake [24]. Alliot-Licht *et al.* [1], Kwong *et al.* [41], and Orly *et al.* [53] have demonstrated that calcium phosphate grains having a size less than 50 μm can be phagocytosed by several kinds of cells such as fibroblasts, osteoblasts, established osteoblast-like cell lines, synovial cells, and macrophages then degraded in intracellular vesicles. Subsequent to the internalization of HA-particles, it has been observed that the growth rate and DNA synthesis of osteoblast were significantly inhibited [1]. The HA-particle size was found by Evans [22] to have an effect on the cytotoxicity of HA *in vitro*. Finely ground powders of HA reduced the growth rate of fibroblast line. The effect was dose-related and only occurred with small particles (3-7 μm). A physical effect could interfere with the cell growth. In particular, the elevated surface at the point of contact of the cell culture medium, when the ceramic powder was finely ground, could induce an ionic modification of the culture medium responsible for the cytotoxicity of the ceramic as demonstrated by Hyakuna *et al.* [35]. However, these results were not consistent with the work of Mitchell *et al.* [50] who reported a mitogenic effect of a mixture of calcium phosphate crystals (HA, calcium phosphate, and calcium pyrophosphate dihydrate) on synovial cells. Surface characteristics and solubility of the materials tested could explain this difference. The increase in free cytosolic Ca^{2+} concentration with a simultaneous release of inositol(1,4,5) triphosphate seems to be necessary for a mitogenic effect [49]. This is consistent with the higher mitogenicity of free Ca^{2+} compared with HA crystals as reported by Praeger and Gilchrest [59]. Based on earlier reports [36-38] published on the implantation of calcium phosphate ceramic, HA-ceramics were supposed to be a non- or slowly-degradable material in a biological environment. HA was considered as non-resorbable, and

β -TCP was considered as slowly degraded. However, the biological effects of the HA on isolated cells related to the release of Ca^{2+} from the ceramic, shows that HA is not absolutely stable and may be degraded in living tissue. It is particularly interesting to know the factors affecting the degradation of HA-coatings which are supposed, in some cases, to last as long as possible, once implanted. Klein *et al.* [39] have demonstrated that the solubility of coatings was much higher than sintered particles. Degradation of calcium phosphate coatings in a buffered solution *in vitro* was shown for tetracalcium phosphate, tricalcium phosphate and HA. Maxian *et al.* [47] recently reported that a greater *in vitro* Ca^{2+} dissolution, and a greater surface, physical, and chemical changes were observed for poorly crystallized coatings compared to amorphous coatings, suggesting that amorphous coatings could be more advantageous if coating longevity is desired. This is not consistent with what has been previously published [61]. When we investigated the *in vitro* behaviour of animal cells grown at the point of contact of HA-coating having different crystallinities, it was found that more Ca^{2+} dissolved from coatings having a low crystallinity (40-70%) compared to the coating having a high crystallinity (100%) [25]. HA-particles were found inside cells grown on both poorly and highly crystalline coatings. The highly crystalline coatings induced a cytostatic effect. It is interesting to note that a coating degradation was found *in vitro* and during short periods of time for all the crystallinities tested.

Microscopic evidence of material degradation: HA is used under different forms: dense, porous, powdered or thin coatings. Gomi *et al.* [29] showed that osteoclast-like cells resorb sintered synthetic hydroxyapatite *in vitro* and that the nuclearity of the resorptive cells is influenced by the surface topography of the substratum. Koerten *et al.* [40] demonstrated that spray dried HA-spheres were degraded when injected into the mouse peritoneal cavity. Müller-Mai *et al.* [51] showed the microscopic degradation of HA-coating. Recently Baslé *et al.* [8] demonstrated that macroporous calcium phosphate elicited, at the ceramic contact, the recruitment of a double multinucleated cell population, giant multinucleated cells and osteoclasts, having a resorption activity. This result is not consistent with that obtained by Glowacki *et al.* [28]; it could be due to the difference in features chosen for osteoclast characterization.

We have been able to perform histological analyses of HA materials implanted in human and animal bone in the form of coating or macroporous ceramics in order to evaluate the material degradation *in vivo*.

Materials and Methods

Fifteen hip prostheses coated with plasma sprayed-HA coatings (characteristics given in Table 1) and implanted for periods from 5 days up to three years (Table 2) were retrieved post-mortem from patients deceased for reasons which were not directly related to the

Table 1. Characteristic of the HA-coatings analyzed.

Plasma-sprayed powder	more than 97%
Crystallographic structure	Ca ₁₀ (PO ₄) ₆ (OH) ₂ , other compounds admitted: tricalcium phosphate (traces), calcium oxide (traces), amorphous phase
Crystallinity	60% ± 10%
Coating thickness	150µm ± 50µm
Trace elements	As < 3 ppm; Cd < 5 ppm; Hg < 5 ppm; Pb < 3 ppm
Mechanical strength (pull-out test)	35 MPa

Table 2. Extreme values of the thickness (in µm) of coating implanted for various period (thickness before implantation: 100-150 µm).

implantation	Metaphysis	Middle	Diaphysis
12 months	99-220	50-164	40-184
24 months	0-183	0-180	0-188
43 months	58-164	104-204	119-163
1.5 months	104-143	123-182	164-119
7 months	163-137		213-155

Table 3. Characteristics of the macroporous HA-ceramics.

Chemical composition	100% HA
Porosity	70%
Pore diameter	500-1500 µm
Grain size	3 µm
All pores interconnected	

hip surgery. The femurs containing the HA-coated prostheses were cut into three parts which were fixed into a 4% formaldehyde solution into PBS (phosphate buffered saline). They were dehydrated in alcohol solutions and embedded into PMMA (polymethyl methacrylate). Two-three mm thick sections were made using a low speed cooled diamond saw. They were re-embedded at the surface of a polystyrene block and polished until a 50-100 µm thickness. Sections observed using a light microscope were stained with a fucsin-toluidine solution after etching the block in a 1% formic acid solution and a 20% methanol solution. Sections observed using scanning electron microscopy (SEM) were stained with a silver methenamine solution [23]. Briefly, sections were stained using a 1% silver methenamine solution (brought to pH 9.6 with NH₄OH) for 4 minutes in a microwave oven at 20% power (2450 MHz). Sections were then examined using the backscattered mode of a SEM operated

at 25 kV. The degradation of the coating was measured on sections of 5 HA-coated prostheses using an image analysis system. The maximum and minimum coating thickness was determined on sections made at three levels on each prosthesis.

On thick sections of two HA-coated prostheses implanted three weeks and one year, the prosthesis was detached from the bone and treated with a 2% NaOCl solution for 24 hours to remove the organic matrix adsorbed on the coating. They were dehydrated and gold-palladium coated, then they were observed in the SEM at 25 kV.

Twenty macroporous HA ceramics composed of pure HA (characteristics given in Table 3) were implanted into 9 mm holes drilled into the cortical bone of the medium diaphysis of sheep femurs for periods from one month up to ten months. Five other ceramics were implanted for two months into defects made into the medium diaphysis of dog's ulnae. Once retrieved, the implants were processed as described above for histological analysis.

Results and Discussion

A decrease in the coating thickness was noted after a few months of implantation (Table 2). This degradation can appear after a few weeks of implantation and was not dependent solely on time. The degradation could be completed after a two years implantation period or was non-detectable after three years. SEM observations of specimens, whose organic matter had been removed by NaOCl, showed that the surface of the one year implanted coating was very rough (Fig. 3) compared to the surface of the three weeks implanted one (Fig. 4). Higher magnifications made it possible to see the crystals of the coating suggesting a dissolution of the amorphous phase between the grains. Grains also showed some degradation marks on some of their facets (Fig. 5).

Multinucleated cells and mononuclear cells were found at the coating contact point in all the sections examined except the sections taken from the prosthesis implanted for five days. These cells were located at the coating contact in regions facing medullary cavities. Most of them, after a few months implantation period, had HA-grains included in their cytoplasm showing a phagocytosis of calcium phosphate grains coming from the coating (Fig. 6). Several types of coating degradation could be described morphologically and were observed both by SEM or light microscopy on prostheses coating implanted for a few months (5 months or more). Several of these types could be observed on the same prosthesis: **a)** coating having a non-altered thickness in contact with multi- or/and mono-nucleated cells containing HA-particles (Fig. 7); **b)** coating that was of an altered thickness in regions facing medullary cavities although coating in contact with bone tissue is not altered (Fig. 8); **c)** coating having an homogeneously degraded thickness; and **d)** coating degraded regions invaded by bone tissue (Fig. 9).

Cell-Degradation of HA-Ceramics

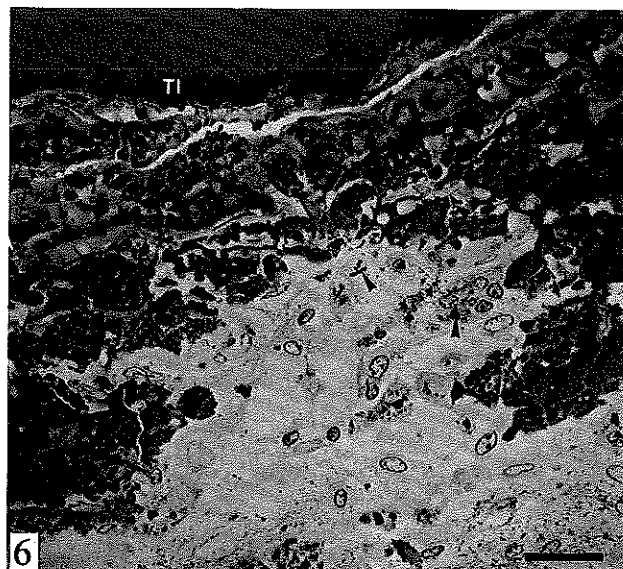
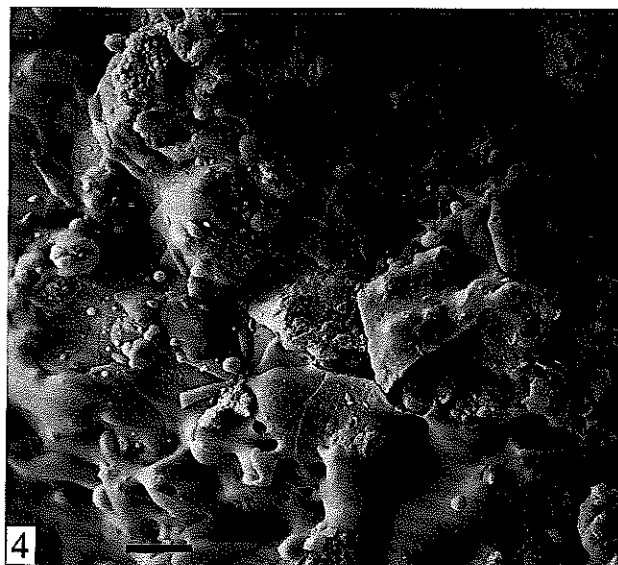
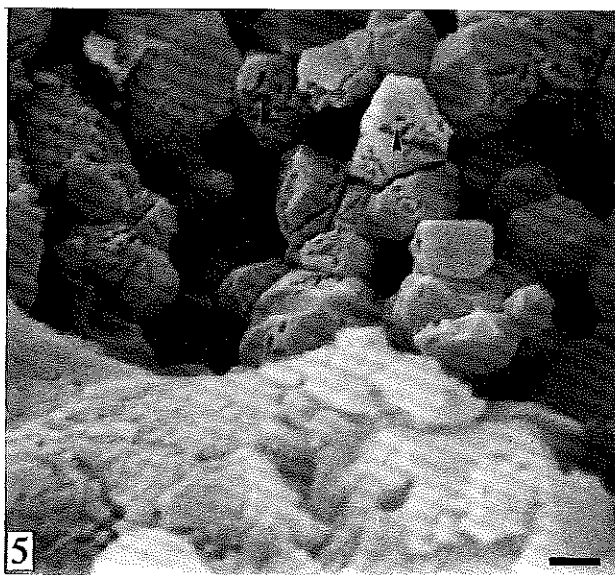
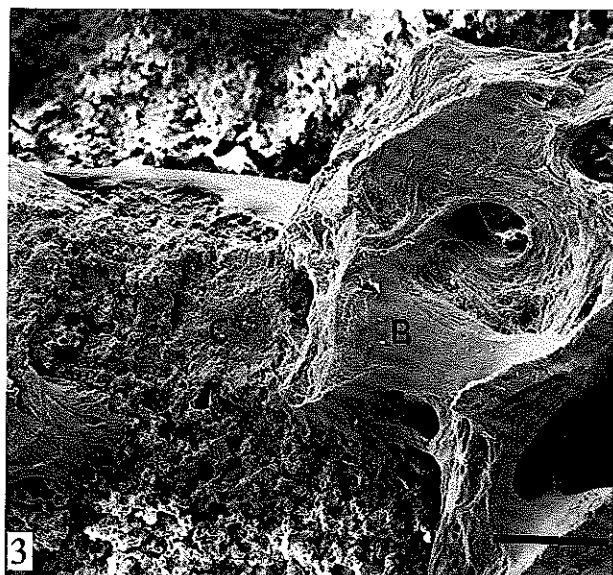


Figure 3. Scanning electron micrograph of a one year implanted HA-coated prosthesis in human. The organic phase in this sample had been removed by using NaOCl. The coating surface (C) is rough suggesting that degradation occurred during the implantation period. Bone trabeculae (B) are apposed on the coating surface. Bar = 550 μm .

Figure 4. Scanning electron micrograph of a three weeks implanted HA-coated prosthesis. The organic phase in this sample had been removed by using NaOCl. The surface is very smooth showing no degradation sign. Bar = 140 μm .

Figure 5. High magnification scanning electron micrograph of the surface of the prosthesis shown in Figure 3. The amorphous phase is resorbed and the grains constituting the crystalline phase of the coating are observed. Some of these grains show resorption marks on some of their facets (arrowheads). Bar = 10 μm .

Figure 6. Back-scattered electron micrograph of a silver methenamine stained section of a HA-coating resorption zone (HA) of a two years implanted prosthesis. Osteoclast and macrophages contains calcium-phosphate particles (arrowheads). Ti: titanium alloy. Bar = 35 μm .

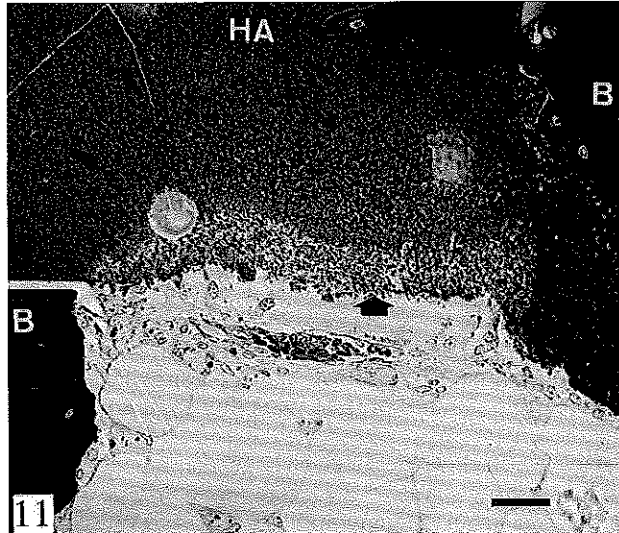
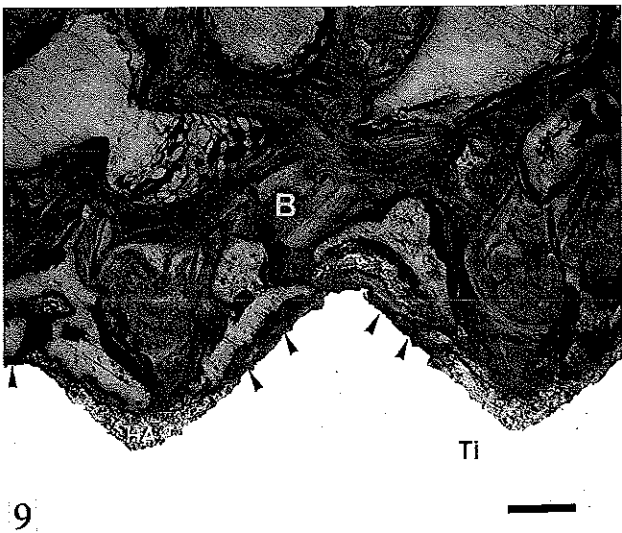
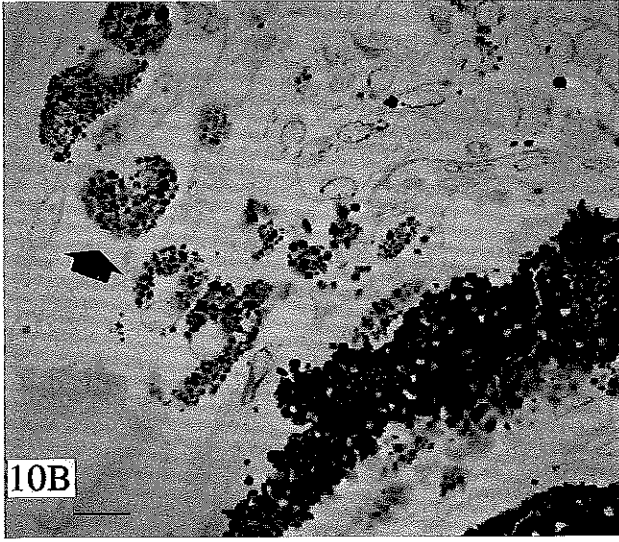
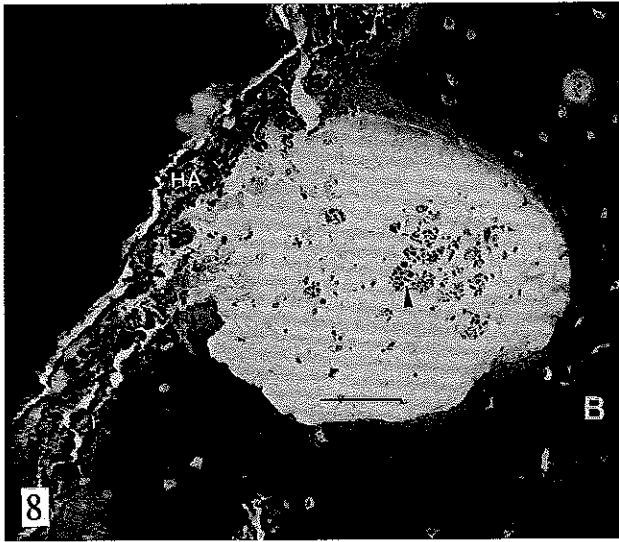
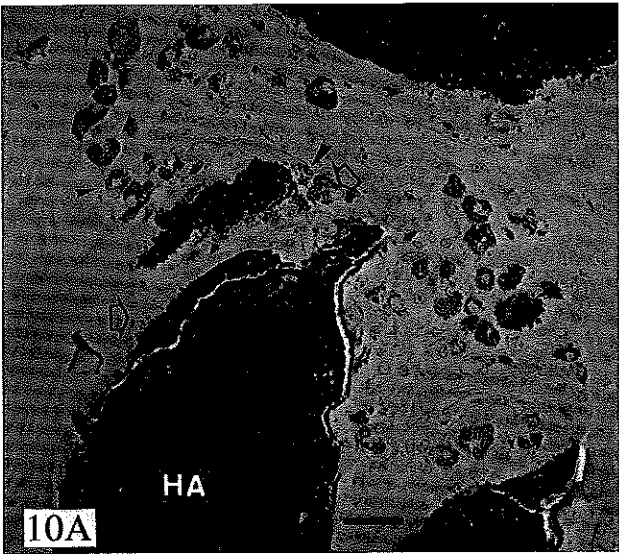
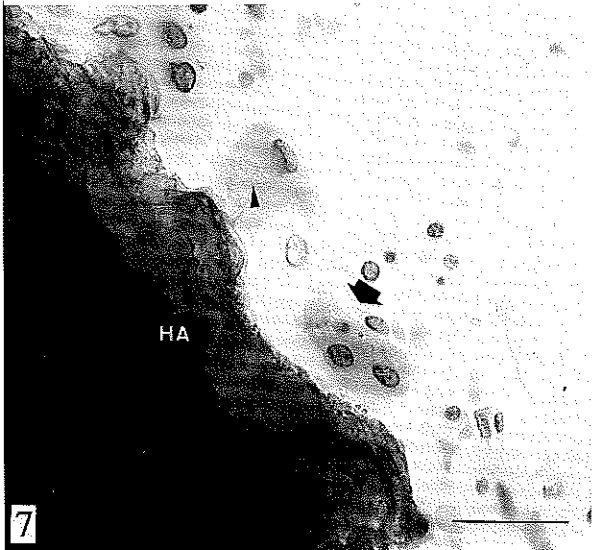


Figure 7. Light micrograph of a one year implanted HA-coating (HA) which does not show any signs of resorption. Some osteoclasts (solid arrow) are at the coating contact and contain HA particles (arrowhead). Fuscine-toluidine stained section (printed in black and white from a color micrograph). Bar = 100 μm .

Figure 8. Back-scattered scanning electron micrograph of a silver methenamine stained section of a one year implanted prosthesis showing a decreased coating thickness in the region facing a medullary cavity (HA). Some cells of the medullary tissue have phagocytosed coating debris (arrowhead). B: Bone. Bar = 150 μm .

Figure 9. Reflected light micrograph of a resorbed coating region of a one year implanted prosthesis. Bone has grown inside the resorbed coating (arrowheads). B: Bone, Ti: Titanium alloy. Bar = 150 μm .

Figure 10A. Back-scattered scanning electron micrograph of a silver methenamine stained section of a soft tissue invaded macroporous HA-ceramic (HA) showing resorption zones at the implant surface (hollow arrows). Cytoplasm of mononucleated cells is filled with calcium phosphate grains (arrowheads) identified by energy dispersive X-ray spectroscopy. Bar = 40 μm .

Figure 10B. Same area as in Figure 10A at a higher magnification. Some cells are filled with calcium phosphate grains (solid arrow). Bar = 10 μm .

Figure 11. Back-scattered scanning electron micrograph of a silver methenamine stained section of an HA-ceramic (HA) implanted into bone (B) for two months. The outside (solid arrow) region of the ceramic facing the medullary cavity is less dense suggesting a dissolution of the ceramic at the grain boundaries, although macrophages at the ceramic contact contain HA particles. Bar = 60 μm .

These micrographs showed that HA-coatings were degraded when implanted into human bones and that the degradation was heterogeneous. HA-particles could be released either by cell activity: osteoclasts acidifying the resorption chamber at the coating contact or by a dissolution process of the amorphous phase as shown *in vitro* [25]. However, the degradation rate of coatings having the same characteristics was not constant and other factors such as the cytology of the implanted tissue should be taken into account.

A coating remodelling process identical to the bone remodelling process described by Frost [26] is suggested by the bone ingrowth into coating resorbed regions. The behaviour of bone at the coating contact could be influenced by protein factors released by coating degrading cells, and by a solution mediated process involving the release of Ca^{2+} affecting cellular activity or mineral precipitation.

A similar process of grain phagocytosis was observed on pure HA crystalline ceramics implanted in bone defects in dogs invaded by loose connective tissue. Mononuclear cells were found at the contact point of the ceramic surface. Some of these cells were filled with many calcium phosphate grains located inside cell vacuoles filling the whole cytoplasm (Fig. 10). They were located in close proximity to the ceramic, suggesting that the solubilization occurs rapidly inside the vacuoles. No morphological sign of cell damage was found. Marks of cell resorption were found in the ceramic zones where the cells were present. The same HA-ceramics implanted in an osseous site in sheep for periods ranging from two months up to ten months and invaded by bone tissue seemed both solubilized and cell resorbed (Fig. 11). Solubilization took place in the surface region in contact with body fluid (in the medullary cavity), and cell resorption took place both in the regions at the bone contact point and in the regions previously partially solubilized. Solubilized regions were identified, on histological sections observed using backscattered electron microscopy as a less dense zone located on the material surface surrounding a dense core of ceramic. The decrease of density was attributed to resorption occurring at the grain boundaries. The evidence of cell resorption of these ceramics was found in some regions where the structure of the ceramic was altered at the contact of some multinucleated cells without HA-grains found in their cytoplasm. This suggested that there was a dissolution process before the grain phagocytosis. Although some evidence of cell mediated dissolution exists, it is difficult to evaluate from this study, which part of the material degradation is due to a solution-mediated process and which part is due to a cell-mediated dissolution.

These results were consistent with those published by den Hollander *et al.* [19] who studied the degradation of ^{45}Ca -labelled macroporous HA-ceramics. HA showed a small decrease in the specific activity of the implant suggesting a degradation of the material once implanted. However, the lack of radioactivity in the adjacent bone normally being an area of increased calcium metabolism, indicated the restricted availability of the liberated ^{45}Ca for neogenesis of bone tissue. The presence of particles of high specific activity was noted in lymph nodes.

Thus, even HA-ceramics which do not contain an amorphous phase are cell degraded both *in vivo* and *in vitro*. HA-macroporous ceramics used in our observations had a very low microporosity (< 2%) with very small pores and could be considered as dense material at the cell level. The molecular mechanism of HA-degradation once implanted can involve surface processes controlling the dissolution rate of HA-crystal and cell excretion of ions, enzymes and proteins modifying the crystal environment. Although the molecular mechanisms ruling the mononuclear precursors of osteoclasts are not known, they are influenced by some of the HA-ceramic characteristic and could be indirectly involved in the HA degradation process.

Conclusions

Dissolution and cell degradation of synthetic HA-ceramic occur simultaneously and it is very difficult to distinguish between them *in vivo*. We could not establish a rate of degradation of these ceramics since their degradation is a very heterogeneous phenomenon. On HA-coatings, the rate of degradation could vary on the same prosthesis depending on the location where the section was made. Also, depending on the tissue (e.g., bone or bone marrow) in contact with the ceramic coating, the thickness of the coating facing these tissues is not the same. Moreover, the resorption varies from one patient to another.

References

- [1] Alliot-Licht B, Gregoire M, Orly J, Menanteau J (1991) Cellular activity of osteoblasts in the presence of hydroxyapatite: an *in vitro* experiment. *Biomaterials* **12**: 752-756.
- [2] Alwan WH, Dieppe PA, Elson CJ, Bradfield JWB (1989) Hydroxyapatite and urate crystal induced cytokine release by macrophages. *Ann Rheum Dis* **48**: 476-482.
- [3] Anderson RGW, Orci L (1988) A view of acidic intracellular compartments. *J Cell Biol* **106**, 539-543.
- [4] Arsenault AL, Frankland BW, Ottensmeyer FP (1991) Vectorial sequence of mineralization in the turkey leg tendon determined by electron microscopic imaging. *Calcif Tissue Int* **48**: 46-55.
- [5] Athanasou NA, Quinn J (1990) Immunophenotypic differences between osteoclast and macrophage polykaryons: immunohistological distinction and implications for osteoclast ontogeny and function. *J Clin Pathol* **43**: 997-1004.
- [6] Athanasou NA, Puddle B, Quinn J, Woods CG (1991) Use of monoclonal antibodies to recognize osteoclasts in routinely processed bone biopsy specimens. *J Clin Pathol* **44**: 664-666.
- [7] Baron R, Neff L, Loward D, Courtoy PJ, (1985) Cell-mediated extracellular acidification and bone resorption: evidence for a low pH in resorbing lacunae and localization of a 100-KD lysosomal membrane protein at the osteoclast ruffled border. *J Cell Biol* **101**: 2210-2222.
- [8] Baslé MF, Chappard D, Grizon F, Filmont F, Delecric J, Daculsi G, Rebel A (1993) Osteoclastic resorption of Ca-P biomaterials implanted in rabbit bone. *Calcif Tissue Int* **53**: 348-356.
- [9] Blair HC, Kahn AJ, Crouch EC, Jeffrey JJ, Teitelbaum ST (1986) Isolated osteoclasts resorb the organic and inorganic components of bone. *J Cell Biol* **102**: 1164-1172.
- [10] Bullough PG (1991) The inflammatory arthritis. In: *Atlas of Orthopedic Pathology*. Hunter J, Rule S (eds.). Gower Medical Publishing, New York. pp. 11.14-11.17.
- [11] Burger E, Van Der Meer J, Nijweide P, (1984) Osteoclast formation from mononuclear phagocytes: role of bone forming cells. *J Cell Biol* **99**: 1901-1906.
- [12] Chambers TJ (1980) The cellular basis of bone resorption. *Clin Orthop Rel Res* **151**: 283-293.
- [13] Chambers TJ (1981) Phagocytic recognition of bone by macrophages. *Pathology* **165**: 1-7.
- [14] Chambers TJ (1989) The origin of the osteoclast. In: *Bone and Mineral Research*. Peck W (ed.). Elsevier, Amsterdam. pp. 1-25.
- [15] Chambers TJ, Thomson BM, Fuller K, (1984) Effect of substrate composition on bone resorption by rabbit osteoclasts. *J Cell Sci* **70**: 61-71.
- [16] Cheung HS, Halverson PB, McCarty DJ, (1983) Phagocytosis of hydroxyapatite crystals by rabbit articular chondrocytes stimulates release of collagenase, neutral protease and prostaglandins E2 and F2. *Proc Soc Exp Biol Med* **173**: 181-189.
- [17] Domon T, Wakita M (1991) The three-dimensional structure of the clear zone of a cultured osteoclast. *J Electron Microsc* **40**: 34-40
- [18] Dieppe PA, Doherty M, Papadimitriou GM (1982) Inflammatory responses to intradermal crystals in healthy volunteers and patients with rheumatic diseases. *Rheumatol Int* **2**: 55-58.
- [19] den Hollander W, Patka P, Klein CPAT, Heidendal GAK (1991) Macroporous calcium phosphate ceramics for bone substitution: a tracer study on biodegradation with ⁴⁵Ca tracer. *Biomaterials* **12**, 569-573.
- [20] Ducheyne P, Beight J, Cuckler J, Evans B, Radin S (1990) Effect of calcium phosphate coating characteristics on early post-operative bone tissue ingrowth. *Biomaterials*, **11**, 531-540.
- [21] Eriksen EF, Melsen F, Mosekilde L (1983) Reconstruction of the resorptive site in iliac trabecular bone: a kinetic model for bone resorption in 20 normal individuals. *Calcif Tissue Int* **35** (suppl): A8 (abstract).
- [22] Evans EJ (1991) Toxicity of hydroxyapatite *in vitro*: the effect of particle size. *Biomaterials* **12**: 574-576.
- [23] Frayssinet P, Hanker J, Hardy D, Giammara B (1991) Novel methods for demonstration osseointegration of hydroxyapatite-coated titanium hip prostheses. In: *Proceedings of the 49th Annual Meeting of Electron Microscopy Society of America*. 1991. San Francisco Press. pp. 34-35.
- [24] Frayssinet P, Hardy D, Rouquet N, Giammara B, Guilhem A, Hanker J (1992) New observations on middle term hydroxyapatite-coated titanium alloy hip prostheses. *Biomaterials* **13**: 668-674.
- [25] Frayssinet P, Tourenne F, Rouquet N, Giammara B, Bonel G (1992) Biological influence of crystallinity of hydroxyapatite coatings for orthopaedic use on cell growth. In: *Thermal Spray: International Advances in Coatings Technology Conference Proceedings*. Brendt C (ed.). ASM International, Material Park, Ohio. pp. 477-486.

- [26] Frost HM (1969) Tetracycline based histological analysis of bone remodeling. *Calcif Tissue Res* **3**: 211-237.
- [27] Glimcher MJ (1984) Recent studies of the mineral phase in bone and its possible linkage to organic matrix by protein-bound phosphate bonds. *Trans R Soc Lond B304*: 479-508.
- [28] Glowacki J, Rey C, Cox K, Lian J (1988) A role for osteocalcin in osteoclast differentiation. *J Cell Biochem* **45**: 292-302.
- [29] Gomi K, Lowenberg B, Shapiro G, Davies JE (1993) Resorption of sintered synthetic hydroxyapatite by osteoclasts in vitro. *Biomaterials* **14**: 91-95.
- [30] Haddad RJ, Cook SD, Thomas KA (1987) Biological fixation of porous-coated implants. *J Bone Joint Surg [Am]* **69A**: 1459-1466.
- [31] Hardy DCR, Frayssinet P, Guilhem A, Lafontaine MA, Delince PE (1991) Bonding of hydroxyapatite-coated femoral prostheses. *J Bone Joint Surg [Br]* **73-B**: 732-740.
- [32] Heersche JNM (1978) Mechanism of osteoclastic bone resorption: a new hypothesis. *Calcif Tissue Res* **26**: 81-84.
- [33] Hench L, Wilson J (1984) Surface active biomaterials. *Science* **226**: 630-635.
- [34] Horowitz MC, Coleman DL, Flood PM, Kupper TS, Jilka RL (1989) Parathyroid hormones and lipopolysaccharide induce murine osteoblast-like cells to secrete a cytokine indistinguishable from granulocyte-macrophage colony-stimulating factor. *J Clin Invest* **83**: 149-157.
- [35] Hyakuna K, Yamamuro T, Kotoura Y, Kakutani Y, Kitsugi T, Takagi H, Oka M, Kokubo T, (1989) The influence of calcium phosphate ceramics and glass-ceramics on cultured cells and their surrounding media. *J Biomed Mater Res* **23**, 1049-1066.
- [36] Jarcho M (1981) Calcium phosphate ceramics as hard tissue prosthetics. *Clin Orthop* **157**: 259-278.
- [37] Jee WSS (1988) The skeletal tissue. In: *Cell and Tissue Biology. A Textbook of Histology*. Weiss L (ed.). Urban & Schwarzenberg, Baltimore. pp. 211-255.
- [38] Klein CPAT, Driessen AA, De Groot K, van den Hoof A (1983) Biodegradation behavior of various calcium phosphate materials in bone tissue. *J Biomed Mater Res* **17**: 769-784.
- [39] Klein CPAT, Blicke-Hogervorst JMA, Wolke JGC, De Groot K (1990) Solubility of hydroxyapatite, tricalciumphosphate and tetracalcium phosphate coatings on titanium in vitro. In: *Clinical Implant Materials. Advances in Biomaterials*, Vol. 9. Heimke G, Soltész C, Lee AJC (eds.). Elsevier, Amsterdam. pp. 277-282.
- [40] Koerten HK, Leenders H, Verhoeven MCH, van der Meulen J (1992) Biodegradation of calcium phosphate ceramics studied in an in vivo model. In: *Proceedings of Fourth World Biomaterials Congress. European Society for Biomaterials*, Berlin, Germany. p. 527 (abstract).
- [41] Kwong CH, Burns WB, Cheung HS (1989) Solubilization of hydroxyapatite crystals by murine bone cells, macrophages and fibroblasts. *Biomaterials* **10**: 579-584.
- [42] Legros R (1984) Apport de la physico-chimie a l'étude de la phase minérale des tissus calcifiés (Application of physical chemistry to the study of the mineral phase of calcified tissues). Doctoral Thesis, Institut National Polytechnique de Toulouse, Toulouse, France.
- [43] Marie PJ (1989) Le remodelage osseux normal et sa régulation (Normal bone remodeling and its regulation). In: *Le Tissu Osseux (Bone Tissue)*. Teot L, Vidal J, Dossa J (eds.). Sauramps Medical, Montpellier, France. pp. 113-139.
- [44] Malone JD, Teitelbaum SL, Griffin GL, Senior RM, Kahn AJ (1982) Recruitment of osteoclast precursors by purified bone matrix constituents. *J Cell Biol* **92**: 227-230.
- [45] Mann S (1988) Molecular recognition in biomineralization. *Nature* **332**: 119-124.
- [46] Magham DC, Scoones DJ, Drayson MT, (1993) Complement and the recruitment of mononuclear osteoclasts. *J Clin Pathol* **46**: 517-521.
- [47] Maxian SH, Zawadsky JP, Dunn MG (1993) In vitro evaluation of amorphous calcium and poorly crystallized hydroxyapatite coatings on titanium implants. *J Biomed Mater Res* **27**: 111-117.
- [48] Mellman I, Fuchs R, Helenius A (1986) Acidification of the endocytic and exocytic pathways. *Annu Rev Biochem* **55**: 663-700.
- [49] Metcalfe JC, Moore JP, Smith GA, Hesketh TR (1986) Calcium and cell proliferation. *Brit Med Bull* **42**: 405-412.
- [50] Mitchell PG, Pledger WJ, Cheung HS (1989) Molecular mechanism of basic calcium phosphate crystal-induced mitogenesis. Role of protein kinase C. *J Biol Chem* **264**: 14071-14077.
- [51] Müller-Mai CM, Voigt C, Gross U (1990) Incorporation and degradation of hydroxyapatite implants of different surface roughness and surface structure in bone. *Scanning Microsc* **4**: 613-624.
- [52] Mundy GR, Yoneda T (1981) Monocytes: interactions with bone cells and lymphocytes during bone resorption. In: *Hormonal Control of Calcium Metabolism*. Cohn DV, Talmage RV, Matthews JL (eds.). Excerpta Medica, Amsterdam. p. 178 (abstract).
- [53] Orly I, Gregoire M, Menanteau J, Dard M (1989) Effects of synthetic calcium phosphates on the ³H-thymidine incorporation and alkaline phosphatase activity of human fibroblasts in culture. *J Biomed Mater Res* **23**: 1433-1440.
- [54] Osdoby P, Oursler MJ, Salino-Hugg T, Krukowski M (1988) Osteoclast development: the cell surface and the bone environment. In: *Cell and Molecular Biology of Vertebrate Hard Tissues (Ciba Foundation Symposium 136)*. Wiley, Chichester, UK. pp. 108-124.
- [55] Parfitt AM (1984) The cellular basis of bone remodeling: the quantum concept reexamined in light of recent advances in the cell biology of bone. *Calcif Tissue Int* **36**: S37-S45.
- [56] Peck WA, Rifas L, Shen V (1985) Macro-

phages release a peptide stimulator of osteoblast growth. *Ann Biol Clin* **43**: 751-754.

[57] Poole B, Ohkuma S (1981) Effect of weak bases on the intralysosomal pH in mouse peritoneal macrophages. *J Cell Biol* **96**: 851-856.

[58] Posner AS (1987) Bone mineral and the mineralization process. In: *Bone and Mineral Research* 5. Peck WA (ed.). Excerpta Medica, Amsterdam. pp. 65-116.

[59] Praeger FC, Gilchrist BA (1989) Calcium, lanthanum, pyrophosphate, and hydroxyapatite: a comparative study in fibroblast mitogenicity (42826). *Proc Soc Exp Biol Med* **190**: 28-34.

[60] Schumacher HR, Somlyo AP, Tse RL, Maurer K (1977) Arthritis associated with apatite crystals. *Ann Intern Med* **87**: 411-416.

[61] Wolke JGC, de Blicq-Hogervorst JMA, Dhert WJA, Klein CPAT, de Groot K (1992) Studies on the thermal spraying of apatite bioceramics. *J Thermal Spray Technol* **1**: 75-82.

Editor's Note: All of the reviewer's concerns were appropriately addressed by text changes, hence there is no **Discussion with Reviewers**.