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Gene expression profiling of bone marrow stromal cells from juvenile, adult, aged and osteoporotic rats: With an emphasis on osteoporosis

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Abstract

Purpose: Osteoporosis is a multi-factorial, age-related disease with a complex etiology and mode of regulation involving a large numbers of genes. To better understand the possible relationships among genes, we fingerprinted genes in a rat model induced by ovariectomy to determine differences among osteoporotic, non-osteoporotic, aged and juvenile rats.
Methods: We applied genome wide cDNA microarray technology to analyze genes expressed in bone marrow mesenchymal stromal cells (BMSC) and compared non-osteoporotic adult vs. osteoporotic, non-osteoporotic adult vs. aged, and non-osteoporotic adult vs. juvenile. Rigorous statistical analysis of functional annotation (EASE program) identified over-represented biological and molecular functions with significant group wide changes ($p \leq 0.05$). Some of the expressed genes were further confirmed by quantitative RT-PCR (reverse transcription-polymerase chain reaction).
Results: Differences in gene expression were observed by identifying transcripts selected by *t*-test that were consistently changed by a minimum of two-fold. There were 195 transcripts that showed an increased expression and 109 transcripts that showed decreased expression relative to the osteoporotic condition. Of these, 75% transcripts were unknown gene products or ESTs (expressed sequence tag). A number of genes found in the aged and juvenile groups were not present in the osteoporotic rats. Functional clustering of the genes using the EASE bioinformatics program revealed that transcripts in osteoporosis were associated with signal transduction, lipid metabolism, protein metabolism, ionic and protein transport, neuropeptide and G protein signaling pathways. Although some of the genes have previously been shown to play a key role in osteoporosis, several genes were uniquely identified in this study and likely play a role in developing aged related osteoporosis that could have compelling implications in the development of new diagnostic strategies and therapeutics for osteoporosis.
Conclusions: These data suggest that osteoporosis is associated with changes of multiple novel gene expression and that numerous pathways could play important roles in osteoporosis pathogenesis.

Keywords: Osteoporosis; Bone marrow stromal cell; cDNA microarray

Introduction

Aging of the human skeleton is characterized by decreased bone formation and bone mass. These changes are more pronounced in patients with osteoporosis. Osteoporosis is a silent epidemic, characterized by low bone density, leading to low trauma fractures (i.e., fragility fractures) among the elderly [1–3]. Fragility is due to intrinsic skeletal factors such as low bone mass, diminished cancellous bone, trabecular fenestrations and an imbalance in bone formation and resorption [4–6].

Osteoporosis is a complex disease with multifactorial determinants that include life style and hormonal influences as well as genetics. Estrogen plays a fundamental role in skeletal turnover and bone homeostasis. Many animal studies on osteoporosis are using ovariectomy to represent an optimal osteoporotic model to investigate the effects of estrogen deficiency. It has been noted that osteoporosis and its associated

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phenotypes are under strong genetic control [7–10]. Identification and characterization of either specific loci or genes involved in determining osteoporosis will contribute to a greater understanding of the pathogenesis of osteoporosis and could lead to the development of innovative diagnostic and treatment strategies.

Consequently, the objective of the study was to use microarray technology to identify gene expression differences in BMSC among juvenile, adult, osteoporotic associated with estrogen deficiency and aged rats. Adult bone marrow stroma contains a subset of non-hematopoietic cells referred to as mesenchymal stem cells (MSC) or mesenchymal progenitors. These cells have the capacity to differentiate to osteoblasts. The osteoporotic condition involves osteoblast dysfunction. Consequently, since MSCs can differentiate into osteoblasts, it is logical to study gene expression in the MSC: an osteoblast precursor cell.

Gene expression and early microarray studies have shown that there are at least 200 genes directly or indirectly involved in bone metabolism which may contribute to either development or prevention of osteoporosis [11]. Recently, the first *in vivo* microarray study directly measuring osteoporosis in human circulating monocytes was reported by Liu et al. [12]. Further studies using microarrays described gene expression in the human osteoclast differentiation [13–16]. Therefore, we decided that microarray techniques would be suitable to study gene expression in osteoblast precursor cells (i.e., MSCs) to determine if different gene expression patterns occurred among non-osteoporotic, osteoporotic, geriatric and juvenile rats.

Materials and methods

Experimental design, tissue culture and RNA isolation

The study was approved by the Institutional Animal Care and Use Committees (IACUC) at the University of Pittsburgh and Carnegie Mellon University and complies with the Animal Care and Use Guidelines of the NIH. The surgical facilities at the University of Pittsburgh where surgeries were performed are AAALAC approved (American Association for Accreditation of Laboratory Animal Care).

Briefly, four types of physiological conditions were emphasized in female Lewis rats. These included: geriatric rats (more than 2 years old), osteoporotic rats (7 months old), non-osteoporotic adult rats (7 months old) and juvenile rats (7 weeks old). Each group contained three animals for individual BMSC isolation and subsequent microarray studies. There were three microarray data generated for each group. The RNA samples left from microarray study were used for real-time PCR to confirm microarray result. Thus, each group generated three sets of data for statistics. The fold of changes of interested gene expression was presented by mean value.

Osteoporosis was induced by ovariectomy of 3-month-old rats, followed by a 30% caloric reduced diet and 4 months to develop osteopenia [5,17]. Histological validation of these animal models demonstrated significant loss of bone density in the rats' tibiae and femurs (Fig. 1), with typical fenestrated trabeculae.

Isolation and culture of rat BMSCs

Three bone marrow samples from each group of rats were isolated by flushing the femurs with 10 ml mesenchymal stem cell culture media (HyClone, Logan, UT), supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 100 U/ml penicillin/100 μg/ml streptomycin, 2 mM glutamine. Clumps of bone marrow were gently minced with a pipette. Cells were centrifuged for 10 min at 1000 rpm, washed by the addition of fresh medium, centrifuged again, re-suspended and plated out in the same stem cell culture media mentioned above at a density of $\sim 2 \times 10^6$ cells/cm^2 in 25 cm^2 plastic culture dishes. The cells were incubated at 37°C in 5% CO_2.

Non-adherent cells were removed by replacing the medium after 7 days. Cells were grown for a further 7 days to confluency, then washed with phosphate-buffered saline (PBS) and lifted by incubation with 0.25% trypsin/2 mM EDTA for 5 min. Non-detached cells were discarded and the remaining cells were regarded as passage 1 of the BMSC culture and further cultured for 7 days before RNA extraction. In our previous study we have demonstrated that all adherent cells do not express hematopoietic markers such as CD34 and CD45, and are positive for CD29, CD 73, CD90, CD105 and CD166 (data not shown).

RNA isolation

Total RNA was isolated using TRIZOL Reagent (Invitrogen, CA, USA). Assessment of the concentration and quality of the total RNA samples were carried out by spectrophotometry and Agilent Bioanalyzer.

Microarray procedure

CodeLink Rat Whole Genome Bioarrays (GE HealthCare, Piscataway, NJ, USA) were used for this study. Biotin-labeled cRNA target was prepared by linear amplification methods as described in CodeLink Protocols. The poly (A)+ RNA subpopulation (within the total RNA population) was primed for reverse

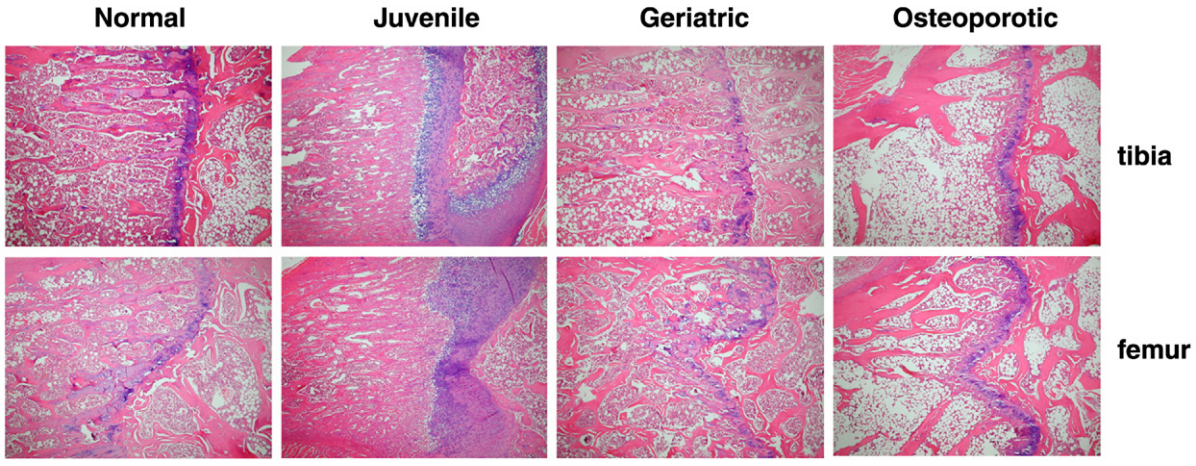

Fig. 1. Histological validation of animal models. Significant loss of bone density in tibia and femur was noted in osteoporotic rats with typical fenestrated trabeculae. All pictures were taken at 40 times magnification from growth plate in epiphysis part of tibia and epiphyseal disk of femur head.