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Osteogenic markers are reduced in bone-marrow mesenchymal cells and femoral bone of young spontaneously hypertensive rats

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ABSTRACT

Aims: Spontaneously hypertensive rats (SHR) and normotensive rats (W) has significant changes in bone metabolism. The purpose of this study was to investigate whether, the genetic predisposition, is sufficient to induce changes in the osteoblast differentiation and osteogenic markers in the BMSCs or in the femoral bone. For this we use young SHR rats without hypertension, but, with genetic predisposition in compared with young W.
Main methods: BMSCs were cultured in a proliferation medium (MEM) or osteogenic medium. Osteogenic differentiation was analyzed by proliferation, total protein, alkaline phosphatase, mineralization, and the mRNA expression of RUNX-2, β-cathenin, osterix, bone morphogenetic protein-2 (BMP-2), osteocalcin (OCN), bone sialoprotein (BSP), collagen type I (Col I), and osteopontin (OPN).
Key findings: Osteoblast differentiation in SHR BMSCs (SHRC) had an increased proliferation compared with W BMSCs (WC). After osteogenic induction, there was greater reduction in proliferation in SHR (SHROM) than in W, in the same condition (WOM). On day 7, although no significant difference in the ALP activity was observed between SHROM and WOM, poor mineralization and osteoblast differentiation was noted in SHROM. The Osterix and β-catenin are involved in the reduced osteoblast differentiation in SHROM. The decreased expression of osteoblast-associated proteins such as OCN, BSP, COL I and OPN revealed poor quality of extracellular matrix (ECM) in SHROM. In the femoral bone, the immunostaining of COL1, BALP, OPN and OCN in SHR was decreased compared with the W. TRAP-positive immunoreactions were observed in major extension in the SHR femur.
Significance: This study is the first to compare osteoblast differentiation in vitro and femoral bone from SHR and W rats. Our results demonstrated that young SHR (4 weeks old), without hypertension, but with genetic predisposition, had alterations in osteoblast differentiation of BMSCs and in the femoral bone when compared with their progenitor strain, W.

1. Introduction

Bone marrow mesenchymal stem cells (BMSCs) are multipotent stem cells that have the ability to differentiation into osteoblasts and other cellular types, such as chondrocytes and adipocytes [1,2]. During in vitro osteogenesis, BMSCs form a multilayer and synthesize extracellular matrix with a bone tissue-like organization [3,4]. This process can be divided into three periods: (i) proliferation, (ii) extracellular matrix maturation and (iii) mineralization [5]. In the active proliferation stage, cells synthesize growth factors, collagen and transcription factors activation. After this stage, there is protein production related to extracellular matrix maturation and later mineralization, such as osteocalcin (OCN), bone sialoprotein (BSP), and alkaline phosphatase (ALP). The cessation of growth and extracellular matrix accumulation are maturation signals of the osteoblast phenotype [6]. All these proteins are expressed at maximal levels in the subsequent stage of differentiation at the onset of mineralization, considered a functional in vitro endpoint reflecting advanced cell differentiation [7].

Spontaneously hypertensive rats (SHR) are a model of human essential hypertension developed by Okamoto and Aoki [8]. This strain was obtained from Wistar rats (W) with a blood pressure above the average. Cardiac hypertrophy, diastolic dysfunction [9], metabolic acidosis [10], constitutive cytokine and chemokine production [11] and significant changes in bone metabolism, such as increased bone turnover and reduction in cortical and trabecular bone mass [12–15] are characteristics of this animals. This strain develops hypertension after 6 weeks of age and is therefore, a model of hypertension associated with bone

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problems. The hypertensive genotypic influence on the hyperproliferation activity on the smooth muscle cells (SMC) of young, four-week-old SHR has been demonstrated in [16]. The purpose of this study was to investigate whether, the genetic predisposition, is sufficient to induce changes in the osteoblast differentiation and osteogenic markers in the BMSCs or in the femoral bone. For this we use young SHR rats (4 weeks old) without hypertension, but, with genetic predisposition in compared with young W. The results demonstrated that there are differences in several parameters: A) SHR BMSCs have increased proliferative rate; B) Mineralization was lower in the SHROM (osteogenic medium) in comparison with the WOM; C) Reduced osteoblast differentiation was observed in SHROM and D) TRAP-positive immunostaining was higher in SHR than in W in the femur.

2. Materials and methods

2.1. Animals and ethical aspects

The Experimental Procedures were reviewed and approved by the Institutional Animal Welfare Committee at the School of Dentistry of Araçatuba (UNESP — Univ. Estadual Paulista, São Paulo, Brazil — Process No. 00716-2012). A total of 12 male W and 12 male SHR, 4 weeks old, weighing between 100 and 160 g, were employed. W rats were used with control, because SHR was obtained from this strain [8]. The age of animals was selected to study the genetic predisposition. Tests with adult animals are part of another study from the laboratory to be published soon. These animals were kept in cages under constant and controlled room temperature (22 ± 2 °C) and humidity (55% ± 3%) in a 12-hour light–dark cycle, and received water and standard food ad libitum. In order to obtain pool of Bone Marrow Mesenchymal Stem Cells (BMSCs) used in this study, the rats were euthanized with an overdose of halothane (Tanohalo; Cristália, Campinas, SP, Brazil).

2.2. Isolation and culture of bone marrow mesenchymal stem cells

Bone marrow was obtained from both femora, and flushed out using 20 mL of minimal essential medium (MEM) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 1% antibiotics (100 U/mL penicillin G, 100 μg/mL streptomycin and 0.3 μg/mL amphotericin B (Sigma).The cell suspension was filtered through a 70-μm size cell strainer and through 22 and 26-gauge needles. After rapid centrifugation, the cell pellet was suspended in culture medium and then seeded in 75 cm^2 tissue culture flasks (Santa Cruz Biotechnology). The cells were grown in a humidified atmosphere containing 5% CO_2 at 37 °C. After 10 days, once confluent, the cells were harvested using trypsin–EDTA solution (Sigma). Nucleated cells were counted and then seeded in a subculture at 4.0×10^5 cells/well in 24-well tissue culture plates (Santa Cruz Biotechnology). Again, under subconfluence (80%), the cells were cultured in a proliferation medium (MEM) or osteogenic medium (MEM supplemented with 10 nM β-glycerophosphate, 50 μg/mL ascorbic acid and 10 nM dexamethasone). Thus, the cells were divided into 4 groups: 1) WC (BMSCs of Wistar cultivated in proliferation medium, without osteogenic induction); 2) WOM (BMSCs of Wistar cultivated in osteogenic medium, with osteogenic induction); 3) SHRC (BMSCs of SHR cultivated in proliferation medium, without osteogenic induction) and 4) SHROM (BMSCs of SHR cultivated in osteogenic medium, with osteogenic induction. These experimental conditions were used for all the experiments.

2.3. Flow cytometry

After obtaining a pool of bone marrow cells of each of the two strains, the cells were cultivated with proliferative medium until the 2nd passage in 75 cm^2 tissue culture flasks (Santa Cruz Biotechnology). Therefore, the cells were harvested after detachment with 0.25% trypsin (Gibco) and were fixed with 4% paraformaldehyde. There was approximately 1×10^6 cells/tube. Next, the cells were incubated for 30 min. at 4 °C with 10 mg/mL of rat anti-mouse monoclonal antibody (CD90:RPE; CD29:RPE; CD54:FITC; CD45:FITC; or CD31:FITC AbDSerotec, Kidlington, Oxford, OX5 1 GE, UK). After this, the cells were centrifuged, the supernatant medium was removed and 200 μL of uncompleted medium was added. Data were acquired with the Attune™ acoustic focusing cytometer system (Applied Biosystems®, Foster City, CA, USA). Negative control IgG was used to exclude the auto fluorescence.

2.4. Markers of osteogenic differentiation

2.4.1. MTT reduction assay

The culture cell proliferation and viability of BMSCs were measured at time intervals of 0, 7 and 14 days using the classical MTT method [17].

2.4.2. Total protein content

The total amount of protein after time intervals of 0, 7 and 14 days was quantified using the Lowry [18] method.

2.4.3. Alkaline phosphatase activity

The Alkaline Phosphatase (ALP) activity was assessed as the release of Thymolphthalein from Thymolphthalein monophosphate using a commercial kit (Labtest Diagnostica SA, Ribeirão Preto, SP, Brazil), a modification of the Roy method [19]. Values were normalized against protein concentration [18].

2.4.4. Mineralization assay

Mineralization in osteoblast cultures was determined by Alizarin Red S staining (Sigma) after 10 and 14 days in culture. Staining was quantified by adaption of the method described by Gregory [20].

2.5. Real-time qPCR

Total RNA were isolated using TRIzol reagent (Invitrogen, Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. The total RNA from each sample was treated with DNAse I and reverse transcribed to complementary DNA (cDNA) using SuperScript™ II Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol.

The relative mRNA levels were evaluated by quantitative RT-PCR using StepOne™ Real-Time PCR System (Applied Biosystems, Life Technologies, United Kingdom) and TaqMan ® (Applied Biosystems, Life Technologies, USA). To use this reaction, inventoried assays labeled with FAM fluorophore provided by the same company: Osteocalcin (Bglap — Rn00566386_g1), osteopontin (SPP1 — Rn00563571_m1), bone sialoprotein (Ibsp — Rn00561414_m1), type 1 collagen (COL1A1 — Rn01463848_m1); Runx2 (Runx2 — Rn01512298_ml), osterix (osterix Rn02769744_s1 Sp7), β-catenin (CTNNB1 — Rn00584431_g1); were used in parallel with fluorophore VIC, provided by the same company: β-actin (ACTB-Rn00667869_m1).

2.6. Immunohistochemistry

The femurs were removed and fixed in 4% neutral formalin solution for 48 h, then demineralized by immersion for 45 d in a solution containing 10% ethylenediaminetetracetic acid. After this procedure, the femurs were dehydrated and embedded in paraffin. Semiserial longitudinal 3-μm thick sections were obtained and stained to determine the production of collagen type 1 (Col1), bone alkaline phosphatase (BALP), osteopontina (OPN), osteocalcina (OCN) and tartrate-resistant acid phosphatase (TRAP). Staining with hematoxylin and eosin was performed, and then analyzed by light microscopy to establish bone characteristics. For immunohistochemistry (IHC), the sections were deparaffinized and rehydrated in phosphate buffered saline (PBS). A primary antibody, diluted 1:250 in PBS and