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## Bone Turnover in Homozygous $\beta_2$ -Microglobulin Knock-Out Mice Does not Differ from That of Their Heterozygous Littermates

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**Summary:**  $\beta_2$ -Microglobulin is a constituent of the class I major histocompatibility complex (MHC) molecule and crucial for its normal function in cell recognition. It has also been isolated from bone and shown to regulate bone metabolism and to be altered in various bone diseases. In order to further investigate the role of the immune system in bone metabolism, we studied basic properties of bone physiology in  $\beta_2$ -microglobulin-deficient mice created by the technique of gene knock-out. Ten week-old male offspring homozygous (non-functional class I MHC molecule) or heterozygous (functional class I MHC molecule) for  $\beta_2$ -microglobulin knock-out gene did not differ in the following measures of bone turnover: femur length, dry and ash weight and calcium content, serum calcium concentration and alkaline phosphatase activity, total vertebral tissue area, trabecular bone volume, osteoid surface, osteoclast surface and mineral apposition rate. These data indicate that the bone turnover in  $\beta_2$ -microglobulin-deficient mice is appropriate for the stage of their skeletal maturation.

### Introduction

$\beta_2$ -Microglobulin was first discovered in human urine and designated according to its electrophoretic mobility ( $\beta_2$ ), size (micro) and solubility (globulin) (1). Later it was found that it is also present in all cells as  $\beta$  chain of class I major histocompatibility complex (MHC) molecules (1).  $\beta_2$ -Microglobulin is structurally homologous to an immunoglobulin constant domain containing a disulfide-linked loop. The interactions of  $\beta_2$ -microglobulin with the  $\alpha_1$ ,  $\alpha_2$  and  $\alpha_3$  segments of the heavy chain are critical for maintaining class I MHC molecules in their native conformation; displacement of the  $\beta_2$ -microglobulin results in the loss of heavy-chain native structure (1).  $\beta_2$ -Microglobulin-deficient mice, created using the technique of gene knock-out, lack functional MHC class I molecules. Since these molecules are instrumental for recognition by CD8 T-lymphocytes,  $\beta_2$ -microglobulin knock-out impairs the effector cellular immune response (2).  $\beta_2$ -Microglobulin knock-out mice

thus provide a useful model for dissecting the regulatory mechanisms and interactions in the immune system.

$\beta_2$ -Microglobulin has also been isolated as a bone-derived growth factor from fetal and adult bone (3) and from bone culture medium (4); it stimulates DNA and collagen synthesis (5), and osteoclastic activity (6) in bone cultures, and regulates bone metabolism (7). In clinical studies,  $\beta_2$ -microglobulin serum concentrations were found to be increased in bone diseases with high remodeling rates, such as postmenopausal osteoporosis (8) and *Paget's* disease (9). Since  $\beta_2$ -microglobulin seems to be crucial both for the immune and bone system, we hypothesized that  $\beta_2$ -microglobulin-deficient mice should have altered bone turnover *in vivo*. The purpose of the present study was to compare basic properties of bone physiology in mice homozygous for  $\beta_2$ -microglobulin knock-out gene and their heterozygous littermates with normal expression of class I MHC molecules.

## Materials and Methods

### $\beta_2$ -Microglobulin-deficient mice

$\beta_2$ -Microglobulin-deficient ( $\beta_2m^{-/-}$ ) mice (129/Sv  $\times$  C57Bl/6, H-2<sup>b</sup>) were kindly provided by Dr. Rudolf Jaenisch (2). Mice were maintained in a pathogen-free environment and monitored daily. They were fed standard rodent chow diet (Pliva Pharmaceuticals, Zagreb, Croatia) and given water ad libitum. The  $\beta_2m^{-/-}$  genome transmitting chimeras were mated to C57BL/6 mouse strain; 25% of mice of the F2 generation displayed the  $\beta_2m^{-/-}$  phenotype. Homozygous mice were then mated with their heterozygous littermates to produce 50% of each genotype. Fourteen ten week-old males homozygous for  $\beta_2m^{-/-}$  genome and 9 male heterozygous littermates from 5 such matings were included in the study.

The genotype of individual mice was determined by cytofluorometric screening of CD8<sup>+</sup> cells in the peripheral blood (10). Briefly, 100  $\mu$ l of tail vein blood were preserved from coagulation with 5  $\mu$ l of 0.5 mol/l EDTA, pH 8.0. FITC-conjugated monoclonal antibodies to CD8 (rat anti mouse Lyt-2 FITC; Becton Dickinson, Mountain View, CA, USA) were added to this suspension and incubated for 30 minutes at 4 °C. After red blood cell lysis by lysis solution (Becton Dickinson), the remaining cells were analyzed by a FACScan cytofluorometer (Becton Dickinson). Individual animals were considered homozygous if CD8<sup>+</sup> cells were completely absent from the sample.

### Physical and bone ash data

Body weight of mice was measured at sacrifice. Right femur from each mouse was carefully cleansed of adherent tissue, weighed and its length measured using a caliper. Bone volume was determined by fluid displacement according to the Archimedes' principle (11) and bone density calculated per gram of fresh weight. Bones were then dried in pre-weighed ceramic vials at 110 °C overnight and again weighed. Dried bones were ashed at 1000 °C for 24 hours and weighed. The ash was dissolved in 1 ml 6 mol/l HCl and diluted with 1 ml of deionized water. The samples were then analyzed for calcium content using atomic absorption spectrometry (ASS, Pye Unicam, USA).

### Bone histomorphometry

For the evaluation of histomorphometric static and dynamic bone properties of bone formation and resorption, proximal lumbar vertebrae were used. Mice received calcein (15 mg/kg body weight) injection 2 and 5 days before sacrifice. Lumbar vertebrae (L1-L3) were dissected out and fixed in 80% ethanol at 4 °C. The bones were dehydrated in increasing ethanol concentrations and embedded undecalcified in methylmetacrylate. Midline longitudinal 3  $\mu$ m-thin sections of vertebrae were cut on a Reichert-Jung microtome and stained with Goldner's stain, or were mounted unstained for fluorescent microscopy (12). Histomorphometric characteristics were evaluated on one entire vertebra according to the system recommended by the American Society for Bone and Mineral Research (13). The following characteristics were evaluated using automated image analysis (14): total tissue area (mm<sup>2</sup> of one vertebra including marrow and bone); trabecular bone volume (percent of the vertebral sample occupied by trabecular bone); osteoid surface (percent bone surface covered by osteoid); and osteoclast surface (percent of the bone surface with visible osteoclasts or remnants of their activity). Mineral apposition rates were calculated from the distance between double calcein labels measured on unstained sections using a fluorescent microscope (14).

### Biochemical analysis of the serum

Serum calcium concentrations were determined by atomic absorption spectrometry. Alkaline phosphatase activity was measured using *p*-nitrophenyl phosphate as substrate (12).

### Statistical analysis

Since the measured data showed normal distribution (15), the experimental groups were compared using the Student's *t*-test.

## Results

Male mice heterozygous and homozygous for the  $\beta_2$ -microglobulin knock-out gene developed normally after birth (data not shown) and had similar body weight at ten weeks ( $30.6 \pm 2.5$  g in homozygotes and  $35.1 \pm 4.7$  g in heterozygotes). Homozygous mice had no apparent abnormalities of the skeleton or other organ systems. Femur lengths were also similar in heterozygous and homozygous male littermates ( $16.12 \pm 0.99$  mm in homozygotes and  $16.59 \pm 1.02$  mm in heterozygotes). Fresh and dry bone weight, as well as the ash and bone calcium content were comparable in the two experimental groups (tab. 1), indicating that both homozygous and heterozygous  $\beta_2$ -microglobulin knock-out mice had bones appropriate for their body size and age.

Static and dynamic bone histomorphometry showed that the indices of bone formation and resorption did not differ in homozygous and heterozygous littermates (tab. 2). Serum alkaline phosphatase, which reflects bone formation activity, as well as serum calcium concentration were not changed in  $\beta_2$ -microglobulin-deficient mice in comparison to the control group (tab. 3).

## Discussion

The data presented in this report indicate that bone turnover of  $\beta_2$ -microglobulin-deficient mice at the tissue and

**Tab. 1** Bone ash evaluation of the femurs from mice heterozygous or homozygous for  $\beta_2$ -microglobulin knock-out gene.

	$\beta_2$ -Microglobulin <sup>-</sup>	$\beta_2$ -Microglobulin <sup>+</sup>
Fresh weight (mg)	$87.23 \pm 9.81$	$88.29 \pm 7.19$
Dry weight (mg)	$51.52 \pm 5.42$	$52.59 \pm 4.27$
Femur density	$1.38 \pm 0.03$	$1.40 \pm 0.04$
Ash weight (% of dry bone)	$69.69 \pm 2.65$	$65.58 \pm 1.84$
Calcium (% of ash weight)	$28.74 \pm 0.97$	$30.51 \pm 1.79$

**Tab. 2** Histomorphometric evaluation of lumbar vertebrae from mice heterozygous or homozygous for  $\beta_2$ -microglobulin knock-out gene.

	$\beta_2$ -Microglobulin <sup>-</sup>	$\beta_2$ -Microglobulin <sup>+</sup>
Total vertebral tissue area (mm <sup>2</sup> )	$2.21 \pm 0.18$	$2.32 \pm 0.16$
Trabecular bone volume (%)	$19.35 \pm 2.05$	$18.92 \pm 1.19$
Osteoid surface (%)	$0.67 \pm 0.23$	$0.71 \pm 0.24$
Osteoclast surface (%)	$13.43 \pm 1.07$	$14.28 \pm 0.93$
Mineral apposition rate ( $\mu$ m/day)	$1.40 \pm 0.22$	$1.54 \pm 0.43$

**Tab. 3** Serum biochemical data in mice heterozygous or homozygous for  $\beta_2$ -microglobulin knock-out gene.

	$\beta_2$ -Microglobulin <sup>-</sup>	$\beta_2$ -Microglobulin <sup>+</sup>
Calcium (mmol/l)	2.37 $\pm$ 0.21	2.55 $\pm$ 0.06
Alkaline phosphatase (U/l)	239 $\pm$ 11	243 $\pm$ 10

organ level do not differ from that of their littermates with functional production of  $\beta_2$ -microglobulin. Bone turnover in both groups of mice was appropriate for the stage of their skeletal maturation and comparable to other, albeit rare reports on bone metabolism in mice (16).

Our results are surprising in the view of the close interdependence of the bone and immune systems. Many experimental and clinical studies have shown that a number of growth factors and cytokines, first isolated from the cells of the immune system, exert effects on bone cells and are produced by bone cells themselves (reviewed in l. c. (17)), indicating complex molecular interactions between the two systems (18, 19).

However, most of the in vivo data on bone turnover in immunodeficient animals are contradictory. Marked decrease in bone formation rate and the number and activity of osteoclasts have been reported in athymic mice

which lack T-lymphocytes (20). Another study showed that athymic mice had physiological bone turnover comparable to that of euthymic mice (16), while we showed that thymectomized rats, which have depression in cellular immunity, had better indices of new bone formation induced by demineralized bone matrix in comparison to the control animals (21). In view of this data, the finding of normal in vivo bone metabolism in  $\beta_2$ -microglobulin-deficient mice is not surprising. Even the reports on  $\beta_2$ -microglobulin in bone diseases are contradictory; e. g. both increased and normal serum  $\beta_2$ -microglobulin levels have been reported for *Paget's disease* (9, 22). The studies of the immune system in  $\beta_2$ -microglobulin-deficient mice indicate that the disturbance in the expression of MHC class I molecules can be compensated in several ways, so that  $\beta_2m^{-/-}$  mice make adequate protective immune responses to foreign antigens in vivo (23). Such plasticity of the immune system and compensatory flexibility in both the immune and bone systems may explain our finding of normal bone turnover in  $\beta_2$ -microglobulin-deficient mice. Detailed and more sophisticated studies of the bone metabolism in immunodeficient animals is needed for a better insight into the relationships between the bone and immune systems.

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