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Osteogenic Efficiency of Demineralized and Lyophylized Xenogeneic Bone and Syngeneic Dentine Implants in Mice

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Intramuscular implantation of demineralized and lyophilized rat bone matrix and murine lower incisors into thigh muscles of BALB/c mice results in deposits of bone adjacent to the implants, a phenomenon termed as ectopic osteogenesis. The yield of induced bone does not critically depend on the mass of implanted matrices, and thus on the quantity of bone morphogenetic proteins (BMPs) present in the implants. A positive correlation between bone matrix implant weight and the yield of induced bone was observed only 28 days post grafting, i.e. when endochondreal osteogenesis is completed and bone resorption has not advanced. A more consistent yield of bone induction wasobserved in the case of demineralized tooth implants. It is postulated that chondro/osteoinduction by demineralized, lyophilized matrix implants is not determined by the range of BMPs presumably released in proportion to implant size, but is rather limited by the population of responsive host mesenchymal cells.

Key words: Bone induction, bone matrix implant, chondro/osteoinduction, demineralized and lyophilized rat bone matrix, dentine implants.

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Bone and dentine are mineralized tissues possessing the ability of triggering mesenchymal cells to differentiate into cartilage and/or bone. This ability is related to the presence of non-collagenous proteins, demonstrated and named bone morphogenetic proteins (BMPs) by URIST (1965). BMPs comprise about 1% of all proteins forming extracellular bone and dentine matrix. On introduction into skeletal muscles these proteins trigger differentiation of mesenchymal cells into chondroblasts and/or osteoblasts (REDDI & CUNNINGHAM 1993; GALUS *et al.* 2006; WŁODARSKI *et al.* 2009) and under physiological conditions are regulators of bone remodeling (ROBEY & BOSKEY 1996; GALLAGHER & DILLON 1997).

Allogeneic demineralized bone matrix has been widely used in orthopaedic surgery because of its inherent osteoinductive properties. This is also a well-established and widely applied experimental model for investigations on heterotopic bone induction because donor age and gender do not influence the osteoinductive properties (TRAIANEDES *et al.* 2004)

However, when grafted into muscles, mineralized bone and dentine usually fails to generate ectopic chondro- and osteogenesis. This is explained by the very slow release of BMPs from mineralized matrices, the concentrations of which do not reach the threshold level of differentiation principles. When bone or dentine is hydrolyzed to remove the mineral phase before implantation, the release of BMPs from matrices is rapid and achieves concentrations adequate to induce chondro- osteogenesis in resident muscle mesenchymal cells. The induced hyaline cartilage is a template for endochondral osteogenesis, but BMP signaling also induces intramembranous ossification.

The phenomenon of cartilage/bone induction by demineralized bone matrix is neither gender nor species-specific, i.e. it works across species (WŁODARSKI *et al.* 2001).

BMPs as signaling molecules trigger the differentiation of responsive resident mesenchymal cells into chondro- or osteoblasts, thus the formed cartilage/bone escapes the immune host reaction.

In this paper we examine the influence of the weight of implants of demineralized rat bone matrix on the yield of bone induction in mice. To this end the samples of demineralized rat bones and demineralized murine lower incisors from the same lot were weighed and implanted intramuscularly into mice and the yield of induced bone was determined 3 to 5 weeks post implantation in order to quantitatively determine the effectiveness of the implanted materials.

Material and Methods

The study was conducted under a research protocol approved by the II Local Ethical Committee at Medical University of Warsaw, nr 26/2006 dated 24.10.2006

Four month-old BALB/c inbred female mice and adult Wistar rats were used in accordance with the Medical University of Warsaw's guidelines for the care and use of laboratory animals.

Demineralized rat bone and murine lower incisor preparation and characterization

Wistar female rats aged 4 months were sacrificed, femoral bones excised, after removal freed of attached soft tissues, the diaphyses were cut off and the bone marrow was flushed out by buffered saline (PBS). Bone shafts were hydrolyzed overnight in an excess of 0.6 N HCl at refrigerator temperature, then extensively washed with sterile distilled water and stored at -70°C in sterile vials until lyophylization. The size of implants is related to their weight and because bone samples were obtained only from the long bone shaft, they were of similar porosity and surface. Other structural features were not considered. No sterilization procedure was used before the implantation.

Before use as implants, lyophilized bones were cut into pieces of various sizes, weighed on an analytical balance with an accuracy of 0.1 ± mg, and implanted into a pocket made in the thigh muscles of mice.

Demineralized murine lower incisors were prepared according to previously described methods (WŁODARSKI *et al.* 2010). Due to the small size of incisors, they were hydrolyzed in 0.6 N HCl for only 5 hours. Lyophilized incisors were implanted into pockets made in thigh muscles of hind legs as were the lyophilized bone chips.

Before implantation the lyophilized incisors were weighed. They were homogenous in size, shape and weight. On average their weight was 0.95 mg ±0.05 and thus were considered as having a standard dose of BMPs when compared with the bone samples of a range of weights.

Implantation of demineralized bone and dentine

Mice were anaesthetized with chlorohydrate (0.15 ml of saline 0.36% solution given intraperitoneally). After shaving, small (3-4 mm) longitudinal skin and muscle incisions were made on the inner side of both thighs and pieces of demineralized and lyophilized rat bones of known weight or murine incisor were inserted into the muscle pockets. Muscles and skin were sutured with 3-0 Dexon S polyglycolic acid suture and the wounds were disinfected with 70% ethanol. Individual recipient mice received bilateral implants from one set of prepared bone or incisors.

Evaluation of bone yields by implanted matrices

Animals were killed by cervical dislocation 22, 28 and 35 days post demineralized bone implantation and 28 and 35 days post incisor implantation. On dissection no evidence of an inflammatory reaction was observed, which speaks for the sterility of the implants. The implants together with the surrounding tissues were excised and hydrolyzed overnight in 0.1N NaOH at 64 °C. The application of this treatment ensures that demineralized matrices and soft tissues including cartilage are completely dissolved, without affecting mineralized tissue. Thus, the undigested material recovered is solely the mineralized component of the induced bone. These undigested deposits were washed in distilled water and dried overnight at 64 °C. The dried mineralized tissue recovered from each implant of known weight was weighed with an accuracy of ±0.1 mg.

The osteogenic effect of the individual implant was determined in order to assess the relationship between the implant weight and its bone yield at different post implantation intervals.

The number of implants, their division into groups according to the type of implanted material, the schedule and the bone induction yields are presented in Table 1.

Statistical analysis

The mean values of the implants and the bone yield weight for each group were calculated and the difference between the smallest standard deviation and the largest was evaluated for initial charcteristics. For initial characteristics of timing groups.

The significance of differences between bone matrix weight between the three experimental groups was analyzed using the ANOVA variance test.

Table 1

The yield of bone formation in BALB/c mice by demineralized and lyophilized rat bone and murine incisors implanted intramuscularly, harvested at various times and implant weights

Duration of implant (days)	No of samples	Implant weight (mg) Average ± SD	Yield of induced bone (mg) Average ± SD
		Demineralized and lyophilized rat bone matrix	
22	18	4.37 ± 1.40	0.46 ± 0.94
28	16	3.44 ± 1.76	0.59 ± 0.58
35	29	3.88 ± 1.75	0.50 ± 0.49
		Demineralized and lyophilized murine lower incisors	
28	32	0.95 ± 0.05	0.48 ± 0.34
35	17	0.96 ± 0.07	0.27 ± 0.34

The relationships between the weight of implanted bone matrices and the weight of induced bone were examined using the Pearson correlation coefficient. Differences among experimental groups were considered significant at a P-value of <0.01. Since the Pearson correlation was statistically significant, linear regression analysis was used to compare the weight of implanted matrices and the weight of induced bone.

The significance of the differences between the weight of bones induced by dentine matrix implantation on day 28 and 35 were analyzed using Student's *t*-test at P<0.05.

Statistical analysis was performed using Statistica 10 (STAT Soft. Inc., USA).

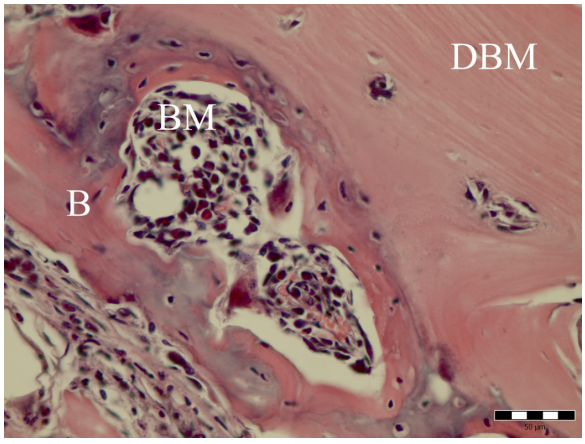

Fig. 1. Bone and bone marrow formation following demineralized bone matrix implantation, 21 days post implantation. Hematoxylin-eosin staining, scale bar: 50 μm. DBM – demineralized bone matrix; B – induced bone; BM – induced bone marrow.

Results

Demineralized rat bone and murine incisors implanted into muscles in mice have induced bone formation at various frequencies and magnitudes (Fig. 1 and Fig. 2) (WŁODARSKI & REDDI 1986).

The results obtained are presented in Table 1.

The analysis of variance (ANOVA) showed no significant differences between the weight of bone matrices among three post implant groups in mice given demineralized, lyophilized rat femoral implants.

However, the Pearson correlation analysis in the 28 day group showed that an increase of weight of bone matrices results in higher yield of heterotopically induced bone (r = 0.757, P<0.01).

The linear regression applied to this group to compare the weight of implanted matrices and the

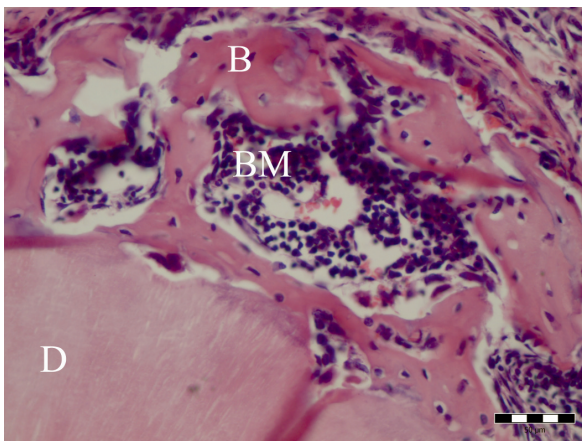

Fig. 2. Induction of osteogenesis by demineralized dentine, 21 days post implantation. Hematoxylin-eosin staining, scale bar: 50 μm. D – dentine; B – induced bone; BM – induced bone marrow.

weight of induced bone revealed that an increase of implanted bone matrix weight by 1 mg increased the yield of induced bone by 0.258 mg.

The difference between the weight of bone induced by dentine matrix evaluated 28 and 35 days post incisor implantation was statistically insignificant.

Discussion

A weak correlation between the decalcified bone matrix size (weight) and the yield of induced bone was found only in samples evaluated 28 days post matrix implantation.

This lack of correlation between bone implant weight and the yield of induced mineralized tissues observed on the 22nd day post grafting can be explained by the observation that at this time endochondral osteogenesis was ongoing and mineralization was incomplete. The induced cartilage, being a template for endochondral osteogenesis, is completely hydrolyzed by NaOH and so does not contribute to the mineralized yield weight. In the 28 day group, endochondral bone formation is complete, the cartilage is replaced by bone, thus the yield of bone induction is higher than at 22 days, and remains almost constant until day 35.

Considerable variation in implant weight and in the bone yield were observed within post-implant groups, and in many cases no mineralized tissue was recovered. A lack of detectable mineralized tissues could be attributed to a delay in induction and /or of an optimal size of implanted bone matrices. In the early (22 days) and late (35 days) group bone matrix implants were bigger (4.37 ±1.70mg and 3.88 ±1.75 mg) than in the 28 day groups (3.44 ±1.70 mg) and in the latter the yield of bone was higher (0.58 ±0.58 mg) than in the former (0.46 ±0.95 mg and 0.50 ±0.49 mg). Thus the smaller bone implants seem to be more effective as bone inducers than larger ones, suggesting an impairment of bone induction by oversized implants. Bone yield by demineralized tooth matrices supports this conclusion.

In this context, the importance of the geometry of carriers controlling BMP-induced osteogenesis is noteworthy (KUBOKI *et al.* 2001). The geometry of the substratum for osteogenin (BMP) combined with porous hydroxyapatite had a profound influence on bone induction (RIPAMONTI *et al.* 1992; RIPAMONTI 2004).

Implants of lower incisors are smaller than the bone chips used and were very homogenous in weight (0.95 ± 0.05 mg) but the yield of induced bone, evaluated on day 28 and 35 post insertion, was 0.48 ±0.34 mg and 0.27 ±0.34 mg, respectively. These values are comparable to those obtained by much bigger bone implants at the same age. On a weight basis the efficiency of bone induction by syngeneic demineralized tooth is superior to that of demineralized rat bone.

Clearly, the efficiency of bone induction does not critically depend on size of implanted rat bone matrices and on the quantity of BMPs present in it. It is suggested, therefore, that the osteogenic response triggered by the release of BMPs is under host control, and that in mice the availability of cells responding to BMPs is limited, thereby limiting the osteogenic response to large implants.

Large variation in the yield of induced bone by bone and dentine matrices implies the existence of as yet unidentified factors other than BMP which limit the degree of bone induction by demineralized bone and tooth. Demineralized lower incisors, very homogenous in terms of shape, size and weight, induced osteogenesis on implantation into thigh muscles at a wide range of intensities, despite the presumed reasonably uniform content of BMPs. Such inconsistency could be explained by the suboptimal dose of BMPs in the implanted matrices used in the present experiment. MUTHUKUMARAN *et al.* (1988) reported that the threshold for bone induction in rats lies between 10 to 25 mg of decalcified rat bone.

Further study is being undertaken to clarify the reason for such inconsistency in osteogenic response by incisors.

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