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Xenogenic demineralized bone matrix: osteoinduction and influence of associated skeletal defects in heterotopic bone formation in rats

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Abstract Demineralized bone matrix (DBM) was ectopically implanted in 36 male Wistar rats. In 18 of the animals a bone defect in the femoral condyles was also created: the left was filled with DBM and the right was left empty as a control. The animals were killed after 2, 4 and 6 weeks and new bone was histologically evaluated, comparing ectopic bone formation with or without distant bone injury. Results showed: (1) osteoinductivity of xenogenic DBM, and (2) earlier mineralization of ectopically implanted DBM in the group with associated skeletal injury. Our results show that xenogenic bone matrix acts as an osteoinductive material and that skeletal injury improves osteogenesis at distant sites.

Résumé Sur 36 rats mâles Wistar, la matrice osseuse déminéralisée (DBM) était ectopiquement implantée. Dans le même temps un défaut osseux au niveau des condyles fémoraux était réalisé sur 18 d'entre eux: à gauche ils étaient remplis avec la DBM et à droite, ils étaient laissés vides. Les rats furent sacrifiés après 2, 4 et 6 semaines et le nouveau tissu osseux était évalué histologiquement en comparant la formation du tissu osseux ectopique avec ou sans la lésion de l'os. Les résultats démontrent: (1) l'ostéoinductivité de la DBM xénogénique, (2) la minéralisation plus rapide de la DBM implantée ectopiquement dans le groupe avec lésion du squelette. Nos résultats démontrent que la matrice osseuse xénogénique agit comme un matériel ostéoinductif et que les lésions du squelette améliorent l'ostéogénèse des sites distants.

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Introduction

Demineralized bone matrix (DBM) is an example of osteoinductive material [1], which has the property to induce differentiation toward an osteoblastic phenotype. The term "induction" refers to the action of some growth factors that may promote a cascade of events that lead from a pluripotent stem cell to a mature osteoblast and to bone tissue formation.

The preparation and demineralization of bone reduces antigenicity and makes the proteins in the bone matrix bioactive and more available for interaction with local cells. In fact, the osteogenic potential of DBM has been ascribed to the presence of bone morphogenetic proteins (BMPs) and some growth factors (transforming growth factor beta, insulin-like growth factor, fibroblast growth factor) that are bound to the extracellular collagen matrix. These proteins promote the chondroblastic differentiation of mesenchymal cells, followed with new bone synthesis by endochondral osteogenesis [4, 5].

The aim of our study was to evaluate both the effects of rabbit DBM powder implanted in rat muscle and bone, and to verify the effects of systemic factors on osteogenesis.

Materials and methods

Demineralized bone powder

Demineralized cortical bone matrix was prepared as described by Reddi and Huggins [2]. The midshafts of the long bones of adult New Zealand rabbits were harvested under aseptic conditions at the killing, cleaned of soft tissue and marrow, washed in sterile distilled water with continuous stirring, then washed three times in 95% ethanol for 15 min, rinsed in ether for 15 min, and finally air dried overnight.

The cleaned and dried bones were then milled to obtain 400–700 µm granules and then demineralized in 0.6 N HCl three times for 1 h (50 ml HCl per g of bone). The bone powder was rinsed with several changes of sterile distilled water to adjust the pH, three times in 95% ethanol and once in ether. The DBM was air dried and stored in sterile plastic containers at 4°C until being used for implantation.

This entire process was performed under sterile conditions (except for the milling) and a sample was cultured to demonstrate that specimens contained no bacterial or fungal contamination.

Animal experiments

Thirty-six 12-week-old male Wistar rats (Charles River, Calco, Lecco, Italy), weighing 300–350 g at the beginning of the study were used. The animals were stabled, three per cage, and fed with a standard pellet diet (Mucedola srl, Settimo Milanese, Milan, Italy) under standard environmental conditions (strictly following Italian and European law on animal experiments D.L. 116/92).

All animals were anaesthetised by means of a subcutaneous injection of ketamine 87 mg/kg (Ketavet, Farmaceutici Gellini, Aprilia Lt-Italy) and xylazine 13 mg/kg (Rompun, Bayer Italia SpA, Milano, Italy) and the dorsal area was shaved. Under aseptic conditions two muscular pouches were created in the two flanks: the right was left empty (sham) and the left was filled with 20 mg of DBM powder. At the same time, the femoral condyles of 18 rats were exposed by means of lateral longitudinal skin incisions and defects of 2 mm diameter and a length of 3 mm were created. Those in the right condyle were untreated and those in the left condyle were filled with DBM.

Two animal groups were obtained: Group I: 18 rats with ectopic implant of DBM, Group II: 18 rats with ectopic implant of DBM plus bone defects. Antibiotic therapy was given during the first 3 postoperative days (flumequine, Flumexil, Fatro, Bologna, Italy: 3 mg/100 g body weight s.c.). Six animals in each group were pharmacologically killed (Tanax, Hoechst, Frankfurt am Main, Germany) after 2, 4 and 6 weeks.

Histology

The implants were removed 2, 4 and 6 weeks postoperatively. Half of the muscular implants from each animal were fixed in 10% formalin, paraffin embedded and sections of 5–6 μ m (Microm HM 340 E microtome) were stained with haematoxylin and eosin.

The remaining samples (muscular and femoral) were fixed in 4% paraformaldehyde, methacrylate embedded, sectioned at 80 $\pm$ 10 μ m on a microtome with a circular diamond saw (Leitz 1600) and stained with Toluidine blue and von Kossa. For the histometric analysis, the femur samples were cut at the same distance from the surface of the bone, in order to make comparison possible between the areas to be observed. Results were evaluated using a semiautomatic imaging program (KS 300 v.2.00, Kontron Elektronik, Munchen, Germany) by an investigator blinded to the study.

Statistical analysis

Values are expressed as means and one standard deviation. To compare data in the same group, the Student's *t*-test for paired data was used and $P < 0.05$ was accepted as showing a significant difference.

Results

Bone implant histology

Regarding the bone implants, measurements of bone defect area after 2, 4 and 6 weeks are reported in Table 1. At 2 weeks there was no statistical difference between the two femurs. However, statistical analysis showed a significant increase in the healing process of the bone at 4 and at 6 weeks, when the femur that received DBM was compared with the one that did not.

Table 1 Bone defect area (mm²) in rat femur (original area of hole 3.14 mm²). Mean $\pm$ SD ($n=18$)

Group II	2 weeks	4 weeks	6 weeks
R (empty)	2.13 $\pm$ 0.45	1.19 $\pm$ 0.51	0.92 $\pm$ 0.41
L (+DBM)	1.92 $\pm$ 0.77	0.72 $\pm$ 0.43	0.53 $\pm$ 0.42
(<i>t</i> -test R versus L)	ns	$P=0.0025$	$P<0.0001$

Muscular implant histology

Light microscopy of DBM muscular implant sections showed that all the samples had similar trends and had the following modification of the original tissue where the DBM was implanted: granulation tissue was observed with polymorphonuclear and mononuclear cells infiltration. These kinds of cells infiltrate the crevices of the demineralised matrix during reabsorption. This phenomenon is more evident at the edges of the fragments where the surface is eroded and has crevices. Fibroblasts penetrate through the vascular channels and form fibrous capsules that surround the implants. The presence of chondroblasts is observed and subsequently newly formed cartilage is mineralized. The DBM undergoes gradual re-mineralization, as shown by dark blue mineral precipitates.

At 2 weeks, the beginning of vascularization inside demineralized bone fragments was observed in Group II samples (Fig. 1). At 4 weeks, chondrocytes, derived from fibroblasts migrated in the implant site, were observed (Fig. 2). At 6 weeks, the chondrocytes were partly replaced by osteocytes (a positive reaction of the von Kossa staining). The presence of newly formed bone was observed under polarized light (Fig. 3). In Group I the same processes could be observed but with a delay of about 2 weeks. In fact, in the first control the DBM seemed almost intact (Fig. 4) and the processes observed in Group II at 30 days was present only at 45 days in Group I. Apparently the bone trauma in the femurs of Group II stimulated the activation and circulation of osteogenic factors that act systemically (via circulation) and thus, influenced an earlier appearance of ectopic bone. None of the muscular samples without DBM showed evidence of the above-described processes.

Discussion

Our study focuses on the osteoinductive properties of DBM, both heterotopically and orthotopically implanted and on the possible influences of distant skeletal injuries in heterotopic mineralization of DBM. Supporting previous reports, all the animals responded to the bone xenogenic demineralised matrix implant, whose efficacy was previously tested in vitro in mouse fibroblasts: L929 cells after contact with DBM showed a chondroblastic phenotype [3].

In Group II the femoral bone defect healed faster than the empty controls: xenogenic DBM was demonstrated

Fig. 1 *Group II*: 2-week sample. Haematoxylin and eosin stain, 20×

Fig. 2 *Group II*: 4-week sample. Toluidine blue stain, 20×

Fig. 3 *Group II*: 6-week sample. Polarized light microscope, 10×

Fig. 4 *Group I*: 2-week sample. Toluidine blue stain, 20×

to be a good osteoinductive material, capable of inducing osteogenesis and accelerate healing. Furthermore, it could be a readily available alternative to autologous bone graft material used in orthopaedic surgery. The use of DBM also avoids a second surgical incision; thus, reducing the morbidity associated with harvesting autologous bone.

An ectopic bone formation was also obtained in the DBM muscular implants, whose development occurred according to the enchondrial osteogenesis model. In both groups of rats studied, the bone formation was formed at different times: in Group II the presence of bone trauma in the femoral condyle bone had notably accelerated the appearance of newly formed bone. The healing of a bone defect is accompanied by the presence of osteogenic factors that are locally stimulated at the site of the bone trauma. The released factors not only act locally, but also systemically; thus, influencing bone remodelling in the whole skeleton. Among the factors involved are the bone morphogenetic proteins, some growth factors as transforming growth factor-beta, fibroblast growth factor,

platelet-derived growth factor, and prostaglandins of the E series. These findings suggest clinical implications in the treatment of non-union fracture or in the aged. The bone-inducing factors seem to be useful for the treatment of local skeletal deficiency and they could be vehicled in association with DBM. It would be interesting to assess how to safely and effectively utilise cytokines in the treatment of disorders of the skeleton.

To summarize, our results show that xenogenic DBM acts as an osteoinductive material, and improves bone repair. In fact, femur bone defects filled with DBM show better healing compared to empty holes. Moreover, the data confirm how skeletal injury improves osteogenesis at distant sites also, as shown by histologic observation of ectopic bone formation: mineralization of DBM implants in Group II (with bone injury) appeared earlier than Group I (without bone injury). In addition, we ob-

served that the presence of more than one bone defect in the same animal should be taken into account when designing experimental animal models.

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