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Streptozotocin-induced diabetes in rats diminishes the size of the osteoprogenitor pool in bone marrow

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ABSTRACT

Aims: Bone formation is reduced in animals and humans with type 1 diabetes, leading to lower bone mass and inferior osseous healing. Since bone formation greatly depends on the recruitment of osteoblasts from their bone marrow precursors, we tested whether experimental type 1 diabetes in rats diminishes the number of bone marrow osteoprogenitors.

Methods: Diabetes was induced by 65 mg/kg streptozotocin and after 4 weeks, femoral bone marrow cells were extracted and cultured. Tibia and femur were frozen for further analysis.

Results: The size of the osteoprogenitor pool in bone marrow of diabetic rats was significantly reduced, as evidenced by (1) lower (~35%) fraction of adherent stromal cells (at 24 h of culture); (2) lower (20–25%) alkaline phosphatase activity at 10 days of culture; and (3) lower (~40%) mineralized nodule formation at 21 days of culture. Administration of insulin to hyperglycemic rats normalized glycemia and abrogated most of the decline in ex vivo mineralized nodule formation. Apoptotic cells in tibial bone marrow were more numerous in hyperglycemic rats. Also, the levels of malondialdehyde (indicator of oxidative stress) were significantly elevated in bone marrow of diabetic animals.

Conclusions: Experimental type 1 diabetes diminishes the osteoprogenitor population in bone marrow, possibly due to increased apoptosis via Oxidative Stress. Reduced number of osteoprogenitors is likely to impair osteoblastogenesis, bone formation, and bone healing in diabetic animals.

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1. Introduction

Osteoporosis is a common complication of diabetes mellitus (DM) and patients with either type 1 or type 2 DM experience a higher incidence of fractures [1,2]. In addition, type 1 DM, induced in rats or mice, results in reduced bone mass [3,4]. Common to all these studies is that DM is associated with a reduction in bone formation rate [4,5]. Systemically, reduced bone formation leads to bone fragility, and locally, it hampers bone healing, such as in femoral fractures [6], bone marrow ablation [7], and insertion of titanium implants [8]. Thus, reduced osteoblastogenesis and bone formation are prominent features of DM.

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The actual mechanism of DM-associated osteopenia is yet unclear. Lack of insulin action and the presence of hyperglycemia per se are some obvious possibilities. Recently, attention has been drawn to AGEs (advanced glycation end products) and oxidative stress (OxS, exaggerated production of reactive oxygen species (ROS)) as major mechanisms of many diabetic complications [9,10].

Accelerated AGE formation due to hyperglycemia in DM [11] leads to their accumulation in bone tissue, which may participate in the pathogenesis of DM-related osteoporosis [2].

Hyperglycemia in DM causes OxS either directly (via glucose overloading of the mitochondria) or indirectly (secondary to signaling pathways such as AGEs and Polyol)) [9]. The increased ROS levels result in cell death, tissue damage and impaired healing. As in many other DM-associated pathologies, OxS has been implicated in the pathogenesis of DM-related loss of bone mass [4,12], possibly via osteoblast apoptosis.

It is well known that bone marrow includes hemopoietic and stromal compartments and that osteoblast precursors reside within the latter [13]. In vitro, explanted bone marrow cells form fibroblastic colonies initiated by cells called CFUf (colony forming units-fibroblastic) [14,15]. Given appropriate culture conditions (such as dexamethasone, organic phosphate, and ascorbate), these cells express bone-associated markers and form mineralized bone-like nodules [16,17]. The number of osteogenic CFUs (CFU-O) declines in conditions characterized by reduced bone formation rate, such as aged rats [18], ovariectomized osteopenic rats [19], IL-10 knockout osteopenic mice [20], and unloaded, osteopenic rat bones [21]. On the other hand, we reported that administration of anabolic doses of PGE_2 into young and old rats increases bone formation and, in parallel, the number of bone marrow osteoprogenitors [18,22]. Taken together, these studies indicate that the rate of bone formation greatly depends on the recruitment of osteoblasts from their marrow progenitors [23] and a lower number of osteoprogenitors within the marrow stroma may impede osteoblast generation and bone formation and will compromise bone mass and healing.

The objective of this study was to test whether type 1 DM in rats, which is known to cause diminished bone formation and lower bone mass, results in a reduced number of osteoprogenitors in bone marrow.

2. Materials and methods

Four-month-old Wistar rats (8–10 per group) were used in all experiments in this study, except where noted otherwise. All animal procedures were approved by the animal use ethics committee of the Faculty of Medicine, Tel-Aviv University.

2.1. Materials

Chemicals for tissue culture were from Biological Industries (Beit Haemek, Israel), unless otherwise stated. Dexamethasone (DEX), streptozotocin (STZ), ascorbic acid, naphthol AS-MX phosphate, fast red violet B, phosphatase substrate, alkaline buffer solution, silver nitrate, sodium carbonate and formalin were from Sigma–Aldrich (Rehovot, Israel). Beta-glycerophosphate (β-GP) was from Calbiochem (La Jolla, CA, USA). Tissue culture dishes were from Nunc (Rosekilde, Denmark). HbA1c levels were measured with the Glyco-tek column kit from Helena Laboratories (Beaumont, TX, USA). Ketamine chlorhydrate was from Rhone-Mérieux (Lyon, France) and Xylazine from Vitamed (Bat-Yam, Israel). Sustained-release insulin implants were from LinShin (Toronto, ON, Canada). OXI-TEK TBARS assay kit was from Enzo Life Sciences (Lausen, Switzerland) and BCA protein determination kit was from Pierce (Rockford, IL, USA).

2.2. Induction of DM and insulin repletion

Diabetes in rats was induced with a single intra-peritoneal (IP) administration of streptozotocin (65 mg/kg of body weight) diluted in citrate buffer (0.01 M, pH = 4.3). Control animals receive the buffer alone. Animals were given food and water ad libitum and body weight was continuously monitored. Blood glucose level was evaluated at regular intervals using a glucometer (Accu-Check, Roche Diagnostics, Basel, Switzerland) and rats with blood glucose level >250 mg/dL were considered diabetic.

In one of the experiments, STZ-injected animals were treated with insulin via sustained-release implants or with identical implants without insulin (sham). Success of insulin repletion was monitored via blood glucose and HbA1c levels.

2.3. Isolation and enumeration of BMSCs

Four weeks after injection of STZ, rats were sacrificed with an overdose of ketamine chlorhydrate (90 mg/kg) and xylazine (10 mg/kg), followed by asphyxiation with carbon dioxide. Blood was collected from the tail vein for final glucose and HbA1C measurements. Femurs were retrieved and bone marrow was expelled and pooled from both femurs of each animal. Cells were counted with a hemocytometer and seeded in 6-well plates at 2×10^7 cells/well in a medium composed of Minimum Essential Medium-Alpha containing 5.5 mmol/L D-glucose in triplicates for each assay. This medium was supplemented with 13% fetal Calf Serum (FCS) + 2 mM glutamine + 100 μ/mL penicillin + 100 mg/mL streptomycin + 12.5 U/mL Nystatin (basic medium). After 24 h, cultures were washed with PBS to remove non adherent cells and attached cells were collected with 0.25 w/v trypsin/0.02 w/v EDTA, counted and their number calculated as percent of the cells seeded.

2.4. Osteoblastic differentiation

Explanted cells were seeded in 6-well plates as described, in basic culture medium supplemented with 10 mM β-GP + 50 μg/mL ascorbic acid + 10 nM DEX (osteogenic medium). Cultures were washed with PBS after 24 h to remove non-adherent cells and were cultured for 10 or 21 days at 5% CO_2 and 37 °C in the same medium, which was changed twice a week. The number of osteoprogenitor cells was evaluated by measuring alkaline phosphatase (ALP) activity and mineralized nodule formation as described below.

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