Osteogenesis in cranial defects and diffusion chambers

Comparison in rabbits of bone matrix, marrow, and collagen implants

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In rabbits, we compared calcification and bone formation by bone marrow, acid-demineralized bone matrix and glutaraldehyde-cross-linked Type I collagen implanted in intramuscular diffusion chambers or in trephine skull defects. The rabbits were killed 4 weeks postimplantation and calcification and osteogenesis were evaluated radiographically and histologically, and by calcium and alkaline phosphatase assays. Bone marrow produced bone and fibrous tissue within the chambers and had high alkaline phosphatase levels. Bone matrix in chambers with intact filters failed to induce bone formation within and outside the chambers, while glutaraldehyde-cross-linked collagen produced only scant calcific deposits following implantation in either diffusion chambers or skull defects. Central areas of skull defects implanted with bone marrow were partially repaired with new bone and had high calcium and alkaline phosphatase levels, but not as high as defects implanted with demineralized bone matrix.

Following intraperitoneal or subcutaneous implantation of whole bone marrow or fractions rich in osteogenic cells in diffusion chambers, stromal cells differentiate into osteoblasts and the hematopoietic cells die (Friedenstein et al. 1966, Budenz and Bernard 1980). The osteogenic stromal cells are either inducible or genetically determined to form bone (Vaughan 1981). On the other hand, acid-demineralized bone matrix contains a bone morphogenetic protein (BMP) that is capable of inducing the differentiation of primitive, genetically undetermined, mesenchymal cells into bone (Urist and Strates 1971). Grafts of glutaraldehyde-cross-linked collagen have been found to promote healing of osteochondral and trephine skull defects in rabbits (Speer et al. 1979, Strates et al. 1987), whereas subcutaneous implants of such collagen calcify prior to being resorbed (Nimni et al. 1988a).

We have found that nonspontaneously healing trephine skull defects in young adult rabbits are repaired centrally by the formation of calcified fibrous tissue when grafted with glutaraldehyde-crosslinked Type I collagen, and by new bone when grafted with demineralized bone matrix (Strates et al. 1987). We have also shown (Palmer et al. 1987) that bone marrow and demineralized bone matrix stimulate repair in cranial defects of old rabbits. We now report comparisons of these three types of implants in diffusion chambers and intracranially.

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Materials and methods

Bone marrow was obtained fresh from the tibiae of young syngeneic animals. Type I collagen was extracted from the skin of young adult animals after digestion with pepsin and cross-linked with glutaraldehyde (Nimni et al. 1987). Cross-linking with glutaraldehyde has been widely used in the preparation of collagenous bioprostheses (e.g., heart valves), because it renders them less biodegradable and clinically nonimmunogenic (Nimni et al. 1987).

Implantation. Diffusion chambers (0.45-μm pore diameter filters; Millipore Corp., Bedford, Mass.) were filled with weighed amounts (~5 mg) of marrow, matrix chips, or collagen and implanted intramuscularly, as previously described (Strates et al. 1971), in 12 young adult New Zealand white rabbits. Control chambers were filled with blood serum. Following anesthesia (ketamine:xylazine, 35 mg: 7 mg/kg body

weight; White and Holmes 1976), shaving of the abdomen, and skin preparation with Betadine®, an incision parallel to the midline was made and the skin retracted to reveal the abdominal muscles. Slits were made in the fasciae of recti abdomini; and four implants, enclosed in chambers, were implanted in an equivalent number of muscle pouches of each abdominal side (two chambers per type of implant and two for controls, a total of eight implants per animal).

Eight-millimeter trephine skull defects were made under anesthesia in the frontal and on each side of the parietal bones of 8 rabbits as previously described (Palmer et al. 1987). Defects of 4-mm diameter heal spontaneously, whereas 8-mm defects fail to heal during a period of up to 6 months. One of three 8-mm-diameter defects in each of 6 animals (18 defects totally) was grafted with weighed amounts (~20 mg) of either marrow, matrix, or collagen. Skull defects in the remaining 2 animals were left unimplanted, and served as controls. The skin was closed with nonabsorbable sutures, and Betadine® was applied to the wound.

Processing of implants. Animals were killed by an overdose of the anesthetic 4 weeks after implantation, and implants were immediately excised and radiographed (mammography OM-1 Kodak film, Rochester, NY; 25 kV, 0.3 sec). A 4-week postimplantation time period was used because it is associated with high alkaline phosphatase activity in intramuscular implants combined with a high implant calcium content (Strates et al. 1971a, 1987). Two of the eight intramuscular and one of the intracranial implants per animal were processed for histology (HE and Goldner's stains). The remaining implants were individually assayed for alkaline phosphatase activity (Frankel and Reitman 1963) and DNA (Ceriotti 1952) determined on samples of fresh implant tissue, and for calcium by atomic absorption spectrophotometry (Willis 1960) after drying at 110 °C and ashing the alkaline phosphatase-DNA residues at 700 °C overnight. Alkaline phosphatase/DNA values were expressed as units of enzyme activity per microgram of DNA phosphorus and interpreted as a measure of enzyme activity per cell (Strates et al. 1971b). Implant calcium content was expressed as milligrams calcium per gram of dry tissue.

The radiographic and histologic findings were compared using the descriptive method. The biochemical data were analyzed for significance by the Student's *t*-test.

Results

Of the three types of intramuscular implants in diffusion chambers, only those of bone marrow were radiographically positive for mineral (Figure 1A) and produced tissue histologically similar to immature bone mixed with fibrous tissue (Figure 1C). In trephine skull defects, both marrow and matrix, applied directly on the defect, produced large amounts of calcified bone, whereas the collagen grafts contained only sparse calcific deposits without bone (Figures 1B and D). Calcium content and enzyme activity were highest in the intraskeletal implants of demineralized bone matrix (Table 1).

Discussion

Osteogenesis in fractures and osseous defects is stimulated by osteoconductive matrices (e.g., collagen and demineralized bone matrix), osteogenic cells (e.g., bone marrow stromal cells) and osteoinductive factors (e.g., BMP; Lane and Sandhu 1987). Several investigators (Nade and Burwell 1977, Lindholm and Urist 1980) implanted allogeneic demineralized bone matrix and bone marrow in osseous defects and reported high yields of new bone. To our knowledge, however, there have been no reports comparing such yields in intramuscular implants, with or without enclosure in diffusion chambers, to yields in intraskeletal grafts. In our experience, bone marrow implanted subcutaneously or in muscle pouches without a carrier produces no bone, presumably because of cell dispersal or failure of the osteogenic stromal cells to adhere to the host tissues.

We have recently reported that bone marrow and calvaria cells, enclosed in chambers made of demineralized bone matrix cylinders, stimulate osteogenesis after subcutaneous implantation in old rats (Nimni et al. 1988b). In our present study, bone marrow grafted in skull defects without a carrier produced more bone than when implanted intramuscularly in diffusion chambers. In variance with reports of cartilage, bone, and connective tissue formation in intraperitoneal bone marrow implants enclosed in such chambers (Friedenstein et al. 1966, Ashton et al. 1980), only bone and fibrous tissue were found after intramuscular implantation, due perhaps to oxygen tension differences in the environment of each of the two implantation sites.

As expected (Urist and Strates 1971, Strates et al. 1987), demineralized bone matrix induced the formation of large amounts of bone following implantation in skull defects and failed to induce the formation of bone outside or within intramuscularly implanted chambers with intact filters. The latter finding supports earlier reports that transmembrane transfer of the bone inductive signal, associated with BMP in the bone matrix (Urist and Strates 1971), is unlikely.

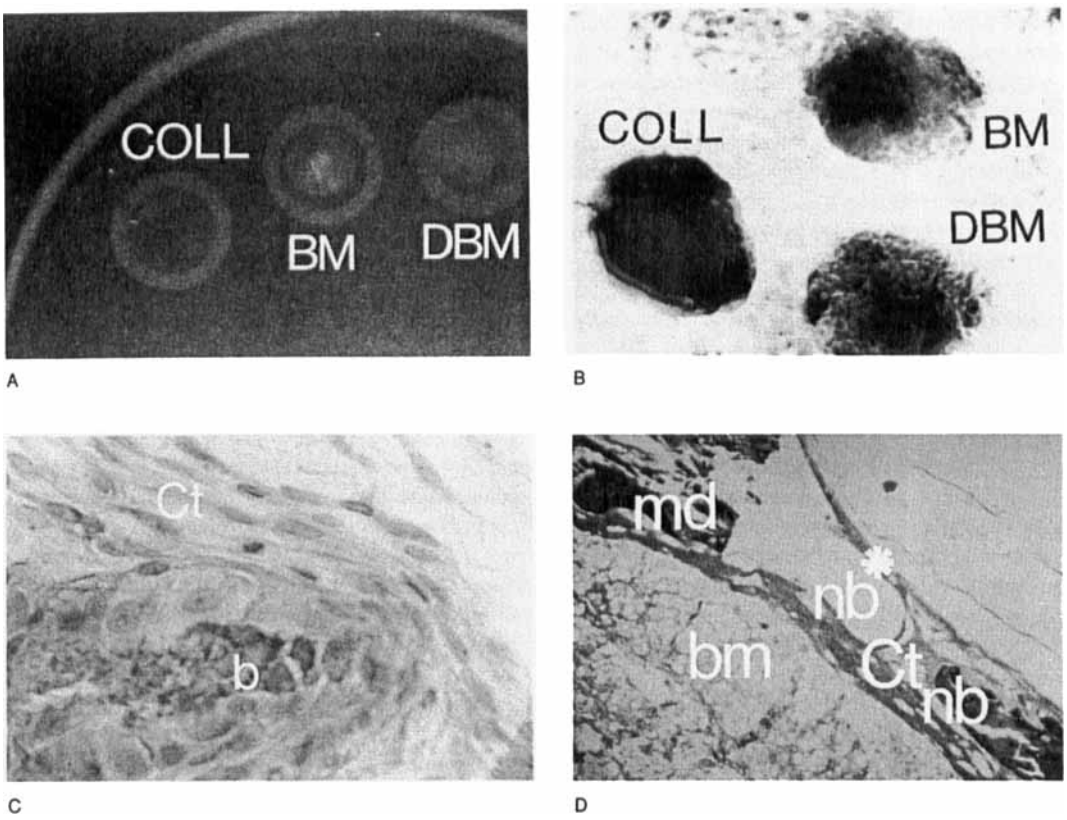

Figure 1. Radiographs and photomicrograhps of bone marrow (BM), demineralized bone matrix (DBM), and glutaraldehyde-cross-linked Type 1 collagen (COLL) 4 weeks after inplantation in young adult rabbits.
A, C. After enclosure in diffusion chambers (HE, x400)
B, D. After grafting in a trephine skull defect (Goldner's stain-methylmetacrylate, x120).
Note fibroblast- like cells and bone in C and nearly complete bridging of the defect in D.
Ct connective tissue; b bone; bm bone marrow; md mineral deposits; nb new bone; nb* broken off bone tissue (methacrylate embedding).

Table 1. Calcium and alkaline phosphatase (AlkPase) levels (expressed as AlkPase/DNA ratio) in intramuscular and cranial implants of demineralized bone matrix (DBM), bone marrow (BM), and glutaraldehyde-cross-linked Type I collagen 4 weeks postimplantation in young adult rabbits (Mean *SEM)*

Type of implant	Intramuscular (diffusion chambers)				Cranial (trephine defects)			
	[Ca] mg/g dry tissue		AlkPase/DNA U/µg DNA-P		[Ca] mg/g dry tissue		AlkPase/DNA U/µg DNA-P	
DBM	0.5	*0.3*	11.6	*4.4*	131	*8.3*	209	*22.8*
Collagen	0.7	*0.2*	9.1	*3.6*	35.4	*6.7*	23.1	*4.6*
BM	49.6	*6.8*	82.7	*13.2*	94.7	*7.3*	133	*14.6*

Intramuscular implants—BM vs DBM or collagen: $P < 0.001$.
Cranial defect implants—DBM vs BM: $P < 0.01$ (calcium), $P < 0.001$ (AlkPase); DBM or BM vs collagen: $P < 0.001$.

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