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BIODEGRADATION OF A NANO-HYDROXYAPATITE/COLLAGEN COMPOSITE BY PERITONEAL MONOCYTE-MACROPHAGES

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Abstract

A nano-hydroxyapatite/collagen composite was prepared by precipitation of hydroxyapatite from an aqueous solution onto collagen. Mouse peritoneal macrophages were used to investigate the *in vitro* biodegradation of the composite. The results showed the mechanism of phagocytosis and extracellular degradation process. The cells that belong to the mononuclear phagocyte system showed some morphological characteristics similar to those of osteoclasts and made pits on the composite surface. The local modification of the material surface by the cell was another phenomenon distinguishable from the degradation process. The degradation and modification made the material porous with a widely varying Ca/P ratio.

Key Words: hydroxyapatite/collagen composite, biodegradation, peritoneal macrophages, mononuclear phagocyte system, phagocytosis, extracellular degradation.

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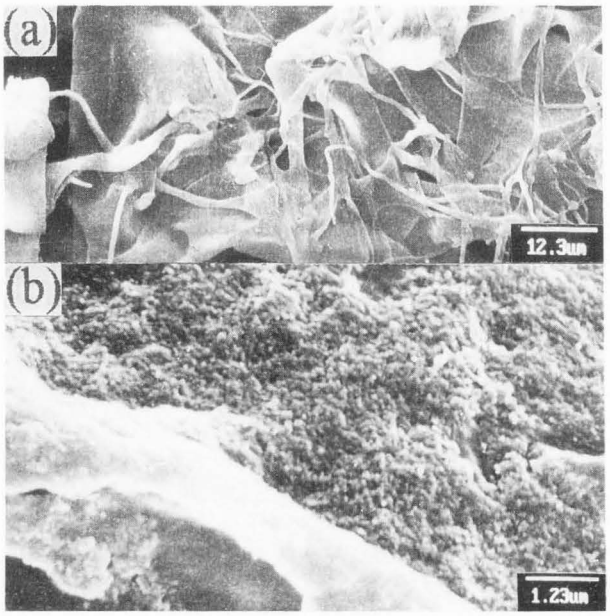
Introduction

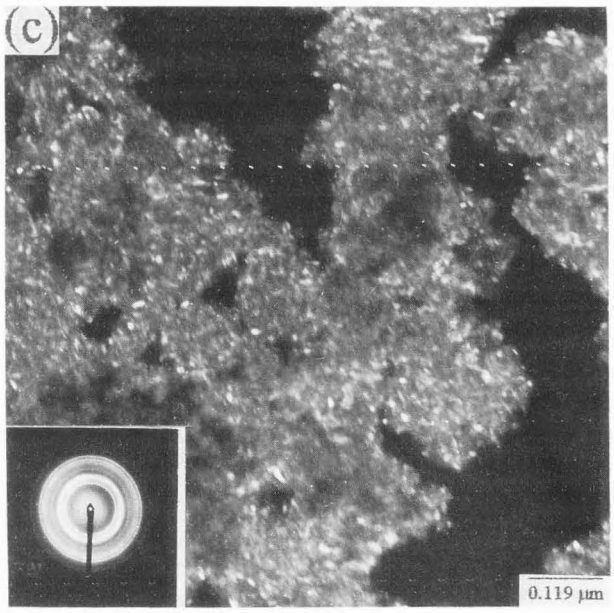
Natural bone can be considered as a composite in which hydroxyapatite (HAp) occurs as nanometer-sized deposits in an orderly manner in collagen matrix. In recent years, HAp/collagen composite has been considered to be a potential biomaterial for bone substitution [8, 19, 22, 24]. We have developed a preparation method of HAp/collagen composite in which nanometer sized HAp is homogeneously deposited in a collagen matrix [24]. Such a composite mimics not only the composition but also the microstructure of natural bone to some extent. It is conceivable that the composite would possess good bioactive properties.

Bearing in mind that living bone permanently undergoes a coupled resorption-reparative process known as bone remodeling, the biodegradation of bioactive bone substitute is an advantage because the implant would not only provide a substrate for bone formation, but, by concurrent resorption or degradation, be gradually replaced by living bone.

Many studies have been published on the degradation *in vivo* and *in vitro* of devitalized bone and dentin [1, 3, 4, 5, 6, 7, 13, 17, 18], which can be considered as natural apatite/collagen composites, of the synthetic calcium phosphate ceramics including HAp [2, 9, 11, 14, 23, 25], and of the artificial HAp/collagen composite [12, 21]. Although contradictory results were obtained concerning the types of cells responsible for resorption (the mononuclear phagocyte system or osteoclasts) and the process through which the material was resorbed (extracellular degradation or phagocytosis), these studies have suggested that cell-mediated resorption is an important process involved in the fate of the implant material.

The aim of the present study was to investigate the *in vitro* biodegradation of the nano-HAp/collagen composite developed in our laboratory by mouse peritoneal monocyte-macrophages.





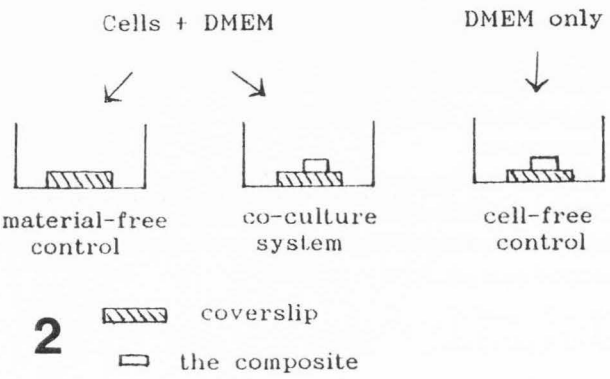


Figure 1. SEM morphology of as-received collagen (**a**) and the composite (**b**). (**c**) the central dark field image showing the nanometer crystals of HAp (inset: selected area transmission electron diffraction pattern) Cultures were incubated at 37°C, in an atmosphere of 5% CO_2 / 95% O_2 and monitored by phase-contrast microscopy.

Figure 2. Culture strategy for the experiment.

Materials and Methods

Nano-HAp/collagen composite

The composite was prepared by precipitation of HAp from an aqueous solution onto collagen. Briefly, 1g HAp was dissolved in 0.1 N HCl and 450 mg type I collagen (Gelfix®, Euroresearch s.r.l., Milano, Italy) was added to the solution which was then ultra-sonicated to obtain a thoroughly mixed slurry. The solution was diluted to 2000 ml with distilled water, which resulted in a 5 mM calcium solution with pH 3.0. Then, the solution was gently stirred at 20°C, and 0.05 M potassium hydroxide solution was added in drops to adjust the pH to 7.4. Finally, the solution was maintained at pH 7.4 for 10 min, after which the composite was harvested by centrifugation and freeze-dried. The material was investigated by means of X-ray diffraction (XRD), infrared spectroscopy (IR), a scanning electron microscope (SEM) equipped with an energy dispersive spectrometer (EDS), and a transmission electron microscope (TEM). The inorganic phase in the composite was determined to be apatitic calcium phosphate and no peaks from other Ca-P materials were present in the XRD pattern. The broadening and overlap of the peaks was similar for the composite and natural bone. In the IR spectra, besides the absorption bands of PO_4, OH in the apatite and NH_2 groups of the collagen, there were bands of CO_3. Furthermore, the spectra were not so sharp as those of well-crystallized HAp. Therefore, the inorganic phase in the composite is carbonate-substituted hydroxyapatite with a low crystallinity. Fig. 1 shows the morphology of the commercially purchased collagen and the composite. HAp precipitates were uniformly distributed on the collagen matrix. From the TEM central dark field image, the crystal size of HAp was measured to be 2-10 nm. Details about this analysis have been published previously [24].

The composite used in this study was compressed to a circular disc (diameter 3 mm, thickness 2 mm) and sterilized by γ-ray irradiation (2.5 mrad).

Cell isolation and culture

Four mice without any stimulation by intraperitoneal injection were sacrificed by cervical dislocation. Peritoneal cells were collected by flushing the peritoneal

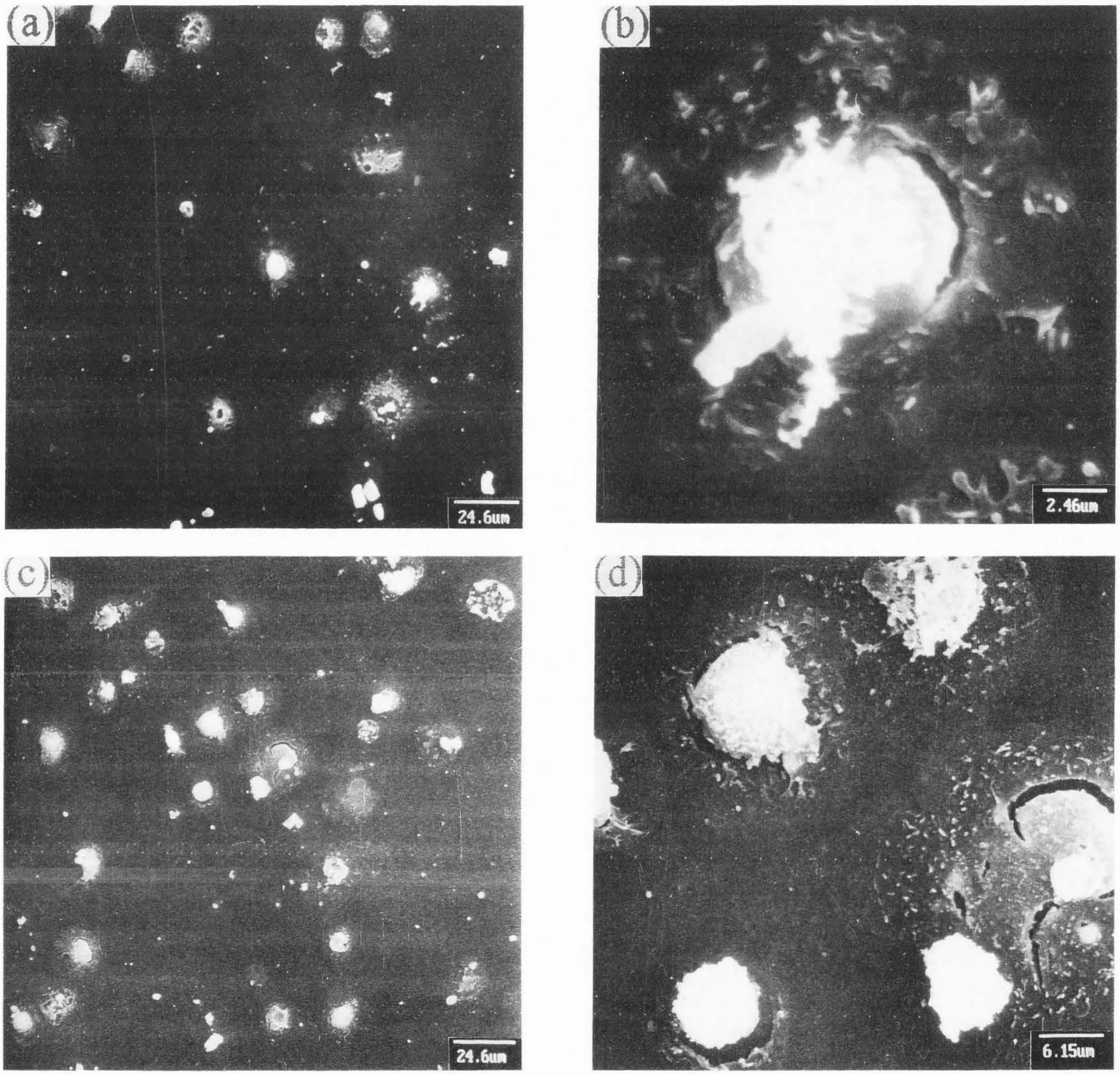


Figure 3. The morphology of cells on the coverslip in material-free control (**a, b**) and in co-culture with the composite (**c, d**) incubated for 2 days.

cavity with Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, St. Louis, MO, USA). The pooled fluids were centrifuged and the cell pellet was washed two times. Then the cells were resuspended in serum-free DMEM and seeded at a concentration of $3x10^5$ cells/ml in tissue culture plate (Linbro®, Hamden, CT, USA) . The culture strategy is illustrated in Fig. 2.

Morphological observations and Ca/P ratio determination

After 2 and 3 days of culture, both the coverslips and the composites were washed with serum-free medium and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), followed by 1% osmium tetroxide in the same buffer. The specimens were then dehydrated through a graded series of acetone and water, critical point dried with CO_2 and gold-coated

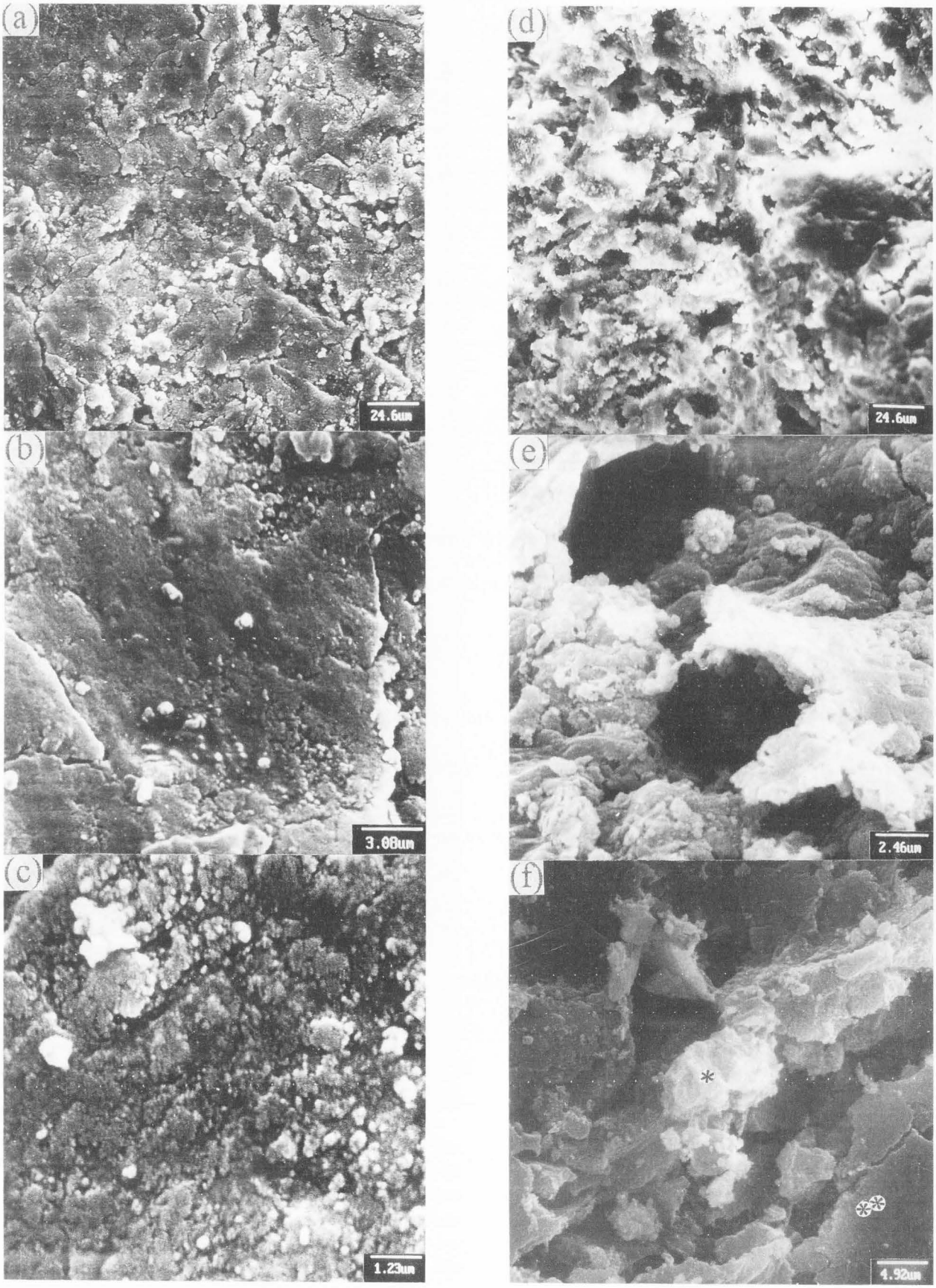

(a)
24.6um
(b)
3.08um
(c)
1.23um
(d)
24.6um
(e)
2.46um
(f)
4.92um

Figure 4 (*on facing page*). The morphology of the composite in the cell-free control (a, b, c) and in co-culture with cells (d, e, f) incubated for 3 days. The cracks (a, b) and microporosity (c) on the surface can be seen. The Ca/P ratio was between 1.1 and 1.3. In the presence of cells, the composite became porous (d) with many deep lacunae (e). The Ca/P ratio varied from 1.11 (asterisk) to 1.92 (double asterisks).

Figure 5 (*to the right*). Two distinguishable types of cell morphology on the composite. (a) The cell with a round body and branched pseudopodia which had extended into micropores of the material surface was spreading out over the substrate and relatively flat. (b) The cell was tall and plump. The membranous structure in the proximity of the material was reminiscent of the "sealing zone" (triangle) and "ruffled border" (between double arrows) of the osteoclast.

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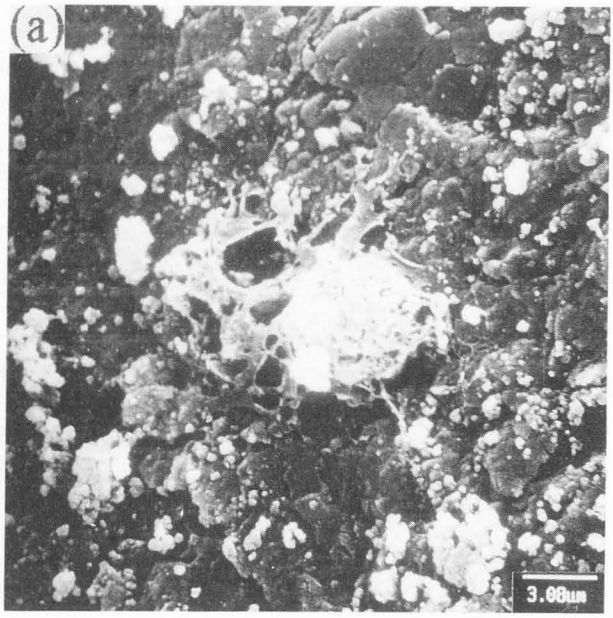
for examination in a SEM (Hitachi S-550, 25 kV), equipped with an energy-dispersive X-ray microanalysis system (EDAX 9100/70). Elemental measurement was performed on selected zones and prominent peaks were identified using computer software. The Ca/P ratio was calculated and the semiquantitative results allowed comparison between different samples or different zones of the same sample.

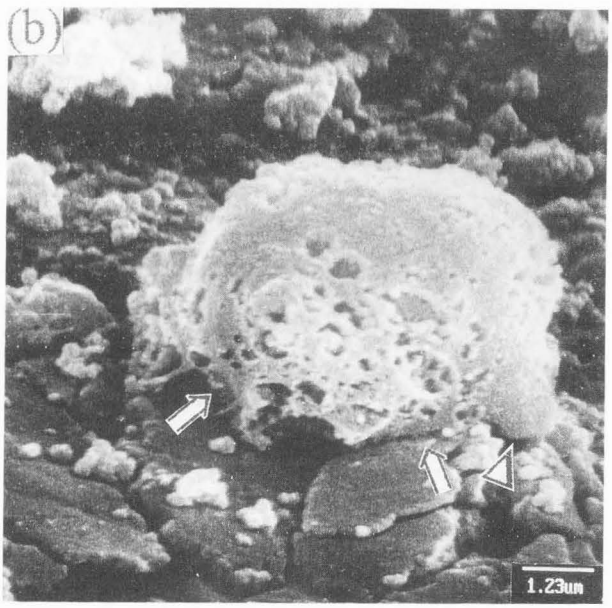
Results

The material was tolerated by cells, as revealed both by phase-contrast microscopy and by SEM. The tolerance was also consistent with several preliminary studies of the same material (data not shown). Under the phase-contrast microscope, it was observed that there were more cells in the vicinity of the material than in other places, which implied a recruitment of resorbing cells by the composite. SEM showed that whether in the presence of the composite or not, cells on the coverslip were round in shape. They were adhering to and fully spreading on the substrate, with many dorsal microvilli and pseudopodia (Fig. 3).

It was clear that the composite was being degraded and this degradation could be attributed to solution- and cell-mediated processes. The solution-mediated process was relatively simple and homogeneous. In contrast, cell-mediated resorption was complex and heterogeneous. In addition, the morphology and behavior of cells on the composite was diverse in contrast with that of cells on the coverslip.

When immersed in culture medium for several hours, the composite swelled slightly due to the rehydration of the collagen. SEM showed that many cracks, long and narrow, had formed on the surface of





the material. At higher magnification, the microporosity of the surface was apparent. Energy-dispersive X-ray analysis showed a Ca/P ratio in the range of 1.1-1.3. This decrease in the Ca/P ratio could be attributed to the transformation of the mineral into calcium-deficient apatite. This degradation was merely the solution-mediated dissolution of the minerals (Fig. 4 a-c).

The size of the cells adhering to the composite was in the range of 6-10 μm, and no cell fusion was observed. Two types of cell morphology could be clearly distinguished. The first was relatively flat, and similar to that of cells on the coverslip. The cells had a round body and branched pseudopodia which had extended into

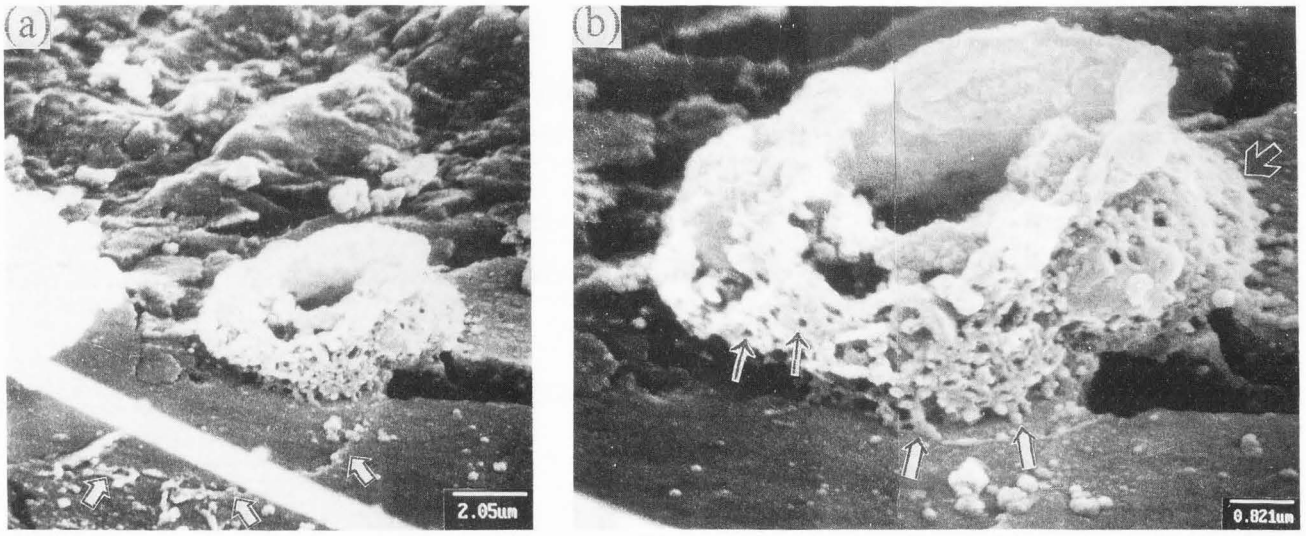


Figure 6. Extracellular degradation of the composite. (**a**) The cell was leaving the lacuna which was shallow and had a well-defined margin (hollow arrows). (**b**) The cell had developed a complex ruffled border-like structure which consisted of numerous filopodial (double arrows) and sheath-like (arrows) membrane foldings or extensions. The cell showed polarization with active filopodia and pseudopodia extending at one pole (solid arrow) over the material surface.

micropores on the material surface (Fig. 5a). The majority of the cells belonged to the second type (Fig. 5b). They were still spherical or oval in shape, but instead of spreading out on the support, they appeared tall and plump with a membranous structure in the proximity of the material reminiscent of the "ruffled border" and "sealing zone" of the osteoclast, a giant (20-100 μm) multinucleated (2-50 nuclei) cell responsible for the bone resorption during bone remodeling.

The degradation of the composite was evident. As shown in Fig. 5a, although neither characteristic resorption lacuna nor phagocytosis could be observed, the surface roughness and the number of small and tiny particles of the material increased when compared with that of samples without cultured cells. The Ca/P ratio of the particles and the material surface was about 0.71-0.94. These results suggested that in addition to solution-mediated dissolution, cell-mediated resorption had occurred.

Extracellular degradation seemed to be the major process through which the cells resorbed the composite. The cells were actively resorbing and migrating and accompanied by resorption lacunae. As shown in Fig. 6, the lacuna was shallow and had a well-defined scalloped margin. It was comparable to the cell in size. The remainders of cell activity, perhaps the secretion of the cell and tiny material particles, were distributed at the margin. The cell leaving the lacuna had developed a complex membranous structure which consisted of numerous filopodial and sheath-like membrane foldings or extensions. The "fingers" and "channels" on the membrane were approximately 80-160 nm in diameter. Although there were differences, this structure showed some morphological similarity with the ruffled border of osteoclasts. For this structure, we suggest the name of "ruffled border-like structure". The cell exhibited polarization. The active filopodia and pseudopodia extended at one pole over the material surface or into the crack. The other pole appeared more quiescent.

The shape, size and deepness of the resorption lacunae varied due to the migration of the cells. Fig. 7 shows the cells accompanied by deep lacunae. These lacunae had a tracklike appearance, indicating the zigzag movement of the cells. The width of the lacunae was slightly smaller than that of the cells, suggesting the sealing of the material surface during the resorption process. The presence of spherical vesicles and their budding from the cell membrane indicated active secretory ability of the cell.

The secretory process is shown in Fig. 8. The topography of the material was very complex and extensive degradation was evident. At higher magnification, the ruffled border-like structure of the cell consisted of filopodia which had penetrated the underlying material, suggesting resorption activity. The Ca/P ratio of the cell body was 1.29. In contrast, composition analysis performed on cells in the material-free control (Fig. 3b) showed abundant phosphorus and negligible calcium. These results suggested an increased level of intracellular calcium in the cells grown on

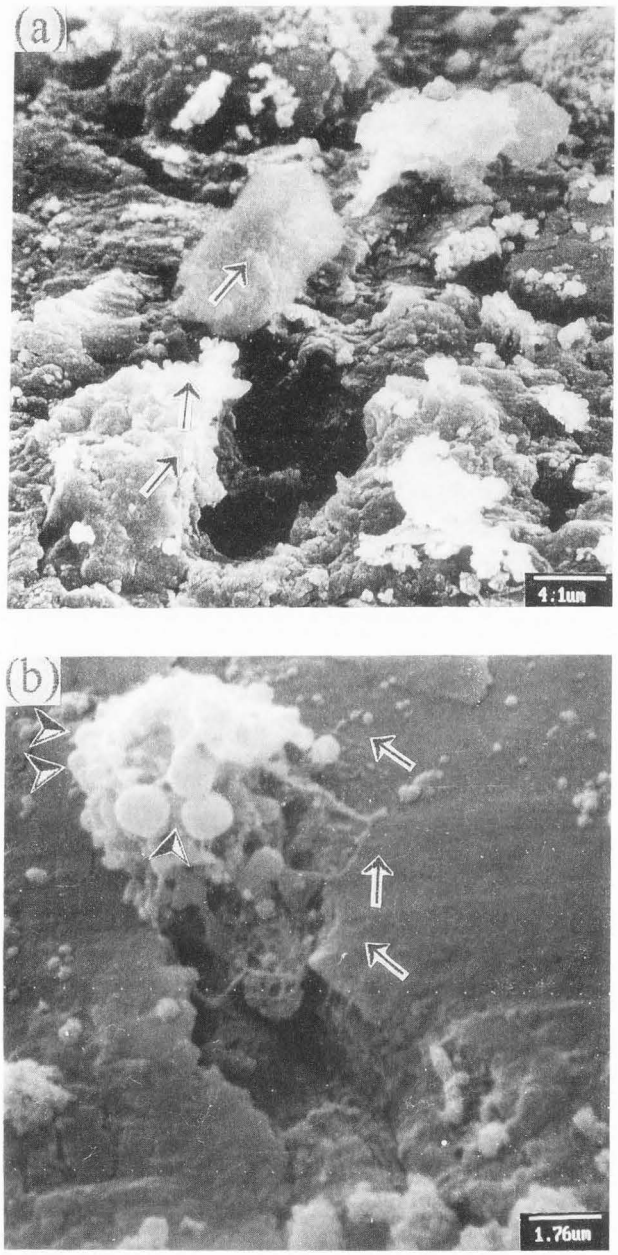


Figure 7. The tracklike lacunae suggested the zigzag movement (arrows) of the cells. The spherical vesicles budding (arrowheads) from the cell membrane suggested the secretory properties of the cell.

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composite. The vesicles appeared to bud from the lateral membrane just above the area of the ruffled border-like structure. The size of these vesicles was approximately 0.2-1.0 μm, and some adhered to the surface of the material.

Another notable phenomenon was the modification of the material surface by the cells. Fig. 9 shows a cell and the locally modified region with a circular profile.

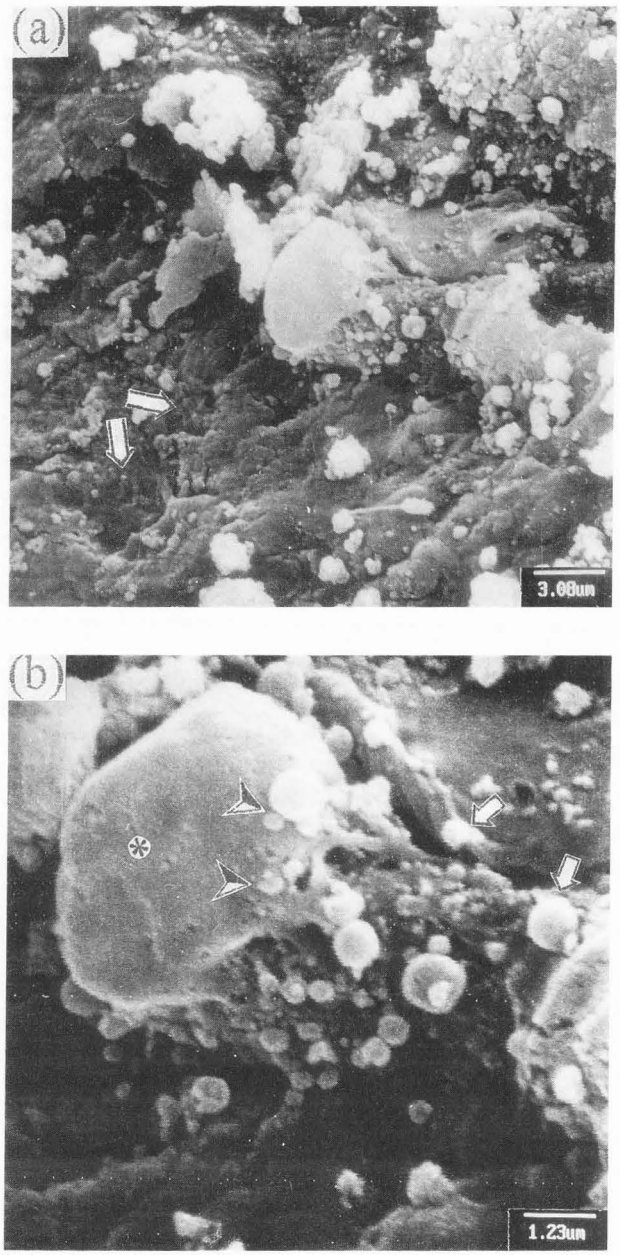

Figure 8. The secretory process of the cell. (**a**) The topography of the material was complex and extensive degradation was evident (double arrows). (**b**) The budding (arrowheads) of the vesicles occurred from the lateral membrane just above the area of the ruffled border-like structure. Some vesicles had adhered to the surface of the material (hollow arrows). The penetration of filopodia into the underlying material suggested resorption activity. The Ca/P ratio of the cell body (asterisk) was 1.29.

The diameter of the cell was about 7 μm, in contrast, that of the area modified was as large as 11 μm. Such

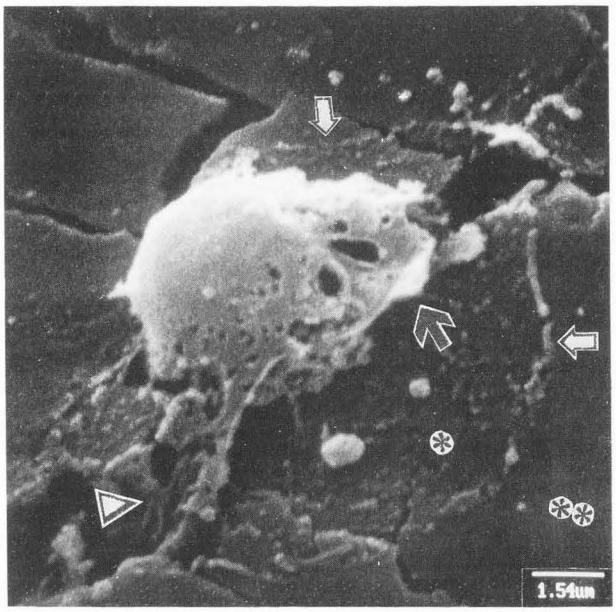

Figure 9. The modification of the material surface by the cells. The modified region had a well-defined margin (hollow arrows), but no pit or excavation. The Ca/P ratio was 1.84 inside (asterisk) and 1.36 outside (double asterisks) the region. The cell showed polarization, extending broad pseudopodia (solid arrow) at one pole and exhibiting fine filopodia (triangle) at the other.

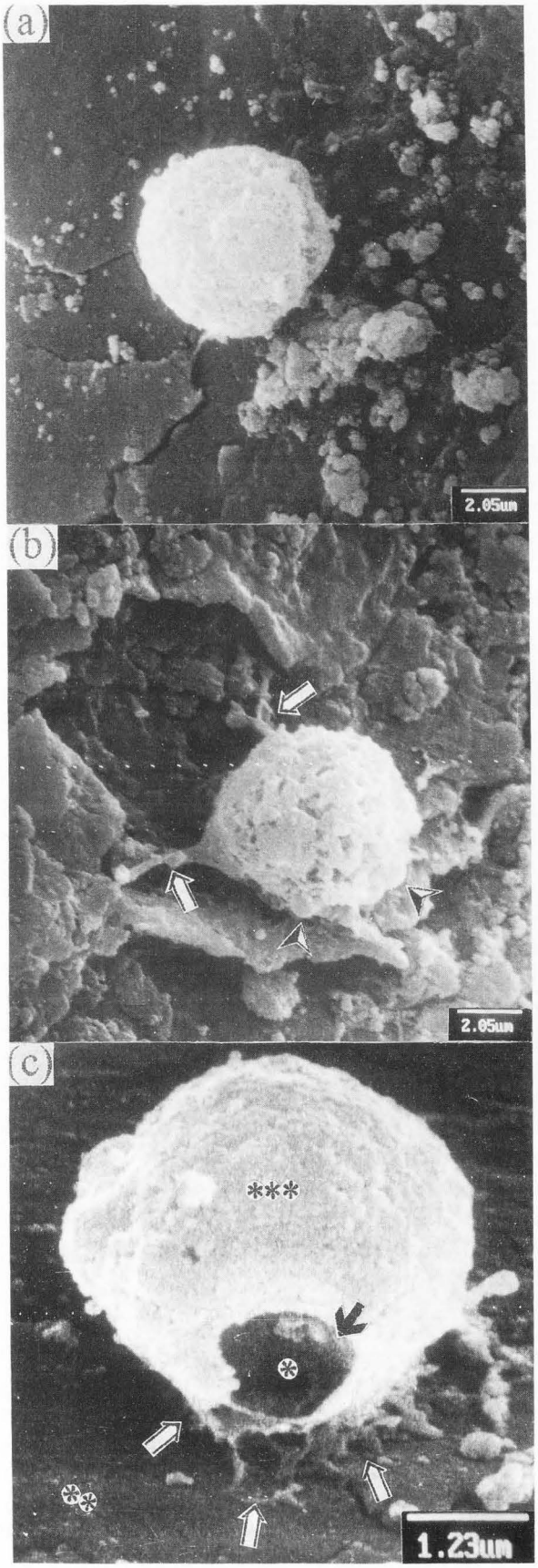

Figure 10. (*to the right*) (**a**) A cell with a round body was adhering to the material surface. (**b**) Resorption lacuna and cell with budding of vesicles (arrowheads) and filopodia (double arrows). (**c**) The phagocytosis (solid arrow) of a cell with ruffled border-like structure (double arrows). The Ca/P ratio was 1.34 for the endocytosed particle (asterisk), 1.43 for the material surface (double asterisks), and 0.98 for the cell body (triple asterisks).

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regions had a well-defined margin, but no pit or excavation, so that they could be distinguished from resorption lacunae. Furthermore, there was a significant change in Ca/P ratio across the margin, with a much higher value (1.84) inside the modified area than outside (1.36). The cell showed polarization, extending broad pseudopodia at one pole over the modified area and exhibiting fine filopodia at the other. Some filopodia remained in the crack which had probably to some extent undergone erosion by the cell.

These peritoneal cells belong to the mononuclear phagocyte system. Phagocytosis followed by intracellular degradation of the material is their main characteristic and function and was also observed in this system. Fig. 10a shows a cell with a round body adhering to the material surface. Fig. 10b shows a similar cell accom-

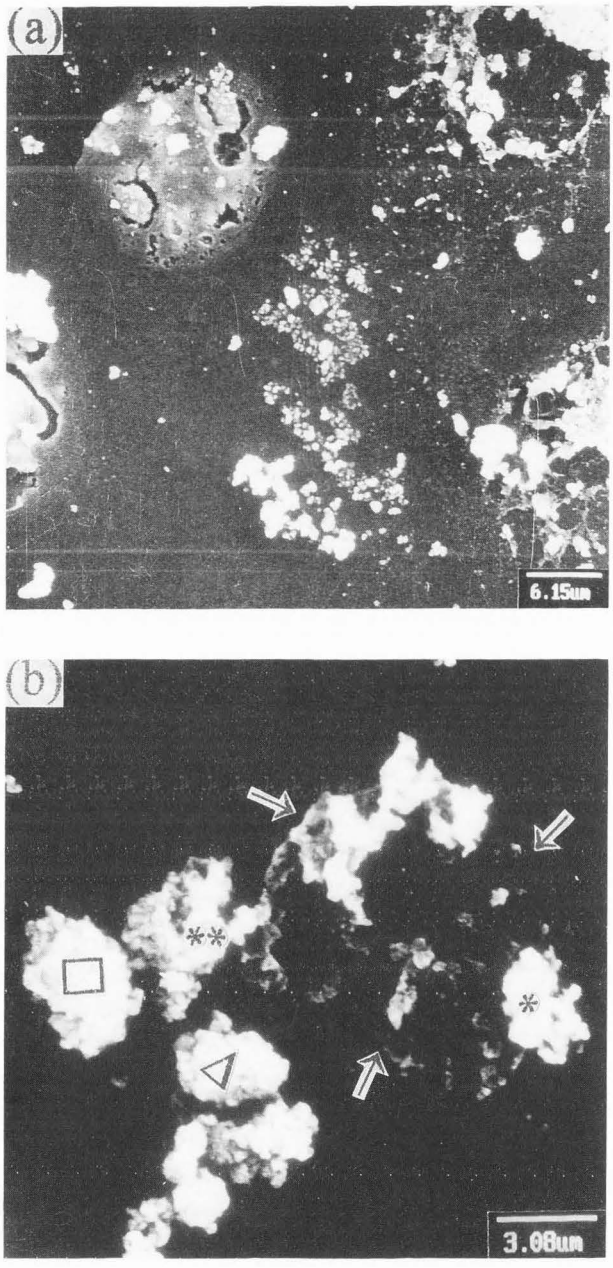

Figure 11. The morphology of cells on the coverslip cultured with the composite for 3 days. (a) The cells were still flat, but many had died. (b) The material particles associated with the debris (between arrows) of cell showed a variable Ca/P ratio, 0.89 (asterisk), 0.96 (double asterisks), 1.12 (triangle), and 1.10 (rectangle). The particle with the lowest value seemed to have been phagocytosed by the cell.

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panied by a lacuna. The budding of the vesicles and the filopodia on the cell body were similar to those demonstrated above, suggesting that extracellular resorption activity had occurred. Fig. 10c shows evidence of phagocytosis. A similar round cell is shown in the process of endocytosis of a material particle with a size of about 1.4 μm. At the same time, the ruffled border-like structure consisting of numerous filopodia can be observed between the cell body and the substrate, but no lacuna is visible. The Ca/P ratio of the endocytosed particle was 1.34, which was slightly less than that of the substrate which was 1.43. An increased level of intracellular calcium was suggested by the Ca/P ratio of the cell body which was 0.98.

The most significant effect of the cellular activity was to convert a relatively dense material into a porous material with a heterogeneous distribution of the Ca/P ratio. Figure 4d-f show the composite co-cultured with cells for 3 days, in striking contrast to composite cultured in cell-free medium (Fig. 4 a-c). The lacunae appear very deep, suggesting extensive resorption. The Ca/P ratio varied widely, from 0.62 to 2.4, which is in contrast to the uniform value of 1.6 before the composite was subjected to cell culture. A high ratio often accompanied a relatively dense surface morphology. The rough surface and many irregularly-shaped particles always had a low Ca/P ratio.

There were sparse cells on the surface of the composite cultured for 3 days, although these cells had a morphology similar to those cultured for 2 days (not shown). Figure 11 shows the cells on the coverslip in the same culture. They still had a flat appearance, but many cells had died. The debris of the cells and material particles could be seen. The Ca/P ratio of the material particles associated with the dead cells was variable, which provides indirect evidence of degradation (Fig. 11b). The particles with the lowest Ca/P ratio probably had been phagocytosed and had undergone incomplete degradation intracellularly. The particle with a medium Ca/P ratio was adjacent to the cell and might have been subjected to extracellular degradation. Because some effects of the cellular activity, such as a significant decrease in pH, can be limited to locations close to the cell, the particles further away from the cell were influenced primarily by the solution and had a relatively high Ca/P ratio.

Discussion

In investigations on the biodegradation of devitalized bone and dentin which can be considered as natural apatite/collagen composites, contradictory evidence has been presented concerning the responsible cells and the mode of process. There is distinct evidence of osteoclast resorbing activity against dentine or bone *in vitro*, showing the characteristic ruffled border and resorption lacuna [3, 4, 5, 6, 18]. However, the capacity of the mononuclear phagocytic system to resorb devitalized

bone has been debated. Mundy *et al.* [17] and Kahn *et al.* [13] demonstrated direct resorption of bone by human monocytes *in vitro*. Furthermore, Kahn *et al.* [13] observed that the cultured monocyte possessed some, but not all of the morphological features of osteoclasts, such as "resorption lacunae" and "clear zones". In contrast, Ali *et al.* [1], Chambers *et al.* [7] and Pazzaglia *et al.* [18] indicated failure of the mononuclear phagocytic system to resorb bone. They found that these cells could not induce detectable changes in the bone surface, even after prolonged incubation.

Controversy also exists regarding the cell-mediated degradation of synthetic calcium phosphate ceramics and calcium phosphate/collagen composites, which have been used as bone substitute because their composition closely resembles that of bone. Kwong *et al.* [14] reported the solubilization of calcium phosphate crystals, including HAp crystals, by bone cells (osteoblast-like and osteoclast-like cells), macrophages and fibroblasts. They suggested the degradation mechanism of phagocytosis followed by intracellular solubilization. Gomi *et al.* [11] showed that osteoclasts could create small hole-like resorption lacunae on sintered synthetic HAp *in vitro*. de Bruijn *et al.* [9] reported that osteoclastic resorption of calcium phosphate took place in cultures with mineralized extracellular matrices formed by previous bone marrow cells culture. They suggested a possible role for bone matrix constituents in cell-mediated resorption. Yamada *et al.* [25] demonstrated that actively resorbing and migrating osteoclasts could produce tracklike resorption lacunae on the bonelike apatite layer formed on an apatite- and wollastonite- containing glass-ceramic by a simulated body fluid. van der Meulen and Koerten [23] reported the degradation of calcium phosphate ceramic spheres (fluorapatite, hydroxyapatite and β-tricalcium phosphate) in the mouse peritoneal cavity. They observed the endocytosis of macrophages or giant cells and globular extracellular deposits. They hypothesized that the formation of extracellular deposits was due to the process of extracellular degradation. Baslée *et al.* [2] demonstrated that resorption of Ca-P ceramics implanted in rabbit bone involved two cell types, multinucleated giant cells and osteoclast-like cells. They suggested that bone proteins adsorbed to ceramics could favor osteoclastic differentiation. St. John *et al.* [21] indicated that multinucleated cells phagocytized particles of ceramic/collagen graft implanted in canine femoral defect for 1 year. The material was composed of bovine type I collagen gel and HAp/TCP ceramic. Hemmerle *et al.* [12] investigated long-term behavior (42 months implantation in a human freshly extracted alveolar socket) of a HAp/collagen-glycosaminoglycan biomaterial which was composed of synthetic HAp powders blended with collagen-glycosaminoglycan. However, they found that the synthetic HAp crystals remained unaltered by long-term implantation.

In the present study, we used peritoneal cells to investigate the *in vitro* biodegradation of the composite. The noteworthy phenomenon is that these cells could degrade the composite through an extracellular degradation process probably by a mechanism similar to that of osteoclasts. It has been established that osteoclasts with a central ruffled border surrounded by a sealing zone could erode resorption lacuna on the substrate by enzyme excretion and temporary acidification of the microenvironment. The ruffled border and resorption lacunae have been considered as the ultrastructural characteristics of the osteoclasts [10]. The present cell developed a complex membranous structure in the proximity of the material. Although it was not limited to the central area and thus could be visualized in SEM, this structure showed some morphological similarity with the ruffled border of osteoclasts, i.e., it consisted of numerous cytoplasmic extensions which were filopodial and sheath-like. We have used the name of ruffled border-like structure to indicate this similarity. While the exact nature of this structure and the relationship between it and the ruffled border should be investigated further, it is clear that such extensive membrane foldings provide many extracellular channels and spaces and an greatly increased surface area for the exchange of materials on the composite-facing side of the cell. The various pits on the composite showed close association with the cells in size, shape and spatial relation. This provides evidence for the extracellular degradation process.

Despite the similarity demonstrated above, the size, shape and endocytotic properties of the cells suggested that they did belong to the mononuclear phagocytic system. They did not lose their phagocytotic function, at least not during three days of culture. However, since a culture medium which did not contain any other factors such as cytokines, hormones and co-cultured osteoblasts was used, the possibility that the cells might differentiate into osteoclast-like cells after a longer period of incubation cannot be excluded. Whether or not the similarity described above represents a partial differentiation requires additional assessment using other techniques such as histochemical and immunocytochemical observations.

Many factors may be involved in the biodegradation of the material, such as the composition and structure of the composite, the topography of the sample, or the concentration of calcium and phosphate ions: both systemic and local. The composition and structure of the material might be the most important factor that modulates the pattern of cell action. The composite used

in the present study mimics not only the composition but also the microstructure of natural bone to some extent. Two points should be emphasized. Firstly, the inorganic phase is nanometer-sized carbonate-substituted HAp with low crystallinity. The structural features of the mineral could make it in many respects different from synthetic ceramic which is often well-crystallized and consists of coarse grains (in the μm range). Differences may occur in properties such as the binding ability or the state of the organic components, or resorbability. Secondly, the mineral is growing on, not blended with the collagen matrix. Collagen-mediated mineralization is an old topic in the study of biomineralization. Numerous studies using fibrillar systems or gel systems or reconstituted collagen fibers suggest that collagen is an active regulator rather than a passive support for the crystals and that some degree of association between the mineral and collagen actually exists. Therefore, it is reasonable that the biological behaviour of the composite produced on the basis of biomimetic growth should differ from that of the mechanical mixture of ceramic and collagen which is just a compositional analogue to natural bone. Also, the differences between the composite and bone should be noted. Firstly, the collagen of bone has an aligned fibrillar substructure and the mineral shows preferential orientation of its c axis with the axis of the collagen fibril. In contrast, the collagen in the composite consists of randomly oriented fibrils and the mineral shows no preferential orientation. Thus, the composite is in a sense a less mineralized material than bone. This feature could promote degradation. Secondly, natural bone, even devitalized bone, is never a pure apatite/collagen composite. Non-collagenous proteins and other organic constituents of bone matrix could influence cell differentiation and activity. For example, it has been found that osteocalcin-deficient bone particles are resistant to resorption when implanted in normal rats [16]. The effect of these non-collagenous organic components could interfere with that of the pure apatite/collagen. However, obviously a different preparation method of bone sample would alter their performance.

Based on a review of the literature and our own results, we propose that some kind of cooperative effect between the organic and inorganic component of the material might play a key role in the modulation of cell action. Such a cooperativity depends on the binding state and interaction of these two components, and thus on the composition, grain size, crystallinity and orientation of the inorganic phase, and the composition, structure, and orientation of the organic constituents.

The secretory activity of cells was another common phenomenon. The size of membrane-bounded spherical vesicles was between 0.2 and 1.0 μm. They appeared to bud from the lateral cell surface just above the area of the ruffled border-like structure and entered the extracellular space by a membrane-shedding process. With regard to the nature of these vesicles, two possibilities exist. One is that they were of lysosomal origin and participated in the extracellular degradation process. This possibility could be supported by the work of Sakaki *et al.* [20] who have provided morphological evidence of secretory process of macrophages. They found that small round structures, which they specified as blebs or knobs, ranging in size from 0.12 to 0.5 μm and budding from the tips of the cytoplasmic processes, showed the cytochemical characteristics of lysosomal enzymes i.e., acid phosphatase reaction. The alternative is that the secretory process in the present study was a kind of active transport of calcium ions whose role was likely to be maintenance of low levels of cytosolic calcium ions. This possibility is suggested by the observation that the secretory process was accompanied by an increased level of intracellular calcium. However, it also should be pointed out that the fixation technique of glutaraldehyde primary fixation followed by osmium tetroxide postfixation used in the present study has intrinsic problems with the formation of membrane blisters, which are artifacts that can be confused with real structures [15]. Thus, further confirmation is needed.

The modification of the material surface seemed to be a process distinguishable from that of extracellular degradation. The striking feature of this process is a much higher Ca/P ratio of the modified area compared to that of other areas. The substitution of phosphate by carbonate or bicarbonate which are by-products of resorption activity could be one of factors contributing to this increase in Ca/P ratio. Although the exact nature of such a modification is not clear at present, it is conceivable that such a process is a local phenomenon and associated with secretion or exocytosis. Furthermore, such modification might in its turn influence the behavior of the cell.

Conclusions

In addition to being degraded by the solution-mediated dissolution, the nano-HAp/collagen composite could be degraded by peritoneal monocyte-macrophages *in vitro*. The mechanisms involved were phagocytosis and extracellular degradation. The cells which belong to the mononuclear phagocyte system showed some morphological characteristics similar to those of osteoclasts and made pits on the material surface. These cells were actively resorbing and migrating. In addition, the local modification of the material surface by the cells was another phenomenon distinguishable from the

degradation process. The degradation and modification converted the relatively dense compressed material to a porous material with a variable Ca/P ratio. The composition, microstructure and interaction of inorganic and organic components of the materials may play a key role in modulating the cell action.

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Discussion with Reviewers

G. Daculsi: The authors suggest that the cells have a ruffled border similar to osteoclasts and a sealed zone. The pictures do not show these properties. The size and shape of the cells still limited and do not resemble that of an osteoclast. The authors indicated that no cell fusion occurs. How is it possible to suggest osteoclast-like resorption of the materials?

Authors: We have indicated that the cultured cells belonged to the mononuclear phagocyte system due to their size, shape and phagocytosis. We have also shown the similarity between the membranous structure of the cultured cells and the ruffled border of osteoclasts. The ruffled border-like structure consisted of numerous filopodial and sheak-like membrane folding or extensions and only developed on the composite-facing side of the cell. Furthermore, the various pits on the composite showing close association with the cells provided the evidence for the extracellular degradation process. Therefore, a mechanism for resorption of the material similar to that of osteoclasts is possible.

G. Daculsi: Numerous experiments indicate that monocyte-macrophages are unable to produce lacunae of resorption. This was demonstrated on various samples of biological origin (dentin, bone) or on synthetic samples (HA, biphasic...). How do the authors explain these lacunae? Could this particular kind of composite be responsible?

Authors: As mentioned in the **Discussion**, many factors may be involved in the biodegradation of the material. Obviously, the composition and structure of the material are two important factors. The comparison of this kind of composite with other materials indicates significant differences in the composition, microstructure and interaction of inorganic and organic components. On the other hand, the culture medium did not contain other factors such as cytokines and hormones which can influence the differentiation and activity of cells. Therefore, we suggest that composite itself could be responsible for these lacunae, in particular the nanometer-sized mineral particles and their nucleation and growth on the collagen.

E. Bonucci: I am rather disturbed by the presence of commercial collagen in the composite. Its use is probably suggested by the resemblance the composite has with normal bone. However, this resemblance is fallacious, because there is no binding between hydroxyapatite and collagen, and this probably behaves as an inert supporting material, unable to modulate macrophagic cell activity. On the other hand, if the composite is used for bone implants, the presence of collagen can induce adverse immunological reactions. Thus, my question concerns the usefulness of using the collagen-hydroxyapatite composite instead of hydroxyapatite alone.

Authors: Collagen-mediated mineralization has been and continues to be investigated extensively. Many *in vitro* studies using gel systems, fibrillar systems or reconstituted collagen fibers have suggested the regulatory role of collagen and some degree of association between the mineral and collagen [26]. Actually, it is one of the aims of biomimetic synthesis to produce biomaterials which have similar biological behaviours to natural tissues as more as possible. The collagen/hydroxyapatite composite for bone implants is just one example. On the other hand, Delustro *et al.* [27] have reviewed the immune responses to allogeneic and xenogeneic implants of collagen and collagen derivatives used for soft-tissue augmentation, wound repair and the repair of bone. Experience indicates that immunity to collagen can exist without impairing the effectiveness of the medical device or resulting in significant adverse clinical sequelae.

G.M. Roomans: You use an accelerating voltage of 25 kV for the energy-dispersive microanalysis. Have you considered that at this voltage the electron beam completely penetrates the cells and also excites the substrate on which the cells grow? How does this affect your conclusions?

Authors: The depth of penetration of the beam electrons into the specimen depends on the energy of the beam electrons and the atomic number of the specimen. The higher the energy of the beam electrons is, the greater the penetration. At an accelerating voltage of 30 kV, the electron beam will typically penetrate 10 μm into a low atomic number specimen such as carbon but only 0.5 μm into a high atomic number specimen such as uranium. While biological specimens are basically composed of low atomic number elements, the samples of the present study were osmium tetroxide treated and gold-coated and the presence of high atomic number elements (Os and Au) can significantly reduce the penetration of the beam electrons. The X-ray microanalysis of cells was performed primarily on those that appeared tall and plump with a size of around 10 μm. This size is comparable to that of X-ray escape volume (in the range of several micrometers) [15]. Therefore, the major information of the X-ray microanalysis came from the cell body. Anyhow, the possible interaction of the electron beam with the substrate on which the cells grow can introduce interference. The smaller the cell is, the greater the interference. Therefore, the values of the Ca/P ratio of the cells have only a qualitative meaning. Actually, it is

due to the same reason that we cannot make a meaningful elemental analysis on the subcellular structures such as the vesicles shown in figure 8. This question gives us a good suggestion and it is helpful for our future work.

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