Cells and Materials

Volume 1 | Number 3 Article 3

1991

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J. M. Sautier
Institut Biomedical des Cordeliers, Paris

J. R. Nefussi
Universite Paris VII, Paris

N. Forest
Universite Paris VII, Paris

CELLS AND MATERIALS, Vol. 1, No. 3, 1991 (pages 209-217) 1051-6794/91$3.00+.00
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

ULTRASTRUCTURAL STUDY OF BONE FORMATION ON SYNTHETIC HYDROXYAPATITE IN OSTEOBLAST CULTURES

J.-M. Sautier*, J.-R. Nefussi, N. Forest

Laboratoire de Biologie-Odontologie,
Université Paris VII, Paris, France

(Received for publication May 5, 1991, and in revised form August 14, 1991)

Abstract

Collagenase isolated rat calvaria cells forming a mineralized matrix *in vitro* were cultured in the presence of synthetic hydroxyapatite. Interactions between bone cells and hydroxyapatite biomaterial were followed by transmission electron microscopy. The appearance of a granular, collagen free, electron-dense layer at the periphery of the material was noted initially. Progressively, an amorphous, granular material formed and extended between the hydroxyapatite aggregates. Osteoblastic cells then synthesized an osteoid matrix which mineralized on the first formed granular collagen free layer, following a classical pattern of calcification. Demineralization of ultrathin sections confirmed the presence of this interface between the material and bone tissue formed *in vitro*.

Key Words: *in vitro*, hydroxyapatite, osteoblast, bone tissue, interface, ultrastructure.

***Address for Correspondence:**
Dr. Jean-Michel Sautier
Laboratoire de Biologie-Odontologie
Institut Biomédical des Cordeliers
Escalier E, 2ème étage
15 rue de l'Ecole de Médecine
F-75270 Paris Cedex 06 France
Telephone: 33 (1) 4326 9496
FAX: 33 (1) 4407 1421

Introduction

Calcium phosphate ceramics (Ca-P) were used as surgical implant biomaterials for a wide range of applications. Ca-P ceramics are available in two major forms: tricalcium phosphate (TCP) and hydroxyapatite (HA). They differ in their composition and in their rate of resorption. HA have been used as filler of osseous defects after resection of bone tumor or for the filling of periodontal intrabony defects. Experiments involving animals with artificial bone defects have demonstrated HA to be biocompatible and more or less resorbable (Levin *et al.*, 1974; Nery *et al.*, 1975). Human studies demonstrated a similar non-foreign body response and direct bone apposition was observed with bone partially or totally embedded the HA particles (Jarcho *et al.*, 1977; Kenney *et al.*, 1985; Ogilvie *et al.*, 1987; Frank *et al.*, 1987). More recently, coating of HA on orthopedic or dental implants has been intended to allow a bioactive ceramic-bone interface (Klein *et al.*, 1991). Among the large number of biomaterials used for bone replacement, a distinction can be made between bioactive and bioinert materials. The most attractive feature of Ca-P ceramics and bioglasses is their capacity to achieve a microscopically direct bond with bone, whereas only a bone contact was observed on bioinert materials like steels, titanium or aluminum (Hench, 1972; Jarcho, 1981; De Groot, 1983; Ducheyne, 1987). However, several groups reported an afibrillar mineralized layer at the interface between bone tissue and the metal oxide surface suggesting titanium was not absolutely non-bioactive (Albrektsson *et al.*, 1983; Lowenberg *et al.*, 1991). Effectively, the anchoring of so-called bioactive ceramics to bone was assigned to a bonding zone ultrastructurally characterized as a granular electron-dense layer (Osborn *et al.*, 1980; Van Blitterswijk *et al.*, 1986; Ganeles *et al.*, 1986). However, this structure was only observed on decalcified sections and little is known on the formation of this bonding zone. To follow interactions occurring at the HA/adjacent cells interface, the use of cell culture was of great interest. In the culture model currently used in our laboratory, bone nodular structures developed *in vitro* closely resembled

nodular structures developed *in vitro* closely resembled bone formed *in vivo* (Nefussi *et al.*, 1985, 1989a, b; Collin *et al.*, 1991). Furthermore, using this culture model, we have shown that bone formation occurred in less than two weeks on coral granules biomaterial (Sautier *et al.*, 1990). The purpose of this study was to use this culture model in the presence of synthetic HA particles and to further analyze at the electron microscopic level bone formation on the material.

This investigation showed: **a**) the formation of a granular, electron-dense, collagen free layer at the periphery of the material; **b**) an amorphous, granular material extending between the HA crystals to the central part of the HA mass; **c**) a matrix synthesis and a mineralization process occurring on this first formed granular, collagen free layer.

Materials and Methods

Synthetic Hydroxyapatite

The material used in this study was a dense synthetic HA (Bioapatite®, Pred, Levallois-Perret, France) prepared according to the double decomposition technique developed by Trombe (1972). Briefly, a solution of diamonium phosphate was mixed to aqueous solution of calcium nitrate and boiled for 15 minutes. The precipitate was then filtered out, dried for 12 hours at 700°C and calcinated in air for 3 hours at 900°C. The HA blocks were powdered in a mortar and 100 to 400 μm particles were selected by sifting. Irregularly shaped, sharp-edged particles (5 mg) were placed in 50 mm diameter culture dishes (Falcon, Oxnard, CA, USA).

Cell isolation and culture procedure

Cells were enzymatically isolated from calvaria of 21 day-old fetal Sprague-Dawley rats, as previously described by Nefussi *et al.* (1985). The parietal and frontal bones of calvaria were dissected aseptically, taking care to remove all the suture area. Bone fragments with periosteum and endosteum were incubated for 2 hours at 37°C in phosphate buffered solution (PBS), with collagenase (180 U/ml) (Sigma type 1). After this period, cells were dissociated by pipetting bone fragments several times, washed three times in PBS with 10% fetal calf serum (FCS), counted, seeded (2 x 10^4 cells/cm^2) and plated on 50 mm culture dishes. The culture medium used was Dulbecço Modified Eagle Medium (DMEM) (Gibco) supplemented with 10 mM β-glycerophosphate (Sigma), 50 μg/ml of ascorbic acid (Sigma), 10% fetal calf serum (FCS) (Boehringer) and 50 U/ml streptomycin (Gibco). Cell culture dishes containing HA particles (1 mg/ml) were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C. The medium was changed every 48 hours and observations of cell cultures were performed using an inverted phase-contrast microscope (Leitz Fluovert). Experiments were performed in triplicate and cultures were stopped after 15 days.

Transmission Electron Microscopy

Cell cultures were fixed *in situ* in Karnovsky solution (4% paraformaldehyde, 1% glutaraldehyde) for 1 hour and 4 bone nodules in contact with HA particle were selected from each culture dish. After several rinses in 0.2 M sodium cacodylate buffer (pH 7.4), cell cultures were postfixed for 1 hour in 1% osmium tetroxide diluted in 0.2 M sodium cacodylate buffer. The cells were then dehydrated in graded series of ethanol and left overnight in a mixture of absolute ethanol and epon (1:1). The next day, the cells were embedded in Epon-Araldite and incubated at 60°C for 1 day. Semithin sections were cut perpendicularly to the cell layers with a diamond knife and mounted on glass slides, stained with toluidine blue (Merck) and examined under light microscopy for orientation purposes. Ultrathin sections were performed, collected on copper grids and stained with 2.5% uranyl acetate in absolute ethanol for 4 minutes and lead citrate for 2 minutes. In addition, a number of ultrathin sections were decalcified in acetic acid (3%) for 3 minutes before staining. Some culture dishes with HA particles were incubated for the same period in cell-free culture medium and prepared for transmission electron microscopy. All sections were examined using a Philips EM-400 transmission electron microscope.

Results

During the culture period, enzymatically isolated rat calvaria cells differentiated and formed in ten days nodular structures (Fig. 1). By day 15 of culture, bone nodules at different stages of maturation were visible in contact with the biomaterial and selected for electron microscopy examinations.

Ultrastructural observations of areas surrounding HA granules but without bone nodule yet formed, showed the presence of the material which appeared as a mass composed of a loose grouping of individual synthetic crystals separated by large electron-lucent spaces corresponding to artifacts introduced during sections through the dense undecalcified material (Fig. 2a). Typical osteoblast were visible at the periphery of the HA aggregates. Furthermore, the presence of numerous cytoplasmic organelles outlined the engagement of the cells in biosynthesis proteins. A collagen matrix had developed between the cells, but the portion between the plasmic membrane of the cells and the biomaterial was devoid of detectable matrix. In addition, some of these cells exhibited coated-pits against the HA mass. At the periphery of the HA aggregates, an electron-dense band of variable thickness was seen between the material and

the adjacent cells. Observed at high magnification, the resulting interface was clearly visible immediately adjacent to the material and appeared as a granular, amorphous electron-dense band (Fig. 2b). In addition, no collagen fibers were visible in this structure and on the external portion dispersed protrusions formed of needle-shaped filaments were continuous to this collagen-free granular layer. On other sections, the amorphous material extended to the center of the HA mass and progressively filled the spaces between the synthetic crystals but without collagen intervening (Fig. 2c). However, when HA particles were incubated under identical conditions, but in cell-free culture medium, such granular electron-dense did not develop (not illustrated).

On ultrathin sections of nodules newly formed on the HA crystals, bone formation was evident, following a classical pattern of calcification (Fig. 3a). Typical osteoblast containing a large nucleus, an abundant rough endoplasmic reticulum and numerous mitochondria were found among an extensive extracellular matrix composed of numerous collagen fibrils densely packed. These fibers were visible either on transversal sections or longitudinally showing characteristic cross-banding, thus confirming their collagen nature. Further inside the collagenous matrix, a mineralized front formed a boundary between uncalcified osteoid matrix and mineralized bone matrix which was in contact with the material. All the intercrystal spaces were filled by an electron-dense material, and the cracks observed were artifacts introduced during sectioning this undemineralized tissue. On other sections, the mineralized front showed round mineralized foci containing needle-like crystallites which scattered among collagen fibrils (Fig. 3b). Matrix vesicles generally associated with mineralization process in this culture model when cells were cultured without material, were not observed. Typical osteocytes enclosed in their lacuna were visible inside the mineralized matrix. High magnification revealed bone mineral apposition directly on the material without interposition of soft tissue (Fig. 3c). However, on such undecalcified sections, the interface between the bone mineralized matrix and the HA was not clearly visible. Incubation of ultrathin sections for 3 minutes in acetic acid led to a demineralization of the calcified bone matrix but not of the synthetic material (Fig. 4a). Observation of the decalcified mineralized bone matrix revealed the presence of a thin electron-dense layer at the bone/HA interface. High magnification of this interface, clearly confirmed the presence of an electron-dense band, granular in appearance and collagen free. Additionally, in decalcified mineralized bone matrix, collagen fibrils showing typical cross-striations could be observed inside this tissue, but no collagen fibers were ever noted in the granular material formed between the HA aggregates (Fig. 4b).

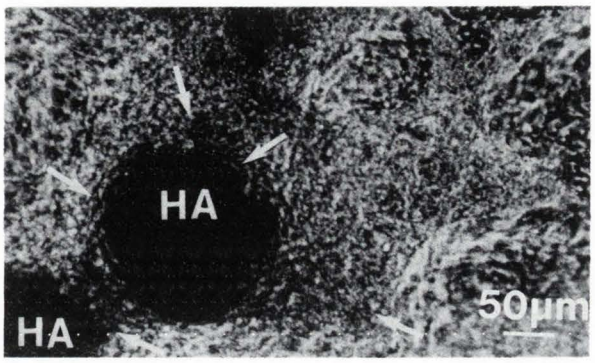

Figure 1 (above). Phase contrast microscope observation of 10 day-old cultures showing a bone nodule (arrows) in contact with 2 hydroxyapatite particles (HA).

Discussion

The present results showed that calvaria cells from 21 day-old rat fetuses produced a mineralized tissue when cultured in the presence of HA particles. TEM observations of the interface HA/adjacent cells revealed first, the formation of a granular electron-dense collagen free layer appearing before the laying down of a collagenous matrix and surrounding the HA mass. Secondly, this granular amorphous material extended to the center of the mass and filled the intercrystalline spaces. Thirdly, a collagen matrix was formed by osteoblast and mineralization occurred on the first formed collagen free structure following a more classical pattern of calcification.

In the present study, we described, for the first time, the step-by-step interactions occurring between a synthetic HA and bone cells, in an *in vitro* mineralization matrix forming system. The first formation observed between the material and the neighboring cells, was the appearance of a granular, collagen free, electron-dense layer. Such interface has been reported by several authors following implantation of HA in osseous sites and regarded as a bonding zone between the bioactive material and bone (Ganeles *et al.*, 1986; Van Blitterswijk *et al.*, 1985, 1986). Similar deposit have been reported culturing rat bone marrow-derived cells on different cell culture substrata (Davies *et al.*, 1991) or on solid titanium (Lowenberg *et al.*, 1991). Likewise, an afibrillar cement-like substance has been reported by Melcher *et al.* (1986) deposited on dentine substrata co-cultured with fetal rat calvaria-derived cells.

To explain the formation and the mineralization of this structure, we speculate that leached material from the surface of the HA could increase the local concentrations of calcium and phosphate ions, causing mineral precipitation. Such degradation of ceramics after

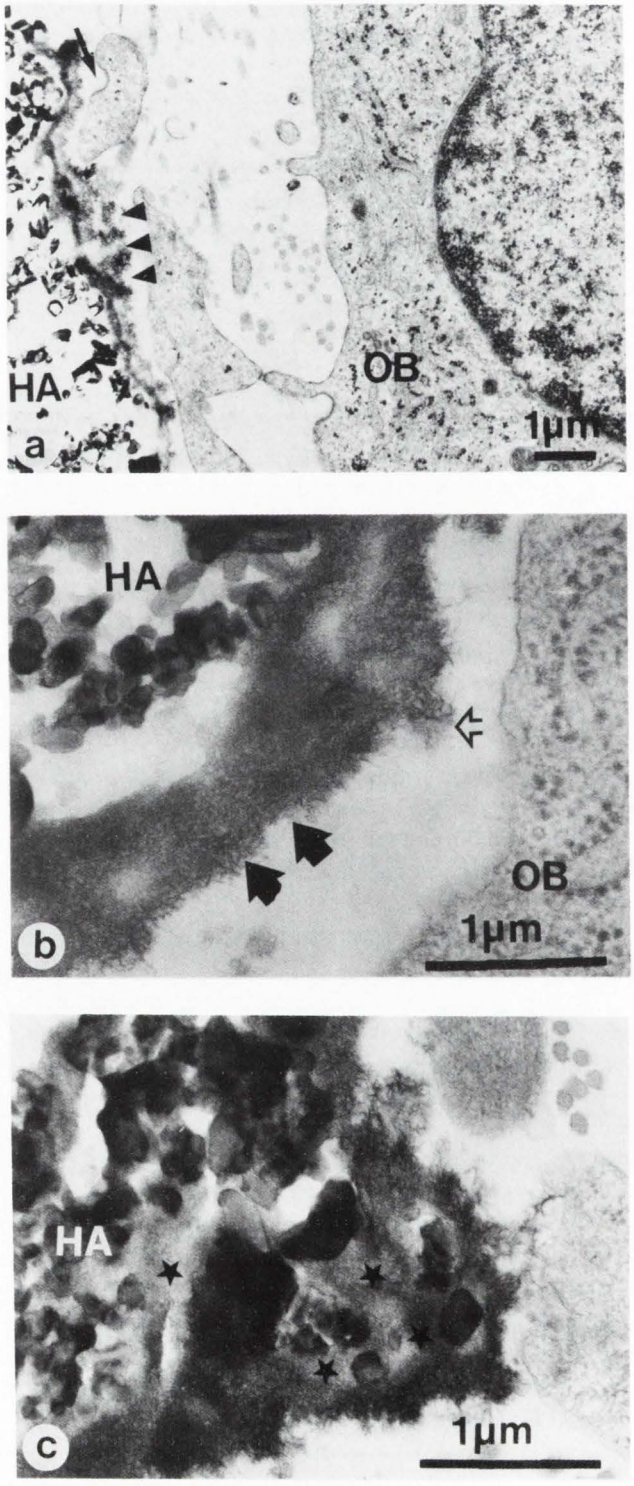

Figure 2 (at left). (**a**) Electron micrograph showing the loose grouping of the synthetic hydroxyapatite crystals (HA). Between the material and the osteoblasts (OB) a thin electron-dense layer is visible (arrow heads). Note the presence of a coated-pit (arrow) on the plasmic membrane of a cell process at the periphery of the material. (**b**) High magnification of the electron-dense band (arrows) formed of a granular material at the periphery of the hydroxyapatite mass (HA). Note at the external portion of this structure dispersed protrusions composed of needle-shaped crystals (open arrow). (**c**) Progressively an amorphous granular material (★) extend to the center of the hydroxyapatite mass (HA).

Figure 3 (at right). Electron micrographs showing a cross-section of bone nodule formed *in vitro* on synthetic hydroxyapatite. (**a**) An osteoblast (OB) forming an osteoid matrix (OS) composed of numerous collagen fibrils (arrows). A mineralized front (wide arrow) forms the boundary between the uncalcified matrix and the bone mineralized matrix (MM) which is directly apposited on the hydroxyapatite (HA). (**b**) The collagenous matrix contains round calcified foci (black open arrow). Note the presence of an osteocyte (white arrow) embedded in the mineralized matrix (MM) which is directly apposited (white open arrows) on the hydroxyapatite (HA). (**c**) High magnification of the interface between mineralized bone matrix (MM) and hydroxyapatite (HA). The spaces between the synthetic hydroxyapatite crystals (HA) are filled by an amorphous granular material.

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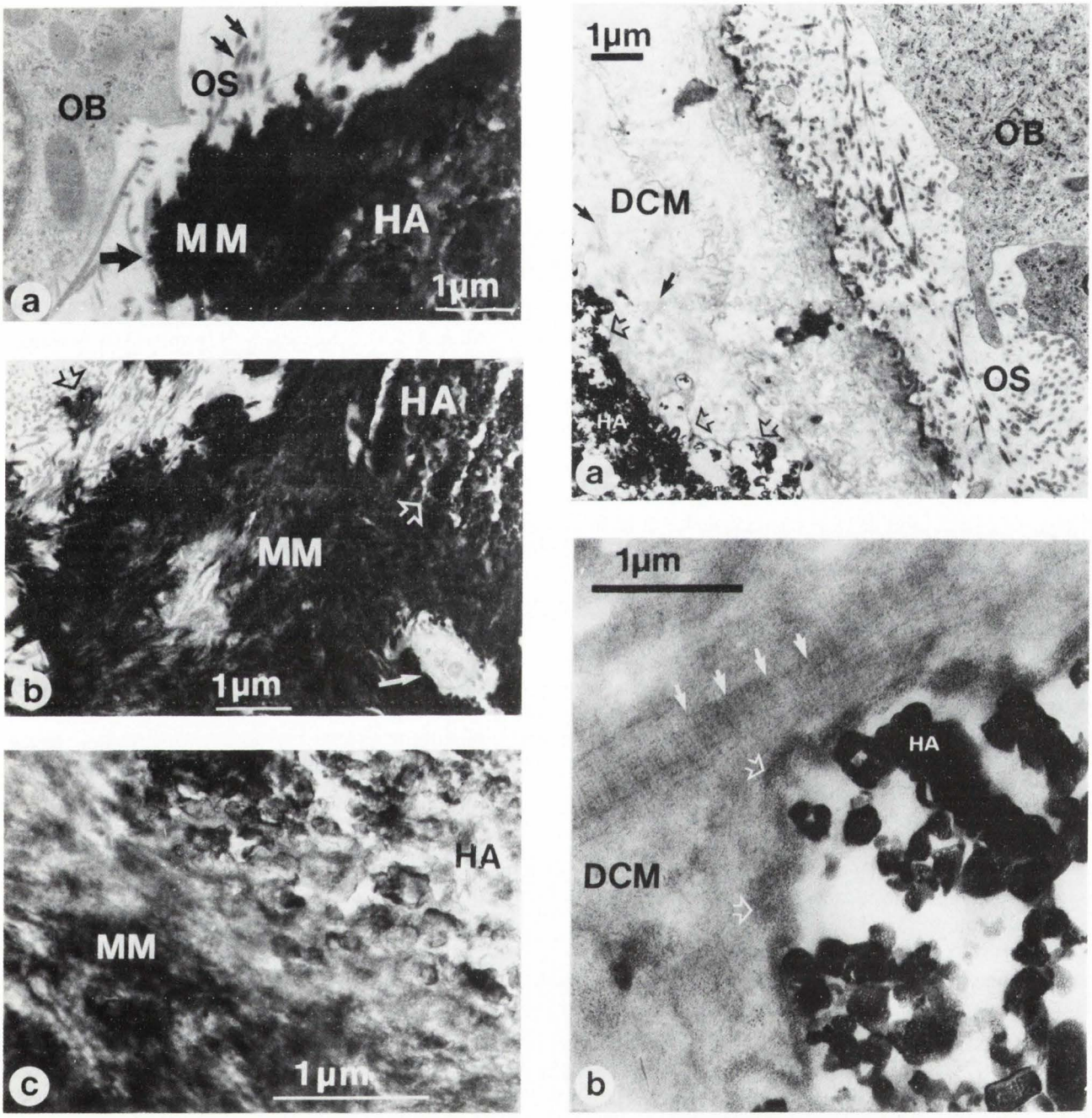
implantation has been reported using light (Tracy and Doremus, 1984) or transmission electron microscopy (Daculsi *et al.*, 1990). In addition, Patka *et al.* (1989), demonstrated *in vivo* a dissolution of Ca-P, using radiolabelled ^{45}Ca. This phenomena was also observed *in vitro* with apatite-wolastonite glass ceramics (Kokubo *et al.*, 1990). Furthermore, using high resolution transmission electron microscopy, it was demonstrated that dissolution started from the crystal defects probably introduced during the sintering process (Daculsi *et al.*, 1989). Such biological degradation was observed during dental caries where dissolution of enamel apatite occurred in screw dislocating sites (Voegel and Frank, 1974, 1977; Tohda *et al.*, 1987; Jongebloed *et al.*, 1975). In addition, after implantation of HA in the rat middle ear Van Blitterswijk (1985) demonstrated by X-ray microanalysis the deposition of calcium phosphate at the bone HA/interface.

However, when HA particles were incubated in the absence of cells for the same period these deposits did not develop. Thus, a dissolution of the material was not sufficient to explain the formation of this interface which arose from a biological cell-mediate effect. This is in line with observations reported by Davies *et al.* (1991) and Lowenberg *et al.* (1991), who did not find a granular material deposit on either solid titanium, polystyrene or fluorocarbon substrata when cell-free culture medium was used. However, Beertsen and Van den Bos (1991)



have recently found a cementum-like structure deposited on dentin slides when incubated in a cell-free culture medium, suggesting that this deposit could result from co-precipitation of mineral with medium-derived components.

In the second phase, the granular material extended between the synthetic crystals to the central part of the mass. This finding agrees with the early work of Ganeles *et al.* (1986) which observed but on decalcified sections after implantation of Durapatite® (dense HA) similar granular amorphous material extending in the intercrystalline spaces and devoid of collagen matrix.

Figure 4. Ultrathin sections decalcified in 3% acetic acid for 3 minutes. (**a**) Lining the plasmic membrane of an osteoblast (OB), the osteoid matrix (OS) with numerous collagen fibers. The demineralized calcified matrix (DCM) containing collagen fibrils (arrows) is in contact with the hydroxyapatite mass (HA). Note at the bone hydroxyapatite interface, a thin electron-dense layer (open arrows). (**b**) High magnification of the interface between the demineralized calcified matrix (DCM) and synthetic hydroxyapatite crystals (HA) which appears as a granular, collagen free, electron-dense layer (open arrows). In contrast, collagen fibers are visible in the DCM (arrows).

In a more advanced step, a mineralization process seemed to occur on this electron-dense, collagen free layer as needle-shaped crystallites and before any collagen matrix was observed. In addition, matrix vesicles generally associated with mineralization process in this culture model were not observed. This result suggested that the small crystals first nucleated on the granular electron-dense layer. This finding was in agreement with Ogilvie *et al.* (1987) which reported after implantation of Bioapatite® in intrabony lesions small needle-like crystals initially in contact with the surface of the synthetic crystals. It was possible that non collagenous proteins adsorbed on the granular collagen free layer could initiate mineralization. It is well known that intimate interactions occurred between proteins and biological apatites (Menanteau *et al.*, 1987; Bonucci, 1987; Aoba *et al.*, 1987) but probably with synthetic ceramics as outlined by Klein *et al.* (1983). Among these molecules, proteoglycans and lipids have been suggested to play an important role in mineralization process of bone (Boskey, 1989 ; Nefussi *et al.*, 1989a, 1991; Boyan-Salyers *et al.*, 1989) and dentin (Chardin *et al.*, 1990). Furthermore, if proteoglycans in solution inhibited mineral nucleation, when immobilized on a stable surface, they can induce mineralization *in vitro* (Linde *et al.*, 1989). Proteoglycans adsorbed on the HA surface, could provide nucleation sites leading to the formation of a mineralized collagen free layer, since a positive reaction using alcian blue staining at the interface between bone and Durapatite® was shown (Jarcho *et al.*, 1978).

Finally, in a third step a collagen matrix was synthesized by osteoblastic cells and mineralized following a classical pattern. In addition, relationship of cell to matrix and matrix to mineral emphasized the true osseous structure of the bone tissue formed *in vitro*. Demineralization of sections confirmed the presence of the granular electron-dense layer at the bone/HA interface and no collagen fibers were visible between the synthetic HA crystals. However, using Bioapatite®, Ogilvie *et al.* (1987) reported after six month of implantation the presence of collagen fibers between the synthetic HA aggregates on decalcified sections. This inconsistent result might be due to different experimental conditions related to the absence of a wound reaction in culture while this will be quite prominent *in vivo*.

It might be concluded from this investigation that bone cell culture system provided a useful method to investigate the surface bioreactivity of materials. Furthermore, this *in vitro* system allowed to study interactions occurring at the interface between bone cells and materials.

Acknowledgements

The authors thank M. Oboeuf for her expert technical assistance, K. Marlin and E. Marie-Rose for secretarial assistance. This work was supported by the University of Paris VII and the Dental Association of Garanciére. This work is a part of Doctorat d'Université submitted by J.M. Sautier to the University of Paris VII.

References

Albrektsson T, Branemark PI, Hanson HA, Kasemo B, Larsson K, Lundstrom I, McQueen DH, Salak R (1983). The interface zone of inorganic implants *in vivo*: titanium implants in bone. Annals. Biomed. Eng. **11**, 1-27.

Aoba T, Fukae M, Tanabe T, Shimizu M, Moreno EC (1987). Selective adsorption of porcine amelogenins onto hydroxyapatite and their inhibitory activity on hydroxyapatite growth in supersaturated solution. Calcif. Tissue Int. **41**, 281-289.

Beertsen W, Van Den Bos T (1991). Alkaline phosphatase induces the deposition of calcified layers in relation to dentin: an *in vitro* study to mimic the formation of afibrillar acellular cementum. J. Dent. Res. **70**, 176-181.

Bonucci E (1987). Is there a calcification factor common to all calcifying matrices? Scanning Microsc. **1**, 1089-1102.

Boskey AL (1989). Non collagenous matrix proteins and their role in mineralization. Bone and Mineral **6**, 111-123.

Boyan-Salyers BD, Schwartz Z, Swain LD, Khare A (1989). Role of lipids in calcification of cartilage. Anat. Rec. **224**, 211-219.

Chardin H, Septier D, Goldberg M (1990). Visualization of glycosaminoglycans in rat incisor predentin and dentin using cetylpyridinium chloride-glutaraldehyde as fixative. J. Histochem. Cytochem. **38**, 885-894.

Collin P, Nefussi JR, Wetterwald A, Nicolas V, Boy-Lefevre ML, Fleish H, Forest N (1991). Expression of collagen, osteocalcin and bone alkaline phosphatase in a mineralized rat osteoblastic cell culture. Calcif. Tissue Int. **49**, in press.

Daculsi G, Le Geros RZ, Mitre D (1989). Crystal dissolution of biological and ceramic apatites. Calcif. Tissue Int. **45**, 95-103.

Daculsi G, Le Geros RZ, Deudon C (1990). Scanning and transmission electron microscopy and electron probe analysis of the interface between implants and host bone. Scanning Microsc. **4**, 309-314.

Davies JE, Chernecky R, Lowenberg B, Shiga A (1991). Deposition and resorption of calcified matrix *in vitro* by rat marrow cells. Cells and Materials **1**, 3-15.

De Groot K (1983). Bioceramics of calcium phosphate: preparation and properties. In: Bioceramics of Calcium Phosphate. CRC Press, Boca Raton, Florida. p. 99-114.

Ducheyne P (1987). Bioceramics: Material characteristic versus *in vivo* behavior. J. Biomed. Mater. Res. **21**, 219-236.

Frank RM, Gineste M, Benqué EP, Hemmerle J, Duffort JF, Heughebaert M (1987). Etude ultrastructurale de l'induction osseuse apres implantation de bioapatite chez l'homme (Ultrastructural study of bone induction after bioapatite implantation in humans). J. Biol. Bucc. **15**, 125-134.

Ganeles J, Listgarten MA, Evian C (1986). Ultrastructure of durapatite-periodontal tissue interface in human intrabony defects. J. Periodont. **57**, 133-140.

Hench LL, Splinter LL, Allen WC, Greenlee TV (1972). Bonding mechanisms at the interface of ceramic prosthetic materials. J. Biomed. Mater. Res. **2**, 117-141.

Jarcho M, Kay J.F, Gumaer Kl, Doremus RH, Drobeck HP (1977). Tissue, cellular and subcellular events at the bone-ceramics hydroxyapatite interface. J. Bioengin. **1**, 79-92.

Jarcho M, Jasty MV, Gumaert KI, Tabata T (1978). Electron microscopic study of bone-hydroxyapatite implant interface. Transactions 4th Annual Meeting, Society for Biomaterials, Algonquin, IL. p. 112.

Jarcho (1981). Calcium phosphate ceramics as hard tissue prosthetics. Clin. Orthop. **157**, 259-278.

Jongebloed WL, Molenaar S, Tanaka N (1975). Morphology and size distribution on sound and acid-treated enamel crystallites. Calcif. Tissue Int. **19**, 109-123.

Kenney EB, Lekovic V, Han T, Carranza FA Jr, Dimitrijevic B (1985). The use of porous hydroxyapatite implant in periodontal defects. Clinical results after six months. J. Periodontol. **56**, 82-86.

Klein CPAT, Driessen AA, De Groot K, Van den Hooff A (1983). Biodegradation behaviour of various calcium phosphate materials in bone tissue. J. Biomed. Mater. Res. **17**, 769-784.

Klein CPAT, Patka P, Van Der Lubbe HBM, Wolke JGC, De Groot K (1991). Plasma-sprayed coating of tetracalciumphosphate, hydroxyl-apatite, and α-TCP on titanium alloy: An interface study. J. Biomed. Mater. Res. **25**, 53-65.

Kokubo T, Kushitani H, Sakka S, Kitsugi T, Yamamuro T (1990). Solutions able to reproduce *in vivo* surface structure changes of bioactive glass ceramic A.W.J. Biomed. Mater. Res. **24**, 721-734.

Levin MP, Getter L, Cutright DE, Bhaskar SN (1974). Biodegradable ceramic in periodontal defects. Oral Surg. **38**, 344-350.

Linde A, Lussi A, Crenshaw MA (1989). Mineral induction by immobilized polyanionic proteins. Calcif Tissue Int. **44**, 286-295.

Lowenberg B, Chernecky R, Shiga A, Davies JE (1991). Mineralized matrix production by osteoblasts on solid titanium *in vitro*. Cells and Materials **1**, 177-187.

Melcher AH, Cheong T, Cox J, Nemeth E and Shiga A (1986). Synthesis of cementum-like tissue *in vitro* by cells cultured from bone: a light and electron microscope study. J. Periodont. Res. **21**, 592-612.

Menanteau J, Gregoire M, Daculsi G, Jans I (1987). *In vitro* albumin binding on apatite crystals from developing enamel. Bone Mineral. **3**, 137-141.

Nefussi, JR, Boy-Lefevre ML, Boulekbache H, Forest N (1985). Mineralization *in vitro* of matrix formed by osteoblasts isolated by collagenase digestion. Differentiation **29**, 160-168.

Nefussi JR, Septier D, Collin P, Goldberg M, Forest N (1989a). A comparative ultrahistochemical study of glycosaminoglycans with cuprolinic blue in bone formed *in vivo* and *in vitro*. Calcif. Tissue Int. **44**, 11-19.

Nefussi JR, Pouchelet M, Collin P, Sautier JM, Develay G, Forest N (1989b). Microcinematographic and autoradiographic kinetic studies of bone cell differentiation *in vitro*: matrix formation and mineralization. Bone **10**, 345-352.

Nefussi JR, Septier D, Sautier JM, Forest N, Goldberg M (1991). Localization of Malachite green positive lipids in the matrix of bone formed *in vitro*. Calcif Tissue Int. **50**, (In Press).

Nery EB, Lynch KL, Hirthe WM, Mueller KH (1975). Bioceramic implants in surgically produced intrabony defects. J. Periodontol. **46**, 328-334.

Ogilvie A, Frank RM, Benque EP, Gineste M, Heughebaert M, Emmerle I (1987). The biocompatibility of hydroxyapatite implanted in the human periodontium. J. Periodont. Res. **22**, 270-283.

Osborn JF, Newesely H (1980). The material science of calcium phosphate ceramic. Biomaterials **1**, 108-111.

Patka P, Den Hollander W, Klein CPAT, De Groot K (1989). Behavior of ceramics, hydroxyapatite and tricalcium phosphate in bone. Transactions 15th Annual Meeting, Society for Biomaterials, Algonquin, IL. p. 103.

Sautier JM, Nefussi JR, Boulekbache H, Forest N (1990). *In vitro* bone formation on coral granules. *In Vitro* Cell & Dev. Biol. **26**, 1079-1085.

Tohda H, Takuma S, Tanaka N (1987). Intercrystalline structure of enamel crystals affected by caries. J. Dent. Res. **66**, 1647-1653.

Tracy BM, Doremus RH (1984). Direct electron microscopy studies of the bone hydroxyapatite interface. J. Biomed. Mater. Res. **18**, 719-726.

Trombe JC (1972). Contribution à l'etude de la décomposition et de la réactivité de certaines apatites hydroxylées ou fluorées alcalino-terreuses (Contribution to the study of decomposition and reactivity of certain hydroxyapatites or fluorinated alkaline-earths). Ph.D. Thesis, University of Toulouse, France.

Van Blitterswijk CA, Grote JJ, Kuijpers W, Blok van Hoek CJG, Daems WT (1985). Bioreactions at the tissue/hydroxyapatite interface. Biomaterials **6**, 243-251.

Van Blitterswijk CA, Grote JJ, Kruijpers W, Daems WT, de Groot K (1986). Macropore tissue ingrowth: a quantitative and qualitative study on hydroxyapatite ceramic. Biomaterials. 7, 137-143.

Voegel JC, Frank RM (1974). Microscopie électronique de haute résolution du cristal d'apatite d'email humain et de sa dissolution carieuse (High resolution electron microscopy of human enamel apatite crystal and its dissolution by caries). J. Biol. Bucc. **2**, 39-50.

Voegel JC, Frank RM (1977). Stages in the dissolution of human enamel crystals in dental caries. Calcif. Tissue Int. **24**, 19-27.

Discussion with Reviewers

C.A. van Blitterswijk: What reason do you have to assume that specific proteins in the calcified layers at the interface would indeed play a vital role in bone/biomaterial interactions? Could it not be that since these proteins are routinely produced during bone metabolism they will automatically be trapped during calcification at the biomaterial surface, but do not have any specific function at the interface?

Authors: To answer your question, we have no evidence for a vital role played by specific proteins at the bone/biomaterial interface. However, favorable interactions qualities of bioactive materials and tight bone bonding seems to be attributed to this electron-dense band. Therefore, we speculate on the formation of this granular collagen-free layer and the possible involvement of non collagenous proteins which could be adsorb on the material surface and so initiate a mineralization process without collagen intervening. We are currently undertaking experiments to localize bone specific proteins in this structure and we hope the results will be presented at the 1992 Biomaterial Session. However the key question is, does this afibrillar mineralized layer represent a normal synthetic activity of osteoblasts or a pathological-like calcifying activity? This is an intriguing question that cannot be answered at present, but could represent a wide field in research of bone cells/materials interactions.

C.A. van Blitterswijk: In relation to the first question. Do you think that adherence and incorporation of so-called "bone-proteins" at the interface is essential for the bone-bonding process, or would the mere leaching of calcium and phosphate from the implant surface be sufficient?

Authors: The surface reactivity of bioglasses or calcium-phosphate ceramics and the leaching of ions into the surrounding intercellular matrix could explain the formation of an electron-dense mineralized deposit at the surface of the so-called bioactive materials. However, such process cannot explain the formation of a similar structure at the bone/copolymer interface due to the absence of calcium and phosphorus as reported by your group (Bakker *et al*, J. Biomed. Mater. Res. **24**, 277-293, 1990). We have also observed a similar interface between a dextran polymer and bone formed *in vitro* (Calcif. Tissue. Int, accepted for publication). It will be interesting to know the composition of morphological similar interfaces between bone and different biomaterials, but in relation to the first question it is reasonable to involve a possible adsorption of anionic proteins allowing epitactic phenomena.

C. Archer: Surely, in the absence of matrix vesicles, calcification in collagen free structure can hardly be described as "classical"- novel perhaps?

Authors: Bone mineralization is a complex process under the control of bone cells involving collagen, lipids, non-collagen proteins, proteoglycans and enzymes. In the present study, the formation of the electron-dense layer occurred without collagen intervening and obviously cannot be describe as a classical pattern of calcification. However, extra collagenous calcifications occur *in vivo* between two zone of bone deposit forming a lamina limitans, but also in the peritubular dentin which contain no collagen. Concerning the second point, matrix vesicles are currently observed in this culture model but not in the presence of calcium-phosphate biomaterials. Since matrix vesicles are associated with primary mineralization, it is possible that synthetic HA act as secondary nucleation sites.

C. Archer: Since only one time point has been analyzed, are the authors sure they are looking at the step by step process? Do all the nodules mature to the same point? This may be unlikely since not all the culture ossifies. Is a critical cell number or shape required for ossification process? These aspects should be clarified since what I think the authors show is a variety of events which occur at one time point.

Authors: In a recent paper (Sautier *et al*, 1990) we studied by direct examination of the culture under phase contrast microscope the time sequence of the formation and development of bone nodule in contact with calcium carbonate granules. Furthermore, bone nodule

formation and osteoblastic cell differentiation were studied in this *in vitro* system without biomaterials using microcinematographic techniques (Nefussi *et al*, 1989). In the present paper experiments were performed in triplicates and the cultures were stopped after 15 days. In the culture dishes, areas of differentiation and bone nodules at different stages of maturations were selected for TEM examination. Taking in account the great number of sections examined, it is reasonable to assume that we have at least an idea of the chronology of events occurring at the HA surface. Concerning the second point, the number of cell divisions per se is a very important factor. *In vitro* the number of cell division can be drastically reduced by the cell density seeding and for example, a 10^3 cells/cm^2 density seeding does not allow mineralization.