Osteoclast adhesion and activity on synthetic hydroxyapatite, carbonated hydroxyapatite, and natural calcium carbonate: Relationship to surface energies

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Abstract: This study investigates the adhesion, cytoskeletal changes, and resorptive activity of disaggregated rat osteoclasts cultured on polished slices of three biomaterials: crystalline synthetic hydroxyapatite (HA), carbonated hydroxyapatite (C-HA), and natural calcium carbonate (C). The surface chemistry of each substrate was defined by X-ray diffraction and IR spectroscopy, surface wettability by the dispersive, and the polar components of the surface energies. Osteoclast adhesion was modulated by the polar component of the surface energy: fewer ($p < 0.01$) osteoclasts adhered to C-HA (97 ± 20/slice, surface energy 9 ± 5 mJ/m^2) than to HA (234 ± 16/slice, surface energy 44 ± 2 mJ/m^2) or to C (268 ± 37/slice, surface energy 58 ± 0.5 mJ/m^2). Actin rings, which are the cytoskeletal structure essential for resorption, developed on all three materials. The area of the actin ring, which is resorbed by local acidification, and the osteoclast area, which reflects osteoclast spreading, were both greater in osteoclasts cultured on HA and C-HA than in those cultured on C. C was resorbed, but HA and C-HA were not. Thus, the surface energy plays an essential role in osteoclast adhesion, whereas osteoclast spreading may depend on the surface chemistry, especially on protein adsorption and/or on newly formed apatite layers. Resorption may be limited to the solubility of the biomaterial. © 1999 John Wiley & Sons, Inc. J Biomed Mater Res, 45, 140–147, 1999.

Key words: osteoclast; adhesion; spreading; surface energies; biomaterials

INTRODUCTION

The materials used for orthopedic implants in bone replacement must be such that they are gradually resorbed and replaced by new bone. It is generally accepted that bone tissue can only be resorbed by osteoclasts,[1] and they also appear to degrade bioactive materials. A recent report indicated that carbonate apatite can only be resorbed by osteoclasts and is not susceptible to phagocytosis by macrophages.[2] Monocytes can degrade calcium phosphate, but only when activated by low liposaccharide concentrations.[3]

The processes involved in bone resorption are not completely clear. The initial adhesion of osteoclasts is mediated by specific membrane receptors, mainly $\alpha_v\beta_3$ integrin, which is the vitronectin receptor that recognizes binding domains [the RGD (Arg-Gly-Asp) sequence] on noncollagenous proteins in the bone matrix (osteopontin, bone sialoprotein, fibronectin, vitronectin). Integrins also interact with cytoplasmic molecules, resulting in recruitment of cytoskeletal proteins to sites of adhesion. The primary attachment sites, termed podosomes, are formed by integrins and cytoskeletal elements, which are actin microfilaments surrounded by vinculin and talin.[1]

The podosomes in osteoclasts preparing for resorption concentrate at the periphery of the cell and form a large circular structure, the actin ring, that surrounds the area to be resorbed. Resorbing osteoclasts undergo profound morphological changes and become polarized. Close to the bone surface each cell develops a ruffled border surrounded by the clear zone that contains the actin ring. Bone resorption takes place under the ruffled border in the resorption lacuna, which is where the osteoclast produces an ex-

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tracellular acidic compartment. This local acidification is the main process responsible for dissolving the bone crystals. The proton gradient between the resorption lacuna and the surrounding extracellular fluid is maintained by the actin ring forming a tight contact with the mineralized surface.[1,4] However, the presence of proteins containing the RGD sequence is not sufficient for actin ring formation, because no rings develop in osteoclasts cultured on collagen gels or demineralized dentine.[5] Some physical properties of the bone surface appear to be essential for tight adhesion and resorption.

Several reports examined the role of the chemical properties, crystal structure, and grain size of biomaterials in their ability to be resorbed,[2,6–12] but little is known about the influence of their surface energies on the adhesion, cytoskeletal organization, and activity of osteoclasts cultured on these materials. The present study analyzes the morphology, cytoskeletal changes, and resorptive activity of osteoclasts cultured on three biomaterials: crystalline synthetic hydroxyapatite (HA), carbonated hydroxyapatite (C-HA), and natural calcium carbonate (C), each with its specific surface chemistry and surface energy.

MATERIALS AND METHODS

Biomaterials

HA powder was prepared by double decomposition between solutions of calcium nitrate and ammonium phosphate adapted from Trombe[13,14] as reported previously.[15] The pellets were obtained by sintering HA at 1200°C for 30 min. The samples were analyzed for calcium and phosphate by chemical analysis and X-ray diffraction.[16] The chemical composition corresponded to that of the stoichiometric compound $Ca_{10}(PO_4)_6(OH)_2$ with a calcium/phosphate ratio of 1.67. No foreign phases were detected by X-ray diffraction and the IR spectrum was free of extra bands. The sintered HA bodies were carbonated by treatment under a dry CO_2 atmosphere for 48 h at 900°C.[14] Dry CO_2, which was generated by sublimation of dry ice, flowed over HA pellets in a silica tube placed in a tubular oven. Powdered HA was used as the reference to check the efficiency of the reaction. The IR spectra confirmed the formation of type A C-HA, $Ca_{10}(PO_4)_6CO_3$, which was identified by the absence of OH^- bands and the presence of carbonate bands at 1560, 1450, and 880 cm^{-1}.[17] Carbonation of the pellet surfaces was also checked by reflexion IR spectroscopy. Dense C, containing 98–99% $CaCO_3$, 0.4–0.5% Na, 0.02–0.03% K, and 0.1–0.2% Sr, was supplied by Inoteb (Saint Gonnery, France). Disks (15-mm diameter, 1-mm thickness) were cut from bulk C (fossils of the bivalve *Tridacna gidas*) using a diamond saw. The disks were subsequently polished: first coarsely with SiC paper (1200 grade), then with alumina (Escil, Chassieu, France). All of the slices of the three biomaterials had exactly the same diameter (15 mm) and surface (177 mm^2), corresponding to the size of a well of a 24-well culture plate.

Surface energies

The surface energies of HA, C-HA, and C were determined before cell culture by the sessile drop method using one liquid phase.[18] Tricresylphosphate and α-bromonaphthalene were used to determine the dispersive component of the surface energy for each substrate; the polar component of the surface energy was linked to the interaction energy of the solid substrate with double-distilled water. Contact angles were determined at 25°C using the Krüss Contact Angle Measurement System (equipped with Krüss G403 software) with five to seven drops placed on each sample.

Osteoclast isolation and culture

Osteoclasts were isolated and cultured by the method of Chambers.[19] French guidelines for the care and use of laboratory animals were observed (87-849, 1987 and 88-123, 1988). Tibias from 1- to 2-day-old rat pups were dissected out in HEPES-buffered medium 199 (Gibco) and scraped into α-minimal essential medium (α-MEM) (Gibco) containing 100 IU/mL penicillin, 100 μg/mL streptomycin, and 10% fetal calf serum (α-MEM-FCS). The resulting suspension was agitated, larger fragments were allowed to sediment, and the supernatant was seeded into wells of 24-well culture plates (0.4 ml/well). Each well contained a sterile slice of biomaterial prewetted in α-MEM-FCS for 90 min. In some experiments the biomaterial was replaced by slices of devitalized bovine bone. The suspensions were allowed to settle for 30 min at 37°C and 5% CO_2, washed, and incubated in α-MEM-FCS for 4 or 48 h.

Osteoclast cytoskeleton

At the end of the incubation period, the cells were rinsed in PBS and fixed in 3.5% paraformaldehyde. Some samples were stained with toluidine blue; the adhering osteoclasts were counted (magnification ×300), and the area of the single cells was measured by tracing the outline of the cell with a drawing stylus connected to the microscope and to a microcomputer (Morphomat 10, Zeiss). Other samples were permeated with 0.1% Triton X-100 for 2 min, washed in PBS, and incubated in 0.3 μ*M* rhodamine-conjugated phalloidin. The samples were washed, mounted in glycerol, and examined with a Zeiss Universal epifluorescence microscope (magnification ×500). The area enclosed by the actin rings was measured by the same method as the osteoclast area, and 100–300 osteoclasts were measured for each experimental point.

Resorption

Cells were removed from the biomaterials and from a bone slice by tryspination. The samples were coated with

gold and examined in a Philips 525 scanning electron microscope.

Micro-Raman spectrometry

OMARS 89 and LABRAM microspectrometers (DILOR) were used.[20]

Statistics

Data are expressed as mean ± standard error of the mean and are compared by analysis of variance or by Student *t* test when appropriate.

RESULTS

Preliminary experiments indicated that the most reproducible responses were obtained 4 h after seeding in our culture conditions. This period was used in all experiments, unless otherwise stated.

Adhesion and surface energies (wettability)

Osteoclast adhesion [Fig. 1(A)] was modulated by the polar component of the surface energy [Fig. 1(B)]: fewer ($p < 0.01$) osteoclasts adhered to C-HA (97 ± 20/slice, surface energy 9 ± 5 mJ/m^2) than to HA (234 ± 16/slice, surface energy 44 ± 2 mJ/m^2) or to C (268 ± 37/slice, surface energy 58 ± 0.5 mJ/m^2). The number of osteoclasts adhering to HA and C was not significantly different. The dispersive components of the surface energy (g^ds) of each material were similar: 34.5 ± 3 mJ/m^2 for HA, 28 ± 5 mJ/m^2 for C-HA, and 36.5 ± 3 mJ/m^2 for C.

Actin rings and osteoclast area

Most osteoclast developed actin rings on all the substrates tested. The actin distribution at the periphery of the cells was typical (Fig. 2). The area enclosed by the actin rings, which is closely related to the cell area, did not depend on surface energies. This area was greater in cells cultured on HA (4758 ± 1341 μm^2) and on C-HA (2971 ± 1141 μm^2) than in those cultured on C (1379 ± 273 μm^2) or on devitalized bone slices (746 ± 128 μm^2) [Fig. 1(C)]. By 48 h of incubation, great changes had occurred in osteoclast spreading. The osteoclast area was expanded, which was greater ($p <$ 0.01) in cells cultured on HA (11,089 ± 455 μm^2) than in those cultured on C-HA (6905 ± 1095 μm^2) or on C (5170 ± 393 μm^2) [Fig. 1(D)]. The osteoclasts on C were disrupted and contained small deposits [Fig. 3(B)].

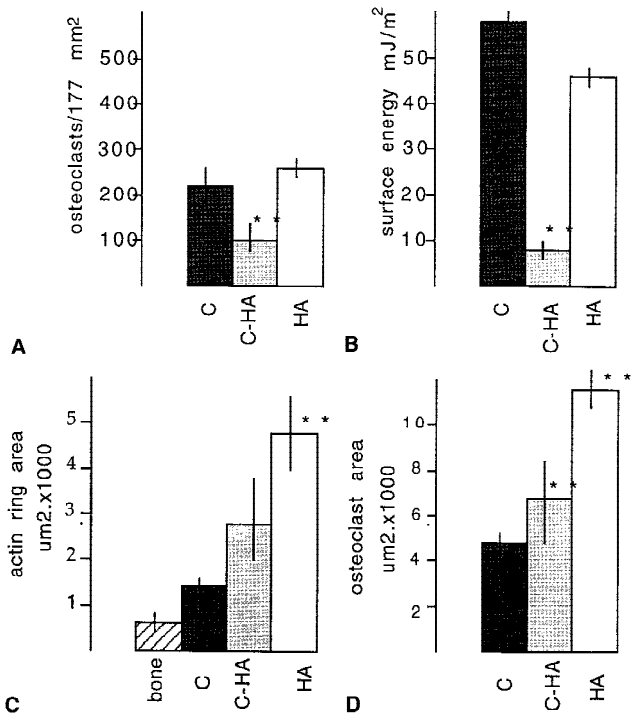

Figure 1. Influence of substratum wettability on the adhesion and spreading of disaggregated rat osteoclasts. (A) Osteoclasts on HA, C-HA, and C were incubated in α-MEM-FCS for 4 h, fixed, stained with toluidine blue, and counted; $^{**}p < 0.01$ vs. HA and C. (B) Interaction energy with water (mJ/m^2) of the biomaterials before cell culture; $^{**}p < 0.01$ vs. HA and C. (C) Osteoclasts were prepared and fixed as in (A), permeabilized with 0.1% Triton X-100, stained with rhodamine-phalloidin, and examined by epifluorescence microscopy. The area of the actin ring (μm^2) was measured (original magnification ×500); $^{**}p < 0.01$ vs. C and bone. (D) Osteoclasts were incubated in α-MEM-FCS for 48 h, fixed, and stained with toluidine blue. The total cell area (μm^2) was measured (magnification ×300). $^{**}p < 0.01$ vs. C.

Resorption

Resorption lacunae were looked for after 48 h in culture after removal of the cells by trypsination. Resorption cavities were seen on the calcium carbonate and on the devitalized bone slice [Fig. 4(C,D)], whereas HA and C-HA surfaces were relatively smooth [Fig. 4(A,B)] with no apparent resorption cavities. At high magnification, however, small lacunae, which were around 0.1 μm in size, were observed on the different crystalline structures of HA and C-HA [Fig. 4(A′,B′)], suggesting that a very thin superficial layer was seemingly affected by the osteoclasts and

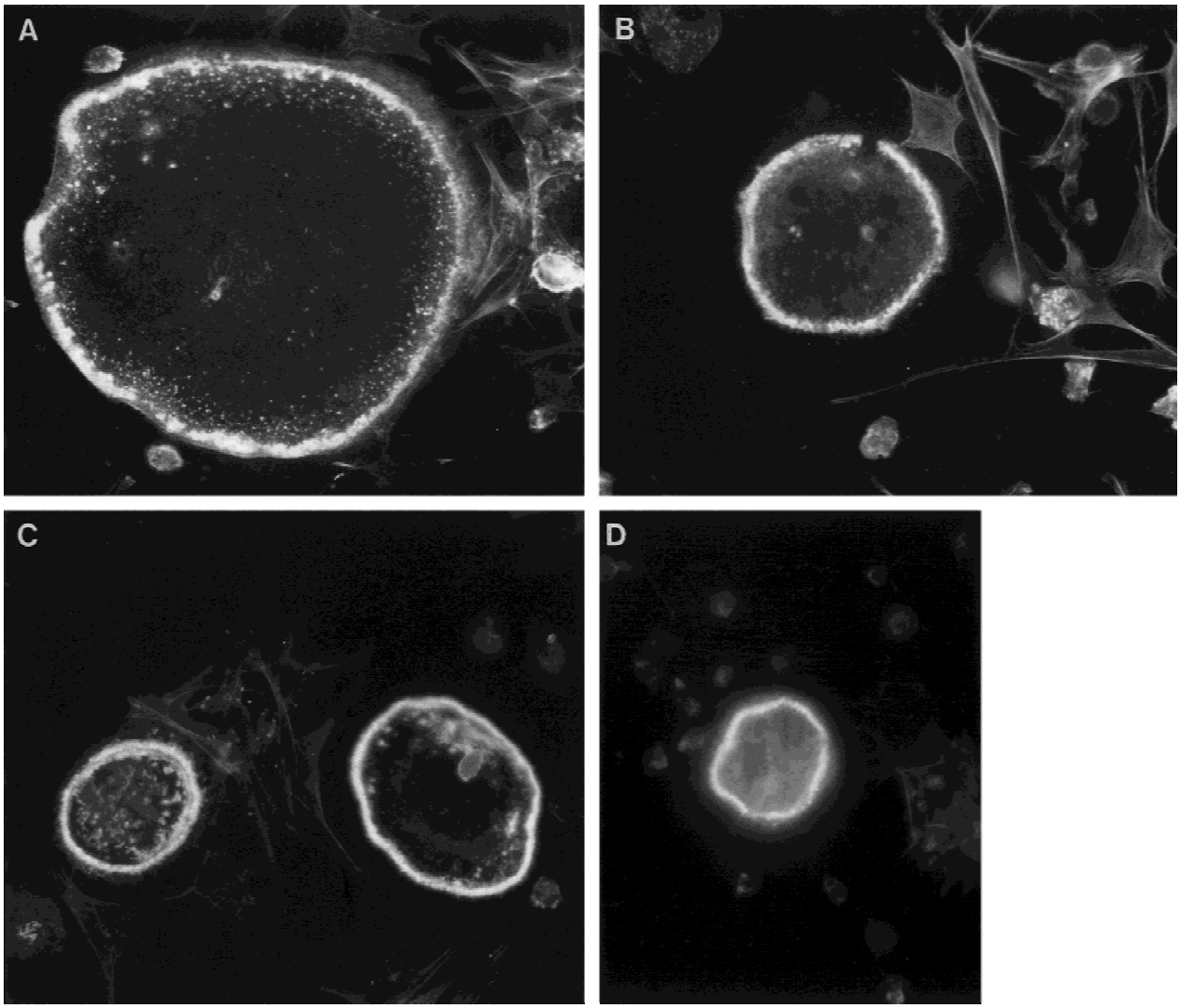

Figure 2. Rhodamine-phalloidin staining of F-actin in osteoclasts cultured for 4 h on (A) HA, (B) C-HA, (C) C, and (D) devitalized bone (original magnification ×500).

dissolved. The lacuna on C contained small crystals, but the bone lacuna did not. Micro-Raman spectroscopy showed that these crystals were type B carbonated apatite (965 and 1071 cm^{-1} bands), which was superimposed to the calcium carbonate of the support (1085 cm^{-1} band; Fig. 5).

DISCUSSION

Osteoclast adhesion depends on the presence of specific extracellular adhesive proteins and on the surface properties of a biomaterial.[21] This study shows that differences in adhesion do not result from differences in the composition of adhesive proteins because FCS, which contains mainly vitronectin, was used in all experiments; but the wettability of the biomaterials differed. Although the presence of proteins is essential, the key parameter appears to be the polar component of the surface energy, which is the interaction energy of each material with water (W^{nd} S/H_20). Cells adhered significantly less well to C-HA with low W^{nd} S/H_20 than to HA or C with much higher W^{nd} S/H_20 values. There were differences in adhesion, in spite of the fact that all samples were preincubated for 90 min in FCS and therefore covered by the adsorbed proteins. The dominant influence of the surface properties, despite the adsorbed protein layer, was also observed by Schakenraad et al.[22] for fibroblasts, muscle cells, and epithelial cells on glass and various polymers. The relationship between adhesion and surface wettability was reported for other cell types, including peripheral blood lymphocytes,[23] erythrocytes,[24] and osteoblasts.[25]

The osteoclast actin ring is a specific feature that develops after osteoclast adhesion. This ring was close to the cell periphery on the biomaterials, and the area enclosed by the ring reflects osteoclast spreading. Our

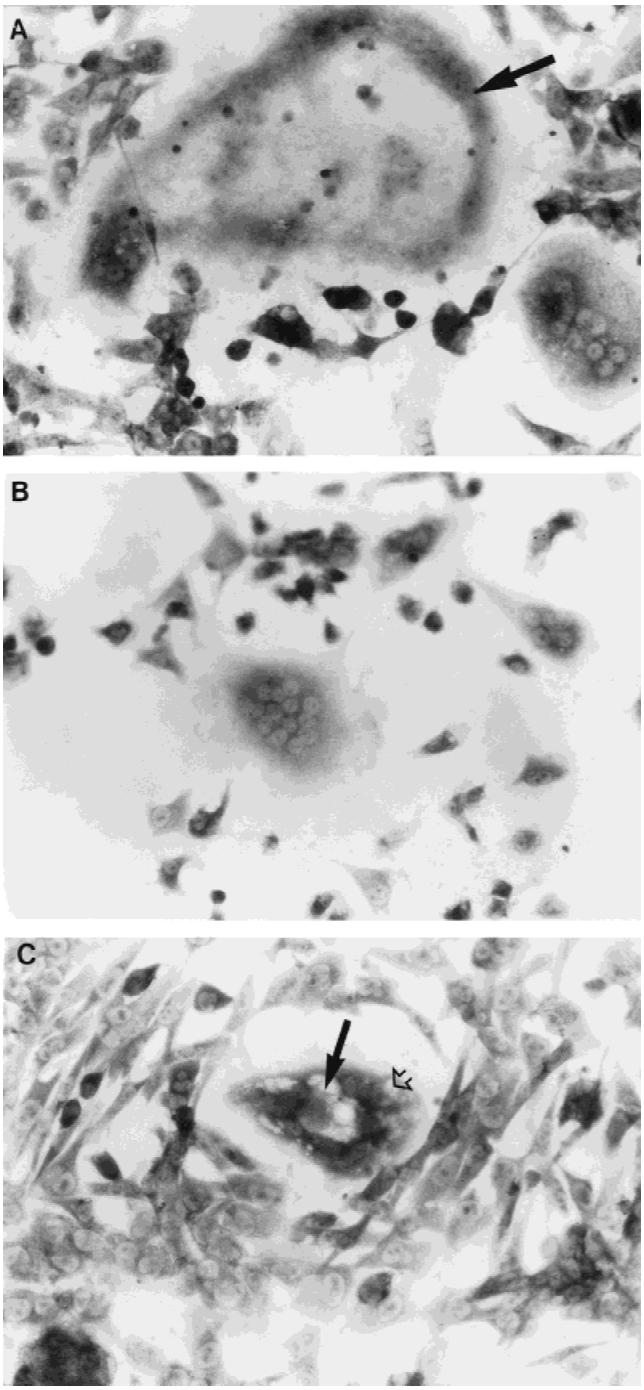

Figure 3. Appearance of osteoclasts cultured for 48 h on (A) HA, the osteoclast in the center of the image is greatly expanded, and several nuclei are seen near the cell periphery (arrow); (B) C-HA, nuclei are concentrated in the center of the cell; and (C) C, the osteoclast is damaged, with large vacuoles (arrow) and small deposits (arrowhead) in the cytoplasm (original magnification ×300).

results show that, in contrast to osteoclast adhesion, the osteoclast spread about to similar extents on HA and C-HA so that they were greatly expanded after 2 days in culture on these materials, suggesting that factors other than wettability, such as the concentration of the adsorbed proteins,[26] their conformation or binding strength, or integrin configuration,[27,28] may be involved. The surfaces of the materials do not remain unaltered in the cell culture medium. HA surfaces may undergo hydrolysis of surface phosphate into hydrogenophosphate with a loss of calcium ions and uptake of several mineral ions such as carbonate, sodium, and magnesium.[29] These reactions probably also occur on the C-HA surface, but they are rapid and cannot explain the changes in osteoclast spreading during 48 h in culture. The major event on all bioactive orthopedic biomaterial surfaces is the precipitation of a newly formed apatite layer analogous to bone mineral.[30–32] These crystals with a very large specific surface area and great reactivity have a stronger affinity for adsorbing molecules than raw HA surfaces.[33] Together these events could explain the changes in osteoclast behavior and spreading.

The relationship between actin ring formation, the development of brush border, and active resorption are complex and not completely clear. Actin ring fomation precedes bone resorption, and the ring surrounds the area to be resorbed. Resorbing activity is due to acid secreted into the area enclosed by the actin ring. We found that osteoclasts clearly recognized adhesive proteins adsorbed onto the biomaterials and actin rings developed on all substrates, although resorption lacunae occurred only on carbonate; there were no lacunae on crystalline HA or carbonated apatite, but only small defects in the 0.1-μm range on the crystalline surfaces. The formation of resorption lacunae could be linked to the rate of substrate removal. The capacity of osteoclasts to dissolve bone depends on the calcium concentration in the resorption pit: calcium released from bone may directly regulate bone resorption.[34] Calcium carbonate is the most soluble of the samples tested, while sintered apatite surfaces with their large crystals and small surface area dissolve very slowly, even in acidic conditions.[35,36] The rate of HA and C-HA dissolution are so slow that only a very thin superficial layer may be affected, and the cells may spread over a larger area than on C or bone in an attempt to increase dissolution.

The resorption pits on calcium carbonate contained small carbonate apatite crystals. These crystals were never observed in the pits formed on bone slices and were probably due to precipitation with the phosphates in the culture medium after cell death and acid release. Thus, calcium carbonate is readily resorbed by osteoclasts.[11] However, carbonate resorption is associated with rapid osteoclast degradation and the local release of acid, suggesting that the biocompatibility of carbonate should be reevaluated.

In conclusion, we showed that in the presence of adhesive proteins the surface energy of HA, C-HA, and C slices is a key factor for osteoclast adhesion; osteoclast spreading may depend on surface chemistry, especially on protein adsorption to newly formed

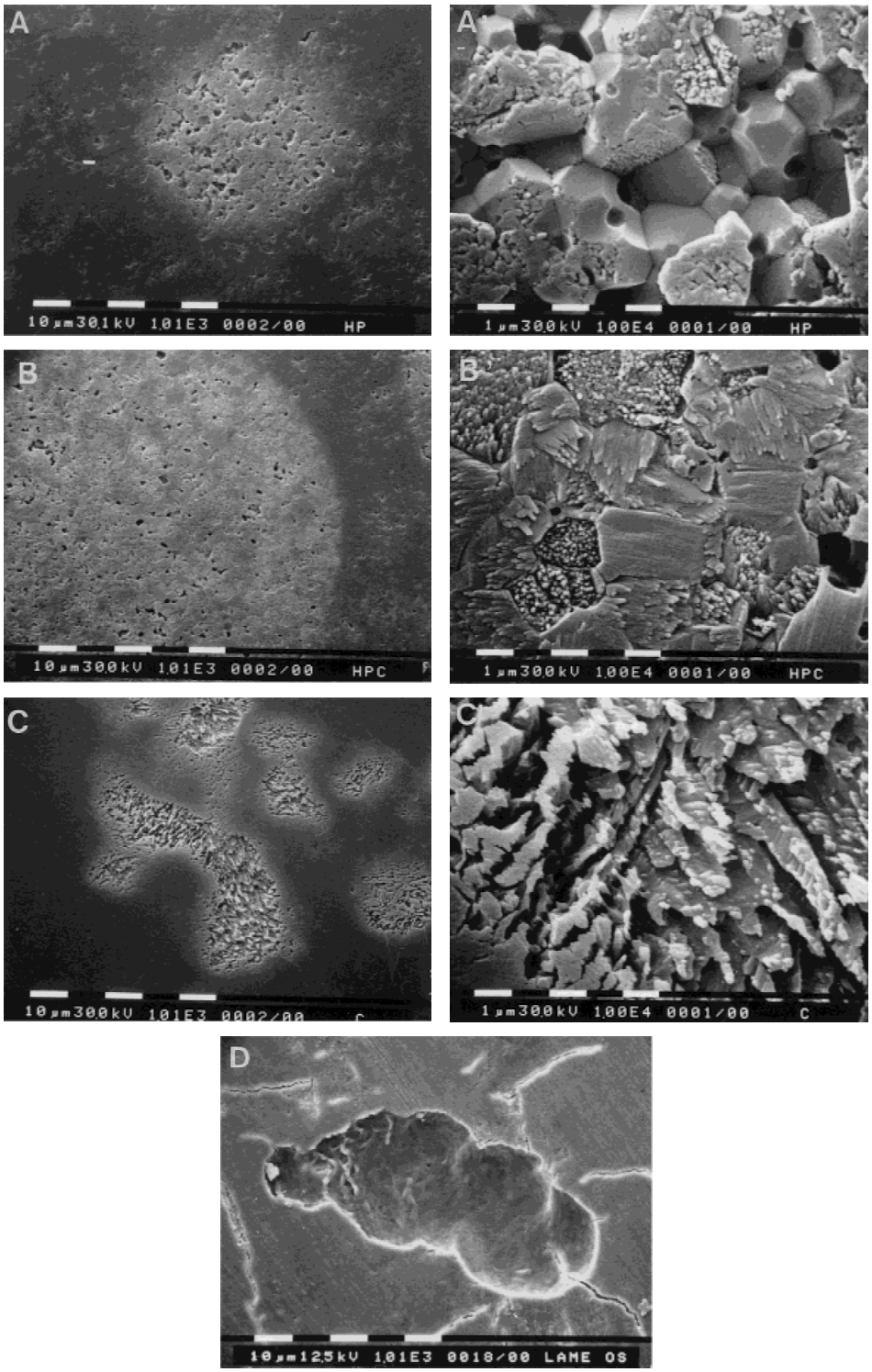

Figure 4. Scanning electron microscopy micrographs of (A,A′) hydroxyapatite, (B,B′) carbonated hydroxyapatite, (C,C′) calcium carbonate, and (D) devitalized bone surfaces after culture for 48 h with osteoclasts (removed by trypsination). Resorption cavities are seen on (D) the devitalized bone slice and (C) calcium carbonate, whereas (A) hydroxyapatite and (B) carbonated hydroxyapatite surfaces are relatively smooth with no apparent resorption cavity. At high magnification, very small lacunae are observed on the different crystalline structures of (A′) hydroxyapatite and (B′) carbonated hydroxyapatite, suggesting that a very thin superficial layer has been affected by the presence of osteoclasts and thus dissolved. (C,C′) The resorption cavity on carbonate contains small deposits. These resorption cavities differ from the well-outlined cavities formed on the bone slice, which do not contain mineral deposits. (A–D) Scale bar = 10 μm; (A′–C′) scale bar = 1 μm.

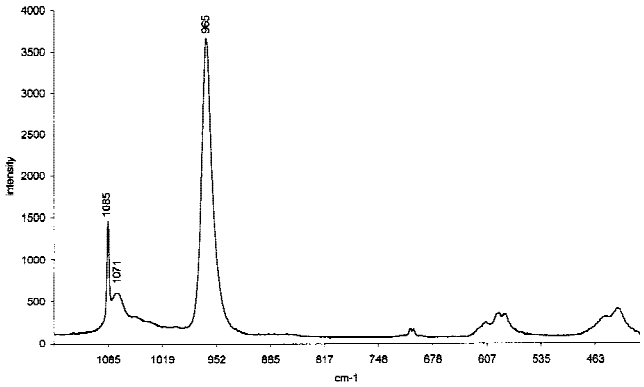

Figure 5. Micro-Raman spectrum obtained in the cavity on C, showing the presence of type B carbonated apatite (965 and 1071 cm^{-1} bands) superimposed on the calcium carbonate of the support (1085 cm^{-1} band).

apatite layers. The formation of resorption lacunae may be linked to the rate at which substrate is dissolved, suggesting that factors other than the osteoclast cytoskeletal organization are involved in their activity.

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