New bone induction by demineralized bone matrix in immunosuppressed rats

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Abstract. Subcutaneous implantation of demineralized bone matrix (DBM) initiates a sequence of developmental events which culminate in endochondral bone formation. To test the effects of T-cell deficiency on new bone formation, the morphology of DBM-induced bone was examined in rats thymectomized at three weeks of age and in thymectomized or nonthymectomized rats lethally irradiated and reconstituted with syngeneic bone marrow. At 24 days after implantation, bone induction in control rats was appropriate for their age, while thymectomized-irradiated-reconstituted rats and thymectomized rats had significantly more new bone and larger bone marrow space than the controls. In non-thymectomized, irradiated and reconstituted rats, bone induction occurred in only 25% of the animals, compared to 95% in other groups.
Key words. Bone matrix; osteoinduction; thymectomy; irradiation, bone marrow reconstitution; immunosuppression.

There is a close interdependence of bone and the immune system[1], but the mechanisms of their interaction are not well understood, especially in vivo. Animals with immunodeficiencies provide a valuable tool for dissecting the role of the immune system in bone growth and turnover. Vignery et al. have reported that bone turnover is markedly altered in athymic (nude) mice lacking T-lymphocytes[2]. They observed a 10-fold slower bone formation rate and 3-fold fewer and less active osteoclasts, and attributed the changes to the indispensability of T-cells for the regulation of bone turnover. However, another study showed that athymic mice had a physiological bone turnover comparable to that of euthymic mice of the same age[3].

In the present study, we investigated the effects of T-cell deficiency on new bone formation induced by subcutaneous implantation of demineralized bone matrix (DBM) in rats. The developmental cascade of DBM osteoinduction includes mesenchymal progenitor cell chemotaxis, proliferation and differentiation into chondrocytes, enlargement of chondrocytes and invasion, calcification of cartilage matrix and formation of bone[4,5]. New bone is finally remodeled by osteoclasts, and bone marrow space is formed[4,5].

Materials and methods

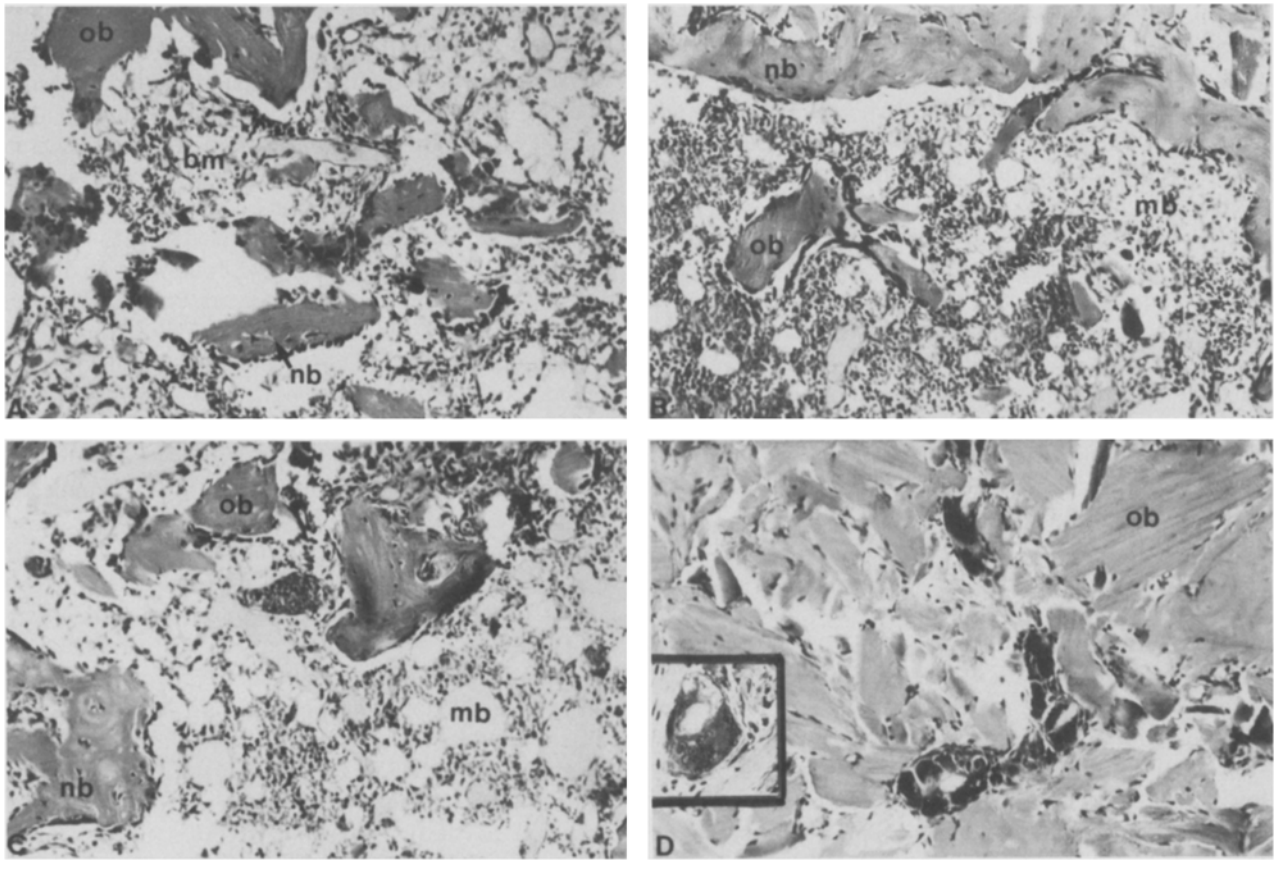
Preparation of demineralized bone matrix (DBM) powder (particle size, 74–420 μm) from normal adult rat diaphyses has been described in detail elsewhere[5]. Age-matched inbred WVM rats (derived from the Wistar stock) of both sexes were divided randomly into the following groups: 1. control, 2. thymectomized, irradiated and bone marrow-reconstituted (TIR), 3. thymectomized (T), and 4. irradiated and bone marrow-reconstituted (IR). Thymectomy was performed at 3 weeks of age, as described elsewhere[6]. After three months, rats were lethally irradiated with 6.5 Gy and reconstituted with 6×10^7 syngeneic bone marrow cells[6]. After recovery of the bone marrow (three months), 25 mg-portions of the DBM powder were implanted subcutaneously in pairs in the parasternal region under light ether anesthesia. Twenty-four days after matrix implantation, animals were sacrificed by cervical dislocation. Implants were excised, one was processed for histology and the other assayed biochemically.

Blood drawn from the tail vein was used for the white blood cell count (WBC). The preparation and measurement of anti-mouse red blood cell hemagglutinin titer and tail-to-tail skin grafting have been described in detail elsewhere[6]. Rats of the inbred Fisher strain were used as donors of full-thickness tail skin allografts. For histological analysis, matrix implants were fixed in 70% ethanol, embedded in methylmetacrylate without prior decalcination, cut into 5 μm-thin serial sections, and stained with modified Goldner's trichrome stain[7]. The percentage of original matrix replaced by new bone and bone marrow was measured by counting points of a Zeiss integrative ocular grid over respective bone compartments[7,8]. Newly formed bone was discriminated from the demineralized bone matrix on the following criteria: (i) demineralized matrix stains pink or light green and newly formed bone stains dark green; (ii) bone matrix is acellular, while new formed bone has osteocytes in the lacunae; and (iii) bone matrix retains a lamellar organization, while newly formed bone has a woven appearance[7,8]. For biochemical analysis, bone matrix implants were lyophilized and assayed for calcium, phosphorus, magnesium and zinc content using inductively coupled plasma argon emission spectrometry (ARL 35000 C ICP; ARL, Ecublens, Switzerland), as described elsewhere[8]. Results were expressed as means ± standard error of the mean (SEM) of 5–8 animals per group. Statistical differences were calculated using the analysis of variance (ANOVA) and Newman-Keuls test for multiple posthoc comparisons.

New bone formation and immunological status of immunosuppressed rats 24 days after subcutaneous implantation of demineralized bone matrix.

	Control	Thymectomized irradiated reconstituted	Irradiated reconstituted	Thymectomized
Bone matrix implants (%)				
New bone	6.2± 1.8	12.7± 2.1[a,c]	1.2± 0.6[a]	12.1± 2.3[a,c]
Old matrix	85.2± 5.5	58.0±10.7[a,c]	97.5± 1.2[a]	73.6± 6.0[a,c]
Bone marrow	8.6± 3.9	29.3±10.5[a,c]	1.2± 0.6[a]	14.3± 3.8
Elements (μg/mg dry weight)				
Ca	41.3± 9.1	61.9± 4.0[a]	42.7± 4.7	52.5± 5.9
P	26.0± 4.9	37.4± 2.6[c]	25.1± 2.3	33.4± 3.3
Mg	0.8± 0.3	1.4± 0.1[a,c,d]	0.7± 0.1	1.0± 0.1
Zn	0.2± 0.1	2.1± 1.7	0.9± 0.7	0.5± 0.3
Immunology				
WBC ($\times 10^9$/L)				
Total	8.5± 0.2	5.8± 0.4[a]	5.8± 0.3[a]	4.8± 0.2[b]
Mononuclears	5.8± 0.1	3.4± 0.2[b,c]	4.1± 0.1	3.4± 0.1[b,c]
Polymorphonuclears	2.7± 0.2	2.4± 0.3	1.8± 0.3	1.5± 0.2
Hemagglutinin titer ($-\log_2$)*	6.4± 0.2	3.4± 0.2[a,c]	5.0± 0.3	4.6± 0.2[a]
Graft survival time (days)**	15.3± 1.7	33.0± 0.9[b,c]	26.3± 1.7[a]	30.0± 0.9[b]

Values are means ±SEM of 5–8 animals. Rats were thymectomized at 3 weeks of age, lethally irradiated with 6.5 Gy and reconstituted with 6×10^7 syngeneic bone marrow cells at the age of 3 months. Demineralized bone matrix from healthy rats was implanted at the age of 6 months. Experiment was repeated twice with similar results. * To mouse (CBA strain) erythrocytes. ** Tail skin donors were allogeneic Fisher rats. [a] $p<0.05$ and [b] $p<0.01$, significantly different from the control; [c] $p<0.01$, significantly different from the irradiated (IR) group; [d] $p<0.05$, significantly different from the thymectomized (T) group.



Demineralized bone matrix implant from control (a), thymectomized-irradiated-bone marrow reconstituted (b), thymectomized (c) and irradiated-bone marrow reconstituted (d) mice, 24 days after subcutaneous bone matrix implantation. ob, old bone matrix; nb, newly induced bone; mb, bone marrow. Magnification 100 x, Goldner's trichrome stain. Inset in (d): single multinucleated giant cell surrounded by particles of demineralized bone matrix.

Results

Immunological data (table) showed that all treated rats had severely depressed immune functions. Cellular immunity (allogeneic skin graft rejection) was significantly depressed in TIR, IR and T rats. TIR rats, and T rats to a small degree, also showed a depression of humoral immunity (hemagglutinin titer).

The implantation of the demineralized bone matrix induced new bone formation in 95% of the implants in TIR, T and control animals. Similar well-formed ossicles with abundant, mostly red bone marrow were observed in control, TIR and T rats (fig. a–c). Significantly more bone matrix was replaced by new bone in TIR and T rats (table). Bone marrow in ossicles from TIR and T rats was predominantly red, while that from the controls contained more fat cells. On the other hand, new bone formation was poor in the IR group and was found in only 25% of the animals (table). The particles of implanted bone matrix were surrounded by a thin fibrous envelope. Occasionally, multinucleated giant cells were found as isolated cells or in clusters among the particles of demineralized matrix (fig. d).

Discussion

The data presented in this study indicate that immune status of the rat influences the induction of new bone by demineralized bone matrix. Apart from the profound depression of cellular immunity, TIR rats also showed a decrease in humoral immunity, suggesting a damage of helper T-cells. The function of T-cells was preserved better in IR and T rats, owing to the maturation of donor bone marrow stem cells in radiation-resistant parts of the thymus in IR rats, and to the presence of helper T-cells developed before three weeks of age in T rats[9]. Slow recovery of the cellular type immunity in IR rats has previously been reported, and has been ascribed to the age of the animals[9]. On the other hand, the profound adverse effects of thymectomy on cellular immunity indicated that the development of rat T cells was not finished at three weeks of age.

The age of the animals also influenced the efficiency of new bone induction: 6.2% of the bone matrix replaced by new bone is significantly lower than over 50% reported in normal young rats[7]. It has been shown that aged rats have decreased potential for new bone induction[4]. However, the morphology of the ossicle was appropriate for the 24th postimplantation day, indicating that this deterioration of new bone formation was not due to the slowing of the osteoinductive sequence, but rather to the slower remodeling of the old bone once the ossicle is formed. The most important finding was the stimulation of the new bone formation in thymectomized animals (TIR and T groups), regardless of the subsequent irradiation and bone marrow reconstitution. This observation is in contrast with the report of either slowed[2] or normal[3] bone turnover in athymic mice. These differences may be species-related, or may depend on the experimental model. Nevertheless, it is clear that T-cells and the immune system in general are crucial for normal bone growth and turnover, and that animals with experimentally-induced immunodeficiencies may be a useful and well-controlled model for studying the interaction of those two systems. Very poor bone induction in IR rats may reflect the effect of whole body irradiation on the mesenchymal progenitor cell proliferation[10]. The appearance of multinucleated giant, osteoclast-like cells among the demineralized bone particles in IR rats is unexplained at the moment. Some authors suggest that such cells can be found in implants of mineralized bone powder, which has no inductive capacity, and that they are not osteoclasts, although they can resorb bone, but rather multinucleated phagocytic cells of macrophage origin (J. Glowacki, personal communication). It is unlikely that the bone powder used in our study was mineralized since it successfully induced new bone formation in other experiments.

The influence of thymectomy and irradiation on systemic hormones affecting bone growth is also important, since it has been shown that both athymic mice[11] and rats thymectomized or irradiated at an early age[12,13] have altered thyroid function, which may in turn affect bone metabolism. However, hypothyroidism usually slows bone growth[10]; this was not observed in TIR and T rats. Moreover, hypothyroidism in irradiated or thymectomized animals can be prevented by reconstitution with normal lymphoid cells[11,12], which was the case in the TIR and IR animals.

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