

1991

Primary Bone Formation in Porous Hydroxyapatite Ceramic: A Light and Scanning Electron Microscopic Study

M. Okumura
Nara Medical University

H. Ohgushi
Nara Medical University

S. Tamai
Nara Medical University

E. C. Shors
Nara Medical University

Follow this and additional works at: <https://digitalcommons.usu.edu/cellsandmaterials>



Part of the [Biological Engineering Commons](#)

Recommended Citation

Okumura, M.; Ohgushi, H.; Tamai, S.; and Shors, E. C. (1991) "Primary Bone Formation in Porous Hydroxyapatite Ceramic: A Light and Scanning Electron Microscopic Study," *Cells and Materials*: Vol. 1 : No. 1 , Article 4.

Available at: <https://digitalcommons.usu.edu/cellsandmaterials/vol1/iss1/4>

This Article is brought to you for free and open access by the Western Dairy Center at DigitalCommons@USU. It has been accepted for inclusion in Cells and Materials by an authorized administrator of DigitalCommons@USU. For more information, please contact digitalcommons@usu.edu.



**PRIMARY BONE FORMATION IN POROUS HYDROXYAPATITE CERAMIC:
A LIGHT AND SCANNING ELECTRON MICROSCOPIC STUDY**

M. Okumura*, H. Ohgushi, S. Tamai, and E.C. Shors¹

Department of Orthopedic Surgery, Nara Medical University,
¹Interpore International, Irvine, California, USA

(Received for publication March 8, 1990, and in revised form July 28, 1990)

Abstract

Porous hydroxyapatite ceramics combined with rat marrow cells were implanted subcutaneously in the back of syngeneic Fischer rats. Fluorochrome-labeling (calcein, tetracycline) was performed post-operatively and the ceramics were harvested 4 weeks after implantation. Undecalcified thin sections of the implants were observed under light microscopy or fluoromicroscopy and the corresponding areas were also analyzed in a scanning electron microscope connected to an electron probe microanalyzer (SEM-EPMA). Many pore areas of the ceramics showed bone and osteoid formation together with active osteoblasts. The bone formation began directly on the surface of the ceramic and proceeded in a centripetal direction towards the center of the pores. SEM-EPMA analysis revealed continuous high levels of calcium and phosphorus in bone/ceramic interface and a gradual decrease of these levels in the osteoid region. These results indicate that the interaction between osteoblasts and ceramic surface resulted in bone formation.

KEY WORDS: Hydroxyapatite, Ceramic, Rat, Bone Marrow Cell, Osteogenesis, Scanning Electron Microscopy, Electron Probe Microanalyzer, Undecalcified Section, Fluorochrome Labeling.

*Address for correspondence:
Motoaki Okumura,
Department of Orthopedic Surgery,
Nara Medical University,
Kashihara, Nara 634,
Japan

Phone No. 07442-2-3051

Introduction

Calcium phosphate ceramics have proven to be non-immunogenic and non-toxic but the ceramic itself cannot induce bone formation^{13, 20, 21, 23, 24}. However, when the ceramic is implanted in orthotopic sites, such as bony defects, bone formation can occur on its surface (osteo-conduction). In addition, calcium phosphate ceramics become strongly bonded to the bone tissue without mechanical interlock¹³. Recently, various analytical methods have been used to characterize the interface between the bone and the ceramic. However, the cause of strong bonding at the bone/ceramic interface has not yet been understood because it is very difficult to identify the primary bone on the ceramic surface by conventional experimental methods, e.g., in implantation of materials in orthotopic sites where bone already exists.

We have developed a new experimental method to show consistent de novo bone formation in porous calcium phosphate ceramics^{23, 24}. The method utilizes the implantation of a composite of the ceramic and rat bone marrow cells into a rat subcutaneous site. It is very simple and suitable for identification of the primary bone in the porous implants because materials/osteogenic cells interaction without any influence of preexisting bone tissues can be observed. By using this method, we analyzed the process of the osteogenesis and the bone/ceramic interface.

Materials and Methods

Implant materials.

Coralline hydroxyapatite ceramics (Interpore International, Irvine, California) were used in this experiment. They are made by conversion of calcium carbonate skeletons of reef-building sea corals into pure hydroxyapatite by means of a hydrothermal chemical exchange reaction²⁷. The solid and porous

components of the microstructure are completely interconnected^{10, 11, 29}. This accounts for the rapid neovascularization and bone incorporation of the implants. The average pore size of this implant is 200 μm in diameter and the void volume averages 66%. In this study we used disk-shaped coralline hydroxyapatite with a diameter of 5 mm and thickness of 2 mm.

Marrow cells preparation and surgical procedure.

Femora and tibiae of five Fischer 344 rats (7 week-old males) were recovered and placed in saline following removal of adherent muscle and periosteum. Both ends of the femur and tibia were cut away from the epiphysis. One of the femur ends was connected with a tygon tube to a 1 ml syringe. The marrow plug was then hydrostatically forced into a test tube containing the heparinized phosphate buffered saline (PBS). The marrow in PBS was disaggregated by sequential passage first through 18 G and then 20 G needles to obtain a cell suspension. The cell suspension was centrifuged at 3000 rpm for 5 minutes and 200 μl of the supernatant was mixed up with the sediment (cell layer) by vortex mixer. To make the composite graft, the porous hydroxyapatite ceramics were soaked in this disaggregated marrow cell suspension at the room temperature and used within 2 hours. Syngeneic Fischer rats were anesthetized by intraperitoneal injection of Nembutal (2.5 mg/100g body weight). Subcutaneous pouches were created in back of the rat following small incisions. The ceramics combined with marrow cells were implanted in subcutaneous sites. We have already reported that the ceramic itself or marrow cells alone when implanted subcutaneously could not show bone formation, however the composite showed consistent bone formation in the ceramic pore regions 4 weeks after implantation^{23, 24}.

To observe the de novo bone dynamics in the pore regions of the ceramic, fluorochrome-labeling was performed. The rats were given one dose each of calcein (15 mg/kg, intravenously) 2 weeks after implantation and tetracycline (50 mg/kg, subcutaneously) 3 weeks after implantation.

Histological evaluation.

Implants were harvested 4 weeks after surgery. For undecalcified sections, the ceramics were immediately fixed in 70% ethyl alcohol and stained with Villanueva bone stain. They were then dehydrated in graded series of ethyl alcohol (70, 95, 95, 100%) and acetone for each 90 minutes, infiltrated in methyl methacrylate monomer for 3 days and in prepolymerized embedding medium (mixed with methyl methacrylate monomer 100ml, polymethyl methacrylate beads 40g, and benzoyl peroxide 1g) for 3 days. After embedding procedure, the surface of the implants in methyl methacrylate was coated with cyano-acrylate and cut into sections of 7 μm using a Jung Model K microtome. These specimens were observed under light microscopy or fluoromicroscopy. For scanning electron microscopy / electron probe microanalysis

(SEM-EPMA), the flat surface of the implant embedded in methyl methacrylate was prepared after another cutting of 7 μm thickness of the implants by using the microtome. The surface of the implant was coated with a thin layer of carbon (~ 10 nm), and the ceramic/bone interface was analyzed by using a scanning electron microanalyzer connected with a wavelength dispersive spectrometer (EPMA8705, Shimadzu Corporation, Kyoto, JAPAN). The ceramic surface was first observed in the backscattered electron (BSE) imaging mode, which provides a useful means for detecting an overall distribution based on the mean atomic numbers of the constitute elements and allows clear observation of the interface between the bone and the ceramic with high contrast. A comparison of the BSE image with the corresponding undecalcified thin section showed the area of primary bone formation on the ceramic. The bone formation areas on BSE images were then observed under secondary electron imaging. Also, the characteristic X-ray images of Ca-K α and Mg-K α for the same area were displayed; and in addition, line scans for three elements (calcium, phosphorus and magnesium) were obtained along a line on the bone ceramic interface. The total scanning time was 4 minutes for each element for the line analyses. Image formation and line scan were performed at an accelerating voltage of 10 kV. An electron beam, below 0.4 μm in diameter, was maintained at 2×10^{-8} amps.

Results

Undecalcified section

Coralline ceramics combined with rat marrow cells showed osteogenesis in the pore regions. There was no intervening fibrous tissue between the ceramic and the de novo bone (Fig. 1a-d). Many pore areas showed the newly formed bone together with active osteoblasts; thus, the bone formation was still progressing. Some pore areas showed primary bone formation on the ceramic surface, i.e., active osteoblasts faced to the ceramic surface then produced osteoid which became mineralized bone (Fig.1d). These histological features indicated that the bone formation began on the surface of the ceramics and proceeded toward the center of the pores (bonding osteogenesis)^{25, 26}. This process was confirmed by fluorochrome labeling. Green colored calcein (administered 2 weeks after implantation) was seen near the ceramic surface and yellow colored tetracycline (administered 3 weeks after implantation) was seen close to the center of pores (Fig. 1c).

SEM-EPMA results

A BSE image of the area corresponding to the undecalcified thin section is shown in Fig. 2a. Ceramic area (white), newly formed bone area (gray) and fibrous tissue area (black) in the pore regions were clearly identified. The higher magnification of secondary electron image allowed to distinguish the de novo

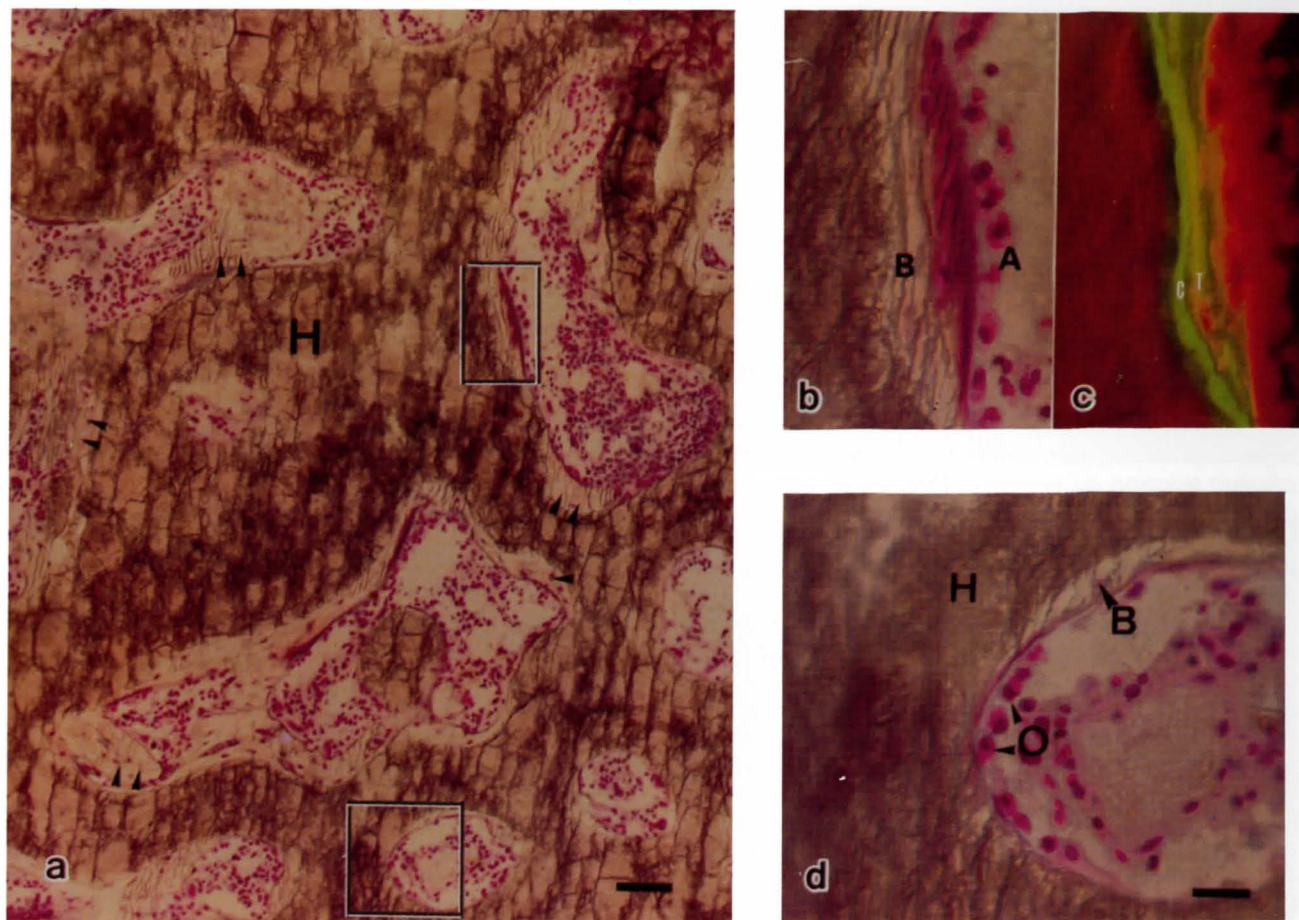


Fig. 1. Ceramic with marrow cells 4 weeks after subcutaneous implantation.

a: An undecalcified section under light microscope. Many pore areas show bone formation. Arrows indicate newly formed bone. H: hydroxyapatite ceramic. Bar = 100 μ m.

b: Higher magnification of the rectangular area (at top right) of Fig. 1a. Active osteoblasts lining (A) together with a small amount of newly formed bone (B) is observed on the surface of pore region. Between the osteoblasts lining and fully mineralized bone (B) a narrow seam of osteoid (light brown ~ purplish red) is seen.

c: The same section as in Fig. 1b under fluoromicroscopy. Green calcein (C) and yellow tetracycline (T) were administrated 2 and 3 weeks after implantation respectively.

d: Higher magnification of the square area (at bottom) of Fig. 1a. Many osteoblasts (O) line up on the ceramic. In addition, a small zone of newly formed unmineralized bone (osteoid) is seen. B: bone; H: hydroxyapatite ceramic. Bar = 30 μ m.

bone from the ceramic. It showed the newly formed bone directly interfaced to ceramic surface (Fig. 2b). Compared with the corresponding undecalcified section, the osteoid region appeared as coarse granular areas. The characteristic X-ray image of calcium showed high content of calcium in both ceramic and bone area (the content in ceramic was slightly higher) and low content of calcium in osteoid/osteoblast lining (Fig. 2e). The magnesium distribution almost exactly

corresponded with the area of newly formed bone. However, low concentration of Mg was also detected in both ceramic and soft tissue areas (Fig. 2f). Fig. 2d shows the line scans of three elements (Ca, P, Mg) from the ceramic area to the bone area (left to right). It revealed the continuity of high levels of calcium and phosphorus at the bone/ceramic interface. The levels of calcium and phosphorus in the bone were slightly lower than those in the ceramic. On the other hand,

the level of magnesium in the bone was much higher than that in the ceramic area and increased moderately across the interface. In the area of osteoid and osteoblasts the contents of Ca, P and Mg gradually decreased. In the fibrous tissue area, the content of these three elements decreased to the base line.

Discussion

Hydroxyapatite ceramics are one of the major types of ceramics having bioactive bonding behavior. Various types of glass ceramics are also well known as bioactive ceramics^{4, 6-9, 16-18, 22}. Several investigations on these ceramics/bone interface have been carried out using a variety of techniques^{3, 8}. Only a few reports observing this interface with the use of transmission electron microscopy (TEM) exist, presumably due to the difficulty of cutting thin sections containing undecalcified bulk bone and brittle ceramics^{2, 5, 7, 14, 28}. In contrast, there has been many SEM studies on the interfacial zone^{1, 7, 9, 12, 15-19, 30}.

Hench⁷ studied the bonding mechanism at the interface between bone and a glass ceramic and found the presence of crystals of hydroxyapatite on the surface of glass ceramic using SEM, TEM and X-ray diffraction analysis. It suggested the direct chemical bonding of hydroxyapatite crystals at the interface. Gross et al.⁵ reported ultrastructure of the interface between a glass ceramic and bone and described areas with bone connection displaying collagen fibers and deposits of apatite crystals in close relationship to the bulk ceramic. Jarcho et al.¹² speculated the direct epitaxial deposition of bone on the apatite surface (chemical attachment) by TEM observation as well as SEM-EPMA analysis. Subsequently many investigations of the interface between bone and bioactive ceramics have been performed using SEM-EPMA^{9, 16-19, 30}. These studies indicate that the Ca-P rich layer is necessary to form a strong bond between the ceramic and bone.

All of these studies of bone/ceramic interface were performed by implantation of samples in orthotopic sites. Our subcutaneous implantation of ceramics/marrow composite allowed us to easily observe initial osteogenesis in the ceramics without any influences of pre-existing bone tissues. Furthermore, comparing the SEM image with its corresponding undecalcified thin section gave us a better understanding of the material/host tissue interaction both morphologically and constitutionally. Thus, the present study is very useful to analyze the bone/ceramic interface.

As shown in Fig. 1, the sequence of osteogenesis on the hydroxyapatite ceramic is: 1) Apposition of osteoblasts on the ceramic surface; 2) Osteoid formation in the appositional area; and 3) Mineralization of the osteoid region resulting in mature bone formation. Thus, unmineralized or partially mineralized areas (osteoid) do not persist on the ceramic surface and do not exist between the ceramic and the bone in the process of osteogenesis. SEM-EPMA analysis of the

corresponding area confirmed this observation as evidenced by the continuous high levels of calcium and phosphorus across the ceramic/bone interface and gradual decrease of these elements in the osteoid area (Fig. 2d). At the interface between the ceramic and bone, Mg content varied in contrast to Ca and P content. It may suggest that chemical compositional variable zone (Ca, P and Mg) existed in the interface as described in other reports^{9, 16-18}. There is also a possibility that this zone may chemically bind to the bone apatite crystal^{4, 16, 26}.

All of these results suggest that osteogenic cells can easily adhere to the surface of the ceramic and the cells' production, such as collagen, proteoglycan and matrix vesicles, can be integrated into the ceramic surface. The process finally leads to fully mineralized bone *via* osteoid formation.

Acknowledgments

The authors wish to express their gratitude to Mr. Komi H. (Shimadzu Co. Kyoto, Japan) for the SEM analysis.

Partial support was provided by JOTF grant (No. 0040, Zimmer Japan Award).

References

1. Brill W, Kattbagen BD (1987) The interface layer between hydroxyapatite-ceramic and newly formed bone in scanning electron microscopy. *Z. Orthop.* 125, 183-187.
2. Denissen H (1979) Dental root implants of apatite ceramics. Vrije Universiteit te Amsterdam. 1979, Boek-en offsetdrukkerij Los-Naarden.
3. Ducheyne P (1987) Bioceramics: material characteristics versus in vivo behavior. *J. Biomed. Mater. Res.* 21, 219-236.
4. Fujiu T, Ogino M (1984) Difference of bond bonding behavior among surface active glasses and sintered apatite. *J. Biomed. Mater. Res.* 18, 845-859.
5. Gross U, Brandes J, Strunz V, Bab I, Sela J (1981) The ultrastructure of the interface between a glass ceramic and bone. *J. Biomed. Mater. Res.* 15, 291-305.
6. Harrell MS, Acree WA, Bates SR, Clark DE, Hench LL, Keane MA, Clark AE Jr. (1978) Thickness of bioglass-bonding layers. *J. Biomed. Mater. Res. Symp.* 2, 111-112.
7. Hench LL, Splinter RJ, Allen WC, Greenlee TK (1971) Bonding mechanisms at the interface of ceramic prosthetic materials. *J. Biomed. Mater. Res. Symp.* 2, 117-141.
8. Hench LL, Ethridge EC (1982) *Bioceramics, an interfacial approach.* Academic Press, New York.
9. Holand W, Vogel W, Naumann K, Gummel J (1985) Interface reactions between

Primary bone formation in porous ceramic

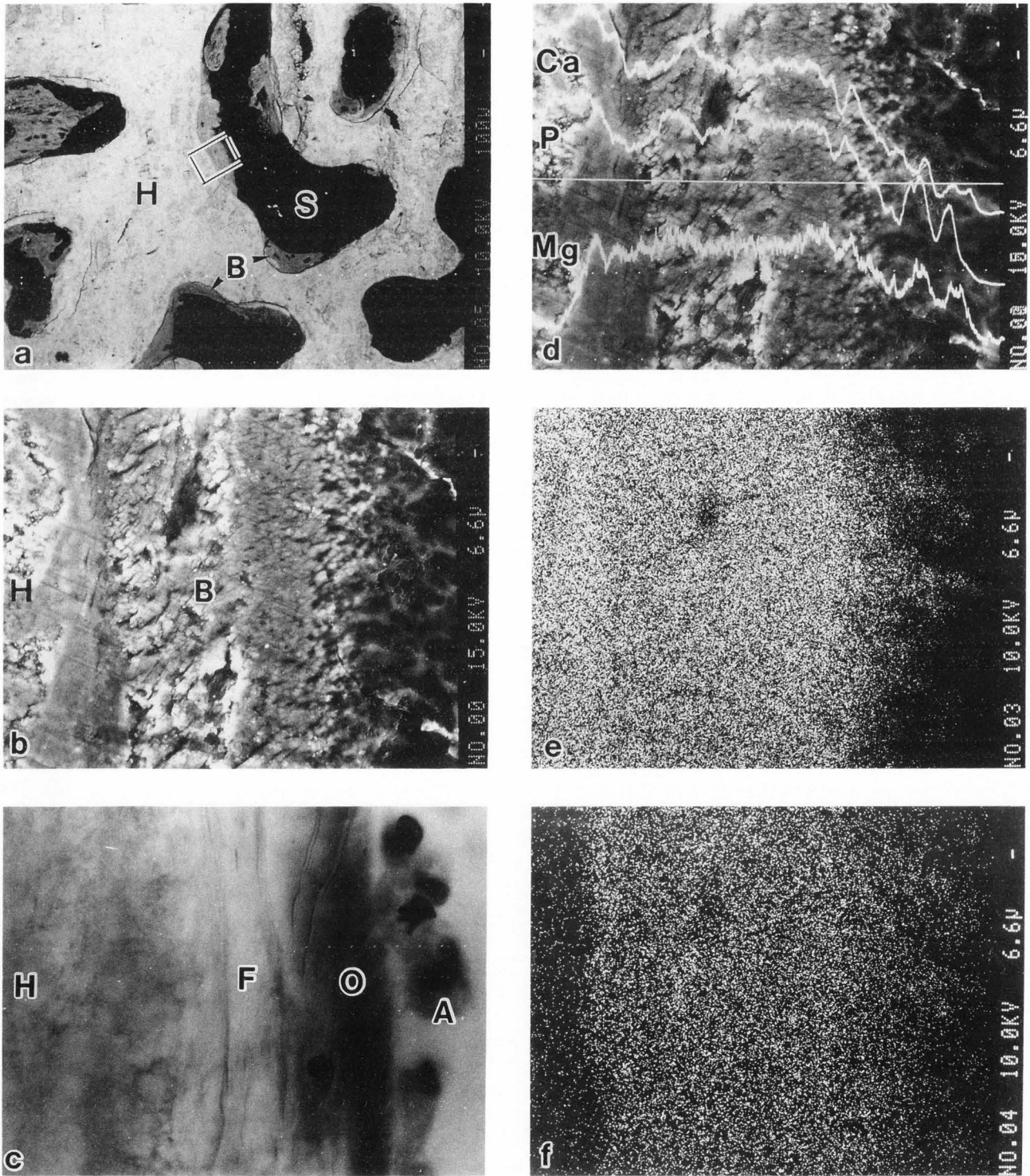


Fig. 2. Ceramic with marrow cells 4 weeks after implantation. **a**: Backscattered electron image of the area corresponding to the upper right area of Fig. 1a. Ceramic (H: white area), bone (B: gray area), and soft connective tissue (S: black area) are seen. **b**: Secondary electron image of the rectangular area of Fig. 2a. Newly formed bone (B) and ceramic (H) are observed. **c**: Light micrograph of an undecalcified thin section of the area corresponding to Fig. 2b; H: hydroxyapatite ceramic, F: fully mineralized bone, O: osteoid, A: active osteoblasts. **d**: Same as Fig. 2b but with superimposed line scans of Ca, P and Mg along the white line indicated at the center of the figure. Full scale for Ca and P is 1000 counts per second; and for Mg is 200 counts per second. **e** and **f**: Characteristic X-ray maps of Ca and Mg respectively for the same area as in Fig. 1d.

machinable bioactive glass-ceramics and bone. *J. Biomed. Mater. Res.* 19, 303-312.

10. Holmes RE (1979) Bone regeneration within a coralline hydroxyapatite implant. *Plast. & Reconstr. Surg.* 63, 626-633.

11. Holmes RE, Bucholz RW, Mooney V (1987) Porous hydroxyapatite as a bone graft substitute in diaphyseal defects. A histometric study. *J. Orthop. Res.* 5, 114-121.

12. Jarcho M, Kay JF, Gumaer KI, Doremus RH, Drobeck HP (1977) Tissue, cellular and subcellular events at a bone-ceramic hydroxyapatite interface. *J. Bioengin.* 1, 79-92.

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

14. Kato K, Aoki H, Tabata T, Ogiso M (1979) Biocompatibility of apatite ceramics in mandibles. *Biomater. Med. Dev. Art. Org.* 7, 291-297.

15. Kenney EB, Lekovic V, Sa Ferreira JC, Han T, Dimitrijevic T, Carranza FA Jr. (1986) Bone formation within porous hydroxyapatite implants in human periodontal defects. *J. Periodontol.* 57, 76-83.

16. Kitsugi T, Yamamuro T, Nakamura T, Higashi S, Kakutani Y, Hyakuna K, Ito S, Kokubo T, Takagi M, Shibuya T (1986) Bone bonding behavior of three kind of apatite containing glass ceramics. *J. Biomed. Mater. Res.* 20, 1295-1307.

17. Kitsugi T, Yamamuro T, Nakamura T, Kokubo T, Takagi M, Shibuya T, Takeuchi H, Ono M (1987) Bonding behavior between two bioactive ceramics in vivo. *J. Biomed. Mater. Res.* 21, 1109-1123.

18. Kitsugi T, Yamamuro T, Kokubo T (1989) Bonding behavior of a glass-ceramic containing apatite and wollastonite in segmental replacement of the rabbit tibia under load-bearing conditions. *J. Bone and Joint Surg.* 71-A, 264-272.

19. Krajewski A, Ravaglioli A, Mongiorgi R, Moroni A (1988) Mineralization and calcium fixation within a porous apatitic ceramic material after implantation in the femur of rabbits. *J. Biomed. Mater. Res.* 22, 445-457.

20. MacDavid PT, Boone ME, Kafraivv AH, Mitchell DF (1979) Effect of autogenous marrow and calcitonin on reactions to a ceramic. *J. Dent. Res.* 58, 1478-1483.

21. Nade S, Armstrong L, McCarthy E, Ceran FI, Baggaley B (1983) Osteogenesis after bone and marrow transplantation. *Clin. Orthop.* 181, 255-263.

22. Nakamura T, Yamamuro T, Higashi S, Kokubo T, Ito S (1985) A new glass-ceramic for bone replacement: evaluation of its bonding to bone tissue. *J. Biomed. Mater. Res.* 19, 685-698.

23. Ohgushi H, Goldberg VM, Caplan AI (1989) Heterotopic osteogenesis in porous ceramics induced by marrow cells. *J. Orthop. Res.* 7, 568-578.

24. Ohgushi H, Okumura M, Masuhara K, Goldberg VM, Davy DT, Caplan AI (1989) Osteogenic

potential of bone marrow sustained by porous calcium phosphate ceramics. *Handbook of bioactive ceramics.* CRC Press Inc. (Eds. T. Yamamuro, J. Wilsonand, L.L. Hench) in press.

25. Osborn JF, Newesely H (1980) Dynamic aspects of the implant-bone interface, in *Dental Implants.* (Ed. G. Heimke), Carl Hanser Verlag, München. pp 111-123.

26. Osborn JF, Newesely H (1982) Bonding osteogenesis induced by calcium phosphate ceramic implants. in *Biomaterials 1980,* (Eds. G.d. Winter, F. Gibbons and H. Plenck), John Wiley and Sons, New York. pp 51-58.

27. Roy DM, Linnehan SK (1974) Hydroxyapatite formed from coral skeletal carbonate by hydrothermal exchange. *Nature* 247, 220-222.

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

29. White E, Shors EC (1986) Biomaterial aspects of Interpore-200 porous hydroxyapatite. *Dent. Clin. North America* 1986, 30, 49-67.

30. Williams KR, Blayney AW, Frootko NJ, Ashton BA (1985) A scanning electron microscopy study of the interface between ceramics and bone. *Biomaterials* 1985, 6, 269-272.

Discussion with Reviewers

J. Appleton: Can the authors speculate on how the osteoblasts become adherent to the surface of the ceramic; and how the developing bone becomes integrated into the surface of the ceramic?

Authors: Our data clearly showed that osteoblasts can adhere to the surface of the ceramic (Fig. 1d). We think the ceramic surface might be overlaid by the Ca-P rich layer having various proteins and speculate that the cell surface of the osteogenic cell has affinity (receptor?) to the reorganized ceramic surface resulting in osteogenic cell adhesion to the ceramic surface. Various models of bone integration could be considered, and as mentioned in the text, there is a possibility that the zone may chemically bind to the bone apatite crystal.