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Osteocyte Lacunae Density in Dentine-Induced Ectopic Bone

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The frequency of osteocytic lacunae, expressed as mean lacunae number per 1000 μm^2 of measured bone, evaluated 65 days post intramuscular implantation of demineralized incisors is higher (1.10 ± 0.19) than in femoral (orthotopic) bone (0.91 ± 0.16). The surface of evaluated bones was measured by means of the "weight of bone picture". These results provide new data on the biology of ectopic bone.

Key words: Demineralized incisors, ectopic bone, osteocytic lacunae.

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Bone induced ectopically by implantation of demineralized bone/dentine matrix or by some epithelial cells can be formed either directly in connective tissue by intramembranous or endochondreal ossification (WŁODARSKI 1985). Heterotopically induced bone is a site of active myelogenesis, and its hematopoietic marrow is gradually replaced by fatty marrow. Such bone is slowly resorbed, but even a year after post induction, foci of haematopoietic bone marrow containing numerous megakaryocytes can be observed (WŁODARSKI *et al.* 2012). Basically, heterotopic bone has the same properties as skeletal bone. Ectopic bone is metabolically active (ZALESKI 1962; KAGAWA 1965) and incorporates radioactive calcium and phosphate more actively than orthotopic bone (OSTROWSKI & WILCZYŃSKI 1958; WŁODARSKI & REDDI 1986). Moreover, the mineralization of heterotopically induced bone as measured by electron spin resonance is similar to that of orthotopic bone (DZIEDZIC-GOCŁAWSKA *et al.*1971). So far the only substantial difference between extraskeletal induced bone and skeletal bone is the lack of a functioning and true periosteal membrane in the former. This was shown by a lack of response to Moloney sarcoma virus of ectopically induced bone by demineralized bone matrix (WŁODARSKI & REDDI 1986). This virus stimulates true periosteum or perichondrium toward rapid proliferation followed by chondro/osteogenic differentiation (WŁODARSKI 1984, 1985). Thus, ectopic bone is unable to regenerate because of the absence of true periosteum.

In this paper we compared the density of osteocytes in bone induced by demineralized murine incisor dentine implanted into thigh muscle with that from orthotopic bone.

Material and Methods

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

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

Preparation of demineralized murine incisors for implantation

Demineralized syngeneic murine lower incisors were prepared according to a previously described method (WŁODARSKI *et al.* 2010). Incisors were hydrolyzed in 0.6 N HCl for 5 hrs, washed in distilled water and lyophilized. Lyophylized incisors were implanted into pockets made in the thigh muscles of 6 mice.

Implantation of incisors

Mice were anaesthetized with chlorhydrate (0.15 ml of saline 0.36% solution) given intraperitoneally. After shaving, small (2-3 mm) longitudinal skin and muscle incisions were made, demineralized and lyophylized incisors were inserted into the thigh
muscle pocket. Muscles and skin were sutured with 3-0 Dexon S polyglycolic acid suture and the wounds were disinfected with 70% ethanol. The animals were killed 65 days later by cervical dislocation and the implants together with surrounding tissues were removed, fixed in Bouin's fixative, demineralized for 4 days in saturated EDTA solution and embedded in paraffin. Then 8 μm sections were stained with haematoxylin-eosin and evaluated microscopically.

Femoral bones of the recipient animals were processed in the same manner as their implants.

The density of bone lacunae, both empty and those containing osteocytes, in the induced bone and in the femoral bone sections were expressed as the number of lacunae observed in areas of comparable size. Evaluation was done on photographs taken at magnification x 400 with a digital camera Olympus E330 using Olympus CX41 microscope and image acquisition software, QuickPhoto Micro 2.2 (Promicra Ltd). Each photo was provided with a marked bar of 50 μm. Photos were printed on Inacopia elite paper sheets (80 g/m^2). The numbers of osteocytic lacunae were counted, and the areas covering bone with counted lacunae were "cut out" and weighed on an analytical balance. From te weight of these cut out fragments of the induced bone trabeculae and of compact femoral bones in which lacunar quantitation were performed the surface of the examined bone slices was calculated and the density of osteocytic lacunaes was established as follows: the weight of the paper of known surface as calculated from the length of the scale bar, equal to 2500 μm^2, was the surface reference. One mg of the paper was equal to 21.7 μm^2. Thus, the surface of cross-sectioned bone with a known number of lacunae was estimated by the weight of the excised paper fragments.

The density of osteocyte lacunae (the number of lacunae per bone surface) in ectopic and femoral bone was evaluated in six specimens of induced bone formed 65 days post dentine inoculation and in three recipient femora. From each case of induction and femoral compact bone, 4-5 microphotographs were taken for lacunar density evaluation. Each microphotograph was analyzed separately and the mean value of the ratio of the bone surface area to lacunae number was calculated. Osteocytic lacunar frequency in the femoral bone was assessed on 11 microphotographs, and on 27 microphotographs for induced bone. Osteocyte lacunar frequency was expressed as the ratio of the weight of the excised paper fragments covering the photograph of bone to the number of lacunae present on it. Obtained data were pooled and the mean ratio value (lacunae frequency LF) of induced and orthotopic femoral bone was calculated and converted into mean lacunae density per 1000 μm^2 (Table). LF= weight of estimated bone surface (mg)/number of lacunae in a cut-out paper fragment. One mg of paper was equivalent to 21.7 μm^2. This value was obtained by weight of scale bar length square taken at magnification of 400 x. The significance of differences in lacunae density between the femoral and heterotopic bone was analyzed by Student's t-test for small groups and the distribution of lacunae was analyzed by a Mann-Whitney U-test. The correlation of osteocyte lacunar number with the weight of bone excised paper fragments (equivalent of bone surface) was measured by the Pearson correlation coefficient.

Results

In all animals the implanted demineralized incisors induced osteogenesis and myelopoiesis to varying degrees. The newly-formed bone was either adjacent to the tooth matrix, forming together a solid block with implanted dentine (Fig. 1), or was present as a bone trabeculae not connected to matrix (Fig. 2), thus it was necessary to isolate induced bone from the implant by excision. No inflammatory reaction around the implants was observed.

The osteocytic lacunar density in the recipient femoral bone and in the induced bone differs. In the femoral bone (Fig. 3) the mean ratio of surface (weight) to lacunae number was 51.66 ± 8.4 (median 53), thus on average, one osteocytic lacuna occupied 1122 ± 181 μm^2, and lacunae density expressed as the number of items per 1000 μm^2 was 0.91 ± 0.16. In the induced bone the weight to lacunae ratio was 42.6 ± 8.3 (median 40.8), thus one lacuna occupied on average 924 ± 180 μm^2, and

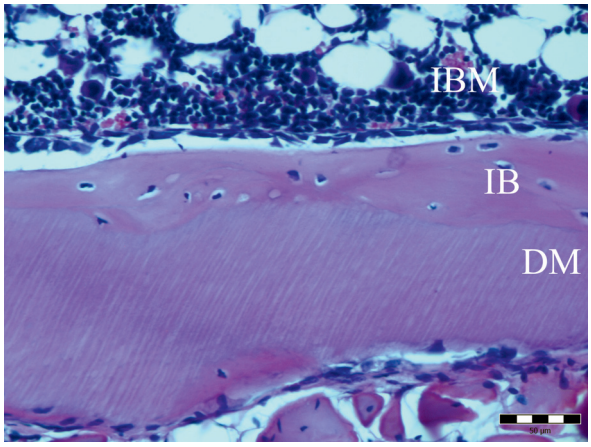

Fig. 1. Induced bone (IB) adjacent to the dentine matrix (DM). Dentine canaliculi are seen. On top – the induced bone marrow (IBM) accompanying induced bone. Hematoxylin-eosin staining. Scale bar = 50 µm.

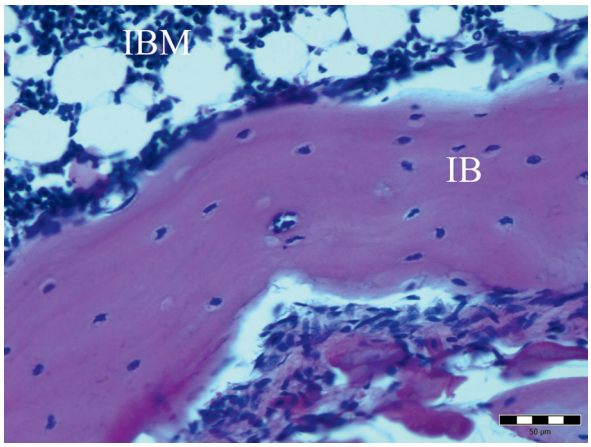

Fig. 2. Separate trabeculae of induced bone (IB). On top – the induced bone marrow (IBM) accompanying ectopic osteogenesis. Hematoxylin-eosin staining. Scale bar = 50 µm.

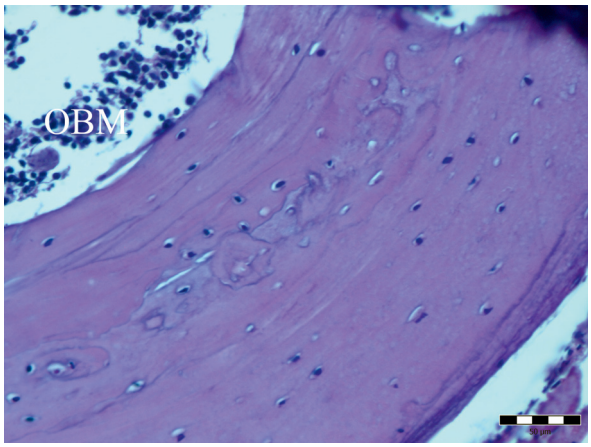

Fig. 3. Diaphysis of femoral bone. On the left orthotopic bone marrow (OBM). Hematoxylin-eosin staining. Scale bar = 50 µm.

Table 1

Osteocyte lacunae density in ectopic and in orthotopic bone. In parenthesis number of items examined

Mean bone surface: lacunae number ratio ± S.D.		Mean osteocyte lacunae density: number of lacunae (per 1000 µm^2) ± S.D.	
Femoral bone	Ectopic bone	Femoral bone	Ectopic bone
51.66 ± 8.4 (11)	42.60 ± 8.3 (27)	0.96 ± 0.16	1.10 ± 0.19

lacunae density expressed as the number of items per 1000 µm^2 was 1.10 ± 0.19 (Table 1). The difference in osteocytic lacunale density in the induced and orthotopic bone is statistically insignifant ($P<0.10$) as evaluated by Student's T-test for two independent groups (T-value 1.42 and P-value 0.08). The Mann-Whitney U-test revealed that the lacunar distribution in the examined bones was approximately normal.

There was a strong positive correlation between the number of osteocytic lacunae with the weight of paper excisions for induced bone ($R= 0.934$) and a moderate positive correlation ($R= 0.615$) for orthotopic (femoral) bone.

Discussion

The density of osteocytic lacunae in ectopically formed bone as evaluated 65 days post induction differs from that in recipient femoral bones. The density, expressed as lacunae number per 1000 µm^2 of bone surface area calculated from the weight of paper for orthotopic and ectopic bone were, accordingly, 0.91 ± 0.16 and 1.10 ± 0.19, the differences being statistically insignificant at $P<0.10$. The distribution of lacunae, expressed as the quotient of bone surface (weight of paper excision) to the number of observed lacunae was approximately normal in both groups.

Quantitave analysis of osteocytic lacunar density in ectopic, 65 days-old bone and in orthotopic host femoral bone revealed eaven distribution in both, while higher, but statistically insignificany, density in ectopic bone. More densely packed osteocytic lacunae in relatively young (65 days old) ectopic bone than in the 3 month old recipient femoral bones could be a manifestation of higher metabolic activity in the former (Ostrowski & Wilczyński 1958). Whether the previously reported lack of a true periosteal membrane in ectopic bone (Włodarski

& REDDI 1986; WŁODARSKI *et al.* 2010) has any influence on lacunar density remains to be elucidated. Until now higher lacunar density in ectopic bone than in orthotopic bone remains another morphological feature of bone induced by demineralized dentine and implicates that increased lacunar density in ectopic bone is a manifestation of remodeling at an accelerated rate (HERNANDEZ *et al.* 2004).

Live osteogenesis by demineralized dentine together with firm coupling with dentine matrix is promising when the practical application of bone induction phenomenon is considered in the healing of bone defects.

References

DZIEDZIC-GOCŁAWSKA A., WŁODARSKI K., STACHOWICZ W., MICHALIK J., OSTROWSKI K. 1971. Quantitative evaluation of the rate of mineralization of induced skeletal tissues by the electron spin resonance technique. Experientia **27**: 1405-1406.

HERNANDEZ C.J., MAJESKA R.J., SCHAFFLER M.B. 2004. Osteocyte density in woven bone. Bone **35**: 1095-1099.

KAGAWA S. 1965. Enzyme histochemistry of bone induction by urinary bladder epithelium. J. Histochem. J. Histochem. Cytochem. **13**: 255-264.

OSTROWSKI K., WILCZYŃSKI M. 1958. Examination of mineral turnover in bone induced by transplantation of urinary bladder mucosa by means of Phosphate-32 isotope. Folia Morphologica (Warszawa) **4**: 343-344.

WŁODARSKI K. 1984. Induction of heterotopic and orthotopic cartilage and bone formation in mice. Acta Biol. Hung. **35**: 205-218.

WŁODARSKI K. 1985. Orthotopic and heterotopic chondrogenesis and osteogenesis mediated by neoplastic cells. Clin. Orthop. Relat. Res. **200**: 248-265.

WŁODARSKI K., WŁODARSKI P., GALUS R., BRODZIKOWSKA A. 2010. Effects of time of initial exposure to MSV sarcoma on bone induction by dentine matrix implants and on orthotopic femora. Int. J. Mol. Sci. **11**: 3277-3287.

WŁODARSKI K., REDDI A.H. 1986. Heterotopically induced bone does not develop functional periosteal membrane. Arch. Immunol. Ther. Exp. (Warszawa) **34**: 583-593.

WŁODARSKI K.H., GALUS R., BRODZIKOWSKA A., WŁODARSKI P.K. 2012. The adipocyte component of bone marrow in heterotopic bone induced by demineralized incisor grafts. Folia Histochem. Cytobiol. **50**: 444-449.

ZALESKI M. 1962. The structure and development of induced bone tissue. Bull. Acad. Pol. Sci. Ser. Sci. Biol. **10**: 555-558.