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Osteogenic capacity of rat and human marrow cells in porous ceramics

Experiments in athymic (nude) mice

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Porous hydroxyapatite ceramics, alone and combined with rat marrow cells, were implanted subcutaneously in 22 nude mice. The ceramics alone were invaded by fibrovascular tissue without any bone formation. In contrast, all the ceramics combined with marrow cells had bone formation in the pores 4 to 8 weeks after implantation. The bone formation began on the surface of the ceramic with direct bonding of the bone to the ceramic and proceeded to the center of the pores.

The ceramics were also combined with bone marrow cells from 7 humans and implanted in nude mice. In five experiments, bone formation occurred after implantation. In addition, the ceramics were combined with in vitro cultured fibroblastic cells, resulting in bone formation in 2/6 cases. Our results indicate that the osteogenic ability of human marrow cells is sustained by porous hydroxyapatite ceramics.

Among synthetic orthopedic implants, calcium phosphate ceramics show no toxicity or immunologic response (Cameron et al. 1977, Kato et al. 1979, deGroot 1980, Jarcho 1981, Holmes et al. 1984). They bond directly to bone (Jarcho 1981, Osborn and Newsely 1982), but have no osteogenic properties (Jarcho 1981, Ohgushi et al. 1989a, 1990). Thus, clinical application is restricted to relatively small osseous defects (Cameron et al. 1977). Cells in rodent bone marrow can differentiate into an osteogenic cell line (Friedenstein 1976, Owen 1985), and a combination of ceramics and marrow cells gives very consistent bone formation in the ceramic pores 4 weeks after implantation (Ohgushi et al. 1989a). Also, ceramics combined with marrow induce healing in rat femur segmental massive bone defects (Ohgushi et al. 1989b). However, for clinical applications, an important question is whether or not human bone marrow cells can show osteogenic ability in ceramics. We report experiments focused on this question.

Material and methods

Porous hydroxyapatite ceramics were prepared using the replamine form process (White and Shors 1986). The materials chosen for this study have a pore volume of 45–55 percent and fully interconnected pores, measuring 190–230 μm in diameter. The ceramics were cut into discs, 5 mm in diameter and 2 mm thick.

The bone marrow was prepared as described previously (Okumura et al. 1990), and the ceramics were soaked in the marrow-cell suspension (6 to 8 discs/suspension).

Twenty-two, 5-week-old, male, nude mice (Jcl:AF-nu, Clea Japan Inc.) were anesthetized by ether inhalation. Two incisions (5 mm) on the back of the mouse were made, and subcutaneous pouches were created by blunt dissection. Ceramics alone and ceramics combined with rat marrow cells were implanted. These ceramics were harvested 2 to 8 weeks after implantation. Ceramics in 2 mice were harvested at 8 weeks, specifically for undecalcified plastic-embedding sectioning after fluorochrome labeling. These mice were given one dose of tetracycline (50 mg/kg intramuscularly) 5 weeks after implantation and calcein (15 mg/kg intramuscularly) 6 weeks after implantation.

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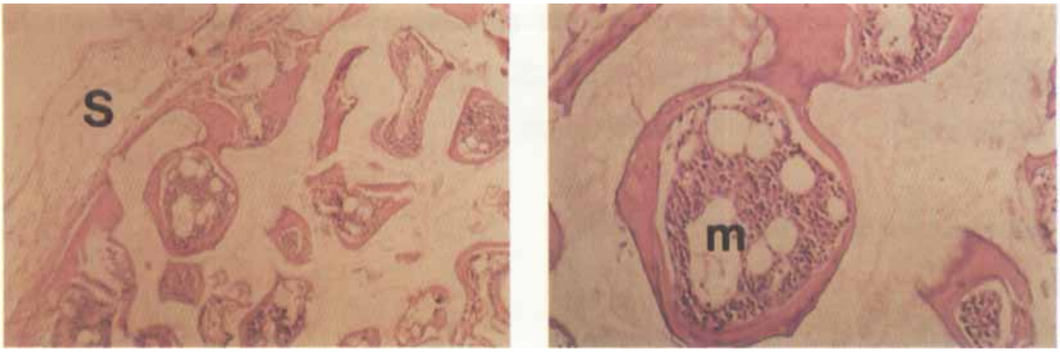
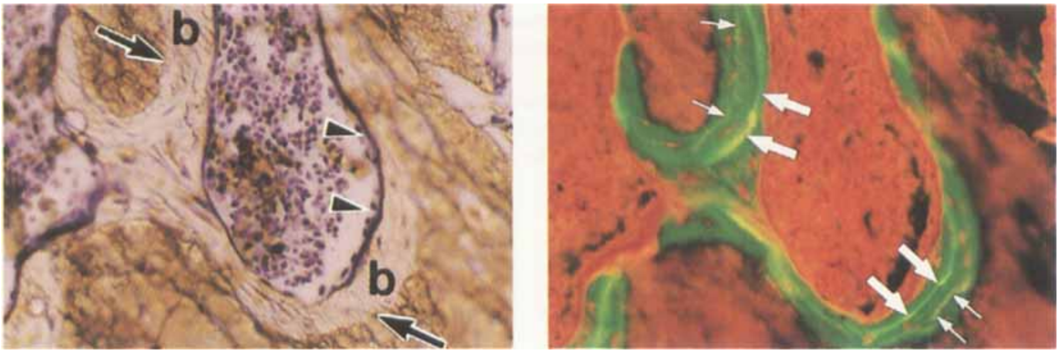


Many ceramic pore areas show bone formation (pink area) together with regenerated bone marrow. S indicates subcutaneous tissue surrounding the ceramic. The ceramic was removed by a decalcification procedure, producing an empty white area. ×28

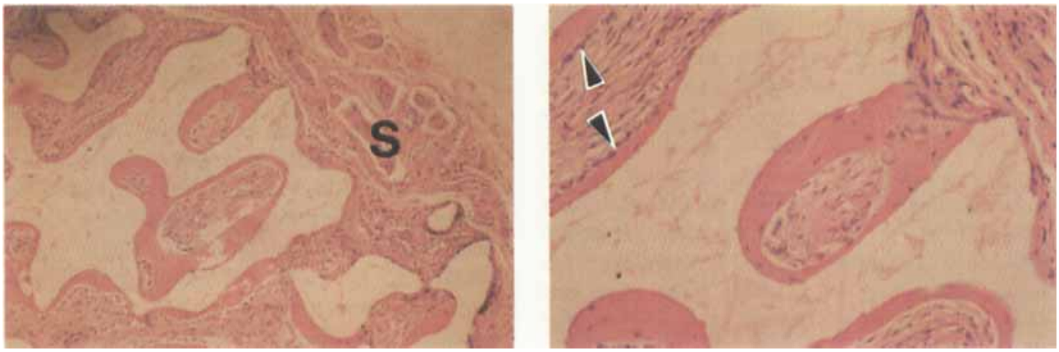
Higher magnification. Regenerated bone marrow (m) is seen in pore region of the ceramic. ×70



Undecalcified section under light microscopy. Arrows indicate the interface between bone (b) and ceramic. Arrowheads indicate active osteoblasts on osteoid streams appearing as a black line. Villanueva's bone stain. ×70

The same section under fluoromicroscopy. Small arrows indicate the tetracycline labeling administrated 5 weeks after implantation and large arrows indicate calcein labeling administrated 6 weeks after implantation.

Figure 1. Eight weeks after subcutaneous implantation of the ceramic with rat marrow cells in nude mice.



Pink areas indicate the bone and S indicates subcutaneous tissue surrounding ceramic. The ceramic was removed by a decalcification procedure, producing an empty white area. ×28

Higher magnification. Arrowheads indicate active osteoblasts. ×70

Figure 2. Six weeks after subcutaneous implantation of ceramic with cultured human marrow cells (from Case 4) in a nude mouse.

Table 1. Bone formation in nude mice following ceramic implantation

Weeks after surgery	Ceramic alone	Ceramic with marrow
2	0/6	1/6
3	0/6	4/6
4	0/4	4/4
8	0/6	6/6

Table 2. Bone formation in nude mice following human marrow cell/ceramic implantation

Case	Age	Sex	Fresh marrow	Cultured marrow
1	4	M	+	–
2	5	M	+	ND
3	13	M	–	–
4	13	F	+	+
5	25	M	+	–
6	46	F	+	+
7	49	F	–	–

ND not done.

Human marrow cells were obtained with informed consent from 7 patients by aspiration through the anterior iliac crest while undergoing reconstructive orthopedic surgery (Table 2). A Komiya needle (1.8 mm diameter, Muraoka Co., Tokyo, Japan) was used for the aspiration. About 2 mL was aspirated and directly applied on the ceramic in a test tube. The marrow-coated ceramics were then implanted in nude mice as described above. In another series of experiments, 1 mL of fresh marrow from the same patients was added to 19 mL of Eagle Minimal Essential Medium supplied with 10 percent fetal bovine serum. Two milliliters of the medium containing marrow cells was then cultured in 35 × 10-mm Falcon tissue culture dish (Becton Dickinson Labware, Lincoln Park, CA, USA) at 37°C in a humidified incubator in 5 percent CO_2 in air. After 2 days of incubation, one-half of the medium (1 mL) was changed. The medium was completely changed thereafter every 2–3 days. After 3–4 weeks, a confluent layer of fibroblastic cells was treated with 0.25 percent trypsin for 5 min at 37 °C. The dispersed cells were then centrifuged and washed with phosphate-buffered saline two times. The ceramics were then immersed in the cultured cells ($10–20 \times 10^6$/mL) and implanted in nude mice as described above.

For decalcified histologic evaluation, the harvested implants were fixed in 10 percent buffered formalin and decalcified (K-CX solution, Falma Co., Tokyo) about 6 hours. Explants were embedded in paraffin, cut into 5-μm sections, and stained with hematoxylin and eosin. For undecalcified histologic processing, harvested implants were fixed in 70 percent ethanol and stained with Villanueva's bone stain. These were then dehydrated in an alcohol series, defatted, embedded in methyl methacrylate, and cut into 7-μm sections using a microtome (Jung Model K). These specimens were examined under light microscopy or fluoromicroscopy.

Results

Bone formation was seen in all the rat marrow soaked ceramic implants after 4 weeks (Table 1 and Figure 1). There was no interposition of fibrous tissue between the newly formed bone and the ceramic surface. Also, regenerated bone marrow was seen in some areas. Undecalcified sections clearly showed that the bone was directly in apposition to the ceramic pore surface Fluorochrome labeling showed tetracycline near the ceramic surface. Calcein was seen close to the center of the pore region. Thus, bone formed from the surface towards the center of the pores by bonding osteogenesis (Osborn 1982). In contrast, there was no bone formation in the pure ceramic implants.

Bone formation was less consistent in implants soaked with fresh or cultured human marrow cells (Table 2). In experiments with fresh marrow, 5 of 7 patients showed bone formation when using marrow directly. There were only two of six positive experiments from human marrow cells that were cultured. The bone that appeared had identical histologic features as the bone from ceramic implants containing rat marrow (Figure 2). The age of the donors did not influence the results.

Discussion

Bone formation was consistently seen in rat marrow soaked ceramic implants in nude mice. The bone directly interfaced to ceramic pore surface. These results were comparable to our previous reports of the implants in syngeneic rats (Ohgushi et al. 1989a and 1990, Okumura et al. 1990). Thus, the present experimental method using subcutaneous implantation in nude mice is useful to evaluate cell-mediated osteogenic potential.

Our results showed that transplantation of young and adult human aspirated marrow cells results in mature bone formation in an extraosseous site. Recently, Bab et al. (1988) reported the production of osteogenic tissue in diffusion chambers inoculated with human marrow cells in nude mice. However, diffusion chambers have little clinical relevance. Also, in their experiment, osteogenic tissue was found from the marrow of only 2 children and in none of the adults.

In contrast to the consistent bone formation that occurred in ceramic implants soaked with rat marrow, 2 out of 7 fresh human preparations did not show bone formation 6 weeks after implantation. The difference may be due to a smaller number of human nucleated cells (less than 10 times) loaded to the ceramics compared with the rat-marrow preparation. However, microfoci of osteogenesis might be present in the negative cases with a potential for bone formation several months later. This is possible because the amount of bone seen in rat marrow soaked ceramics in syngeneic rats increased drastically after 6 months compared with 1 or 2 months after implantation (Okumura et al. 1990).

Friedenstein (1976) reported that rodent fibroblasts derived from bone marrow cultures are capable of bone formation in diffusion chambers. Thus, we tried to answer whether cultured human-marrow fibroblasts would show osteogenic response in the presence of ceramics. As shown in Table 2, the results were inferior compared with fresh rat-marrow preparations. This is probably due to our tissue culture conditions, because in culture the cells easily differentiate into a nonosteogenic clone, resulting in a low incidence of bone formation. Even so, our results indicate that undifferentiated cells in human bone marrow can survive in a tissue culture and differentiate into an osteogenic cell in porous ceramics in vivo.

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