Biochimie 95 (2013) 793–798

Contents lists available at SciVerse ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

Biochemical changes induced by strontium ranelate in differentiating adipocytes

Christopher Vidal [a], Krishanthi Gunaratnam [a], Jessica Tong [a,b], Gustavo Duque [a,*]

[a] Ageing Bone Research Program, Sydney Medical School Nepean, The University of Sydney, Penrith, NSW 2750, Australia
[b] Faculty of Medicine, University of New South Wales, Kensington, NSW 2052, Australia

ARTICLE INFO

Article history:
Received 21 June 2012
Accepted 15 November 2012
Available online 24 November 2012

Keywords:
Adipogenesis
Mesenchymal stem cells
Strontium ranelate
Osteoblastogenesis
Adipokines

ABSTRACT

Low bone formation in osteoporosis is associated with a shift from osteoblastic to adipogenic differentiation of mesenchymal stem cells (MSC) inducing a concomitant lipotoxic milieu within the bone marrow. Strontium ranelate (SrRN), a treatment for osteoporosis, has both anti-resorptive and anabolic effects on bone. The anabolic effect of SrRN has been associated with its effect on both osteoblastogenesis and adipogenesis. However, the effect of SrRN on the potentially lipotoxic factors produced by differentiating marrow adipocytes remains poorly understood. To expand the knowledge on the effect of SrRN treatment on the bone microenvironment, we assessed changes in adipogenic factors and adipokine expression in adipocytic differentiation of MSC *in vitro*. Primary human MSC were induced to differentiate in adipogenic conditions in the presence or absence of SrRN (1–2 mM). We tested the dose-dependent effects of SrRN on adipocyte differentiation including changes in the expression of adipogenic markers and adipokines. We report that adipogenesis was negatively affected in the presence of SrRN with a concomitant dose-dependent decrease in the expression of adipogenic markers and changes in adipokine profile. Taken together, our data suggests that SrRN induces biochemical changes in differentiating adipocytes that could generate a favorable osteogenic effect within the bone marrow milieu.

1. Introduction

Bone is a dynamic organ that is constantly changing throughout life. With aging, bone resorption significantly exceeds bone formation leading to decreased bone mineral density (BMD) and strength, and thus to increasing risk of fracture. Aging also induces a shift from osteoblast to adipocyte differentiation from mesenchymal stem cells (MSC) leading to an increase in adipocyte numbers within the bone marrow [1,2]. This increase in marrow fat not only negatively affects osteoblastogenesis but also has a lipotoxic effect exerted through the secretion of adipokines and fatty acids within the bone marrow milieu, thus affecting mineralization and bone formation [3–5].

Strontium ranelate (SrRN) has been used for the treatment of osteoporosis for the last six years and unlike other drugs it has both anti-resorptive and anabolic effects [6]. Studies show that SrRN affects osteoclasts, osteoblasts and osteocytes rebalancing bone turnover and increasing bone formation [7–9]. Clinically, SrRN reduces the incidence of non-vertebral fractures including hip fractures in high-risk post-menopausal women (age >74 years) and increases BMD at both femoral and total hip [10–12].

Recent studies showed that the effect of SrRN treatment goes beyond its known effects on bone cells. Adipogenic differentiation of *ex-vivo* MSC harvested from ovariectomized SrRN treated rats was lower when compared to those from untreated controls [13]. Another study showed lower metaphyseal adipose tissue and higher trabecular volume in SrRN treated mice [14]. In terms of the mechanism of action of SrRN on adipocytes, Saidak et al. have recently reported that SrRN rebalances bone marrow adipogenesis and osteoblastogenesis in senescent osteopenic mice through the regulation of Wnt signaling pathways [15]. Taken together, these studies in murine cells indicate that SrRN has an inhibitory effect on adipogenesis while favors osteoblastogenesis both *in vitro* and *in vivo*. However, whether this effect is also seen in human

Abbreviations: PPARγ2, peroxisome proliferator activated receptor gamma 2; HGF, hepatocyte growth factor; IGFBP, insulin-like growth factor binding protein; BMD, bone mineral density; RANKL, receptor activator of nuclear factor kappa beta ligand; OPG, osteoprotegerin; CaSR, calcium-sensing receptor; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2*H*-tetrazolium; aP2, adipocyte protein 2; C/EBPα, CCAAT/enhancer-binding protein alpha; ENA-78, epithelial neutrophil activating peptide-78; TIMP, tissue inhibitor of metalloprotease; BMP, bone morphogenic protein.

* Corresponding author. Sydney Medical School Nepean, Level 5, South Block, Nepean Hospital, Penrith, NSW 2750, Australia. Tel.: +61 2 47344278; fax: +61 2 47341817.
E-mail addresses: chris.vidal@sydney.edu.au (C. Vidal), krishanthi.gunaratnam@sydney.edu.au (K. Gunaratnam), jessica.tong@student.unsw.edu.au (J. Tong), gustavo.duque@sydney.edu.au (G. Duque).

0300-9084/$ – see front matter © 2012 Elsevier Masson SAS. All rights reserved.
http://dx.doi.org/10.1016/j.biochi.2012.11.008

adipocytes and the implications of SrRN treatment in the biochemistry of differentiating human adipocytes remain untested. It would be expected that at the same time SrRN stimulates a differentiation shift into osteoblastogenesis [15], as part of its anabolic effect SrRN would also affect the level of adipokines produced by marrow fat, an effect that would induce a bone marrow milieu friendly to osteoblast differentiation and function [2].

In this study, we treated human MSC in adipogenic conditions with or without SrRN. Our results show that SrRN inhibited adipocyte differentiation by decreasing expression and activity of major adipogenic factors. A protein expression profile obtained from SrRN treated MSC helps to understand the effect of SrRN on the biochemistry of differentiating adipocytes.

2. Materials and methods

2.1. Cell culture

Bone marrow derived human MSC were plated at a density of 5×10^5 cells in 6 well plates in MSC growth medium at 37 °C in humidified atmosphere of 5% CO_2. Both MSC and MSC growth medium were bought from Lonza Australia Pty, (Mt Waverley, VIC, Australia). These primary cells are commercially available and are obtained from bone marrow of healthy young male donors (age 24–30 year old). At 90% confluency, MSC were induced to differentiate to adipocytes using adipogenic media for up to 21 days, with day of induction taken as day 0. Differentiation was done using three cycles of alternating adipocyte induction medium (AIM) and adipocyte maintenance medium (AMM), with media being changed every 3–4 days. AIM was prepared with Dulbecco Modified Eagle's Medium (DMEM) containing 4.5 g/L glucose (SIGMA-Aldrich Pty, Castle Hill, NSW, Australia; cat# D6429), 10% Fetal Bovine Serum (FBS), 100 Units/ml penicillin, 100 μg/ml streptomycin sulfate, 0.25 μg/ml amphotericin B (Invitrogen Australia Pty, Mulgrave, VIC, Australia; cat# 15240–062), 10 μg/ml insulin (Sigma cat# I-5500), 0.2 mM Indomethacin (Sigma cat# I7378), 0.5 mM 3-isobutyl-1-methyl-xanthine (Sigma cat# I5879) and 0.1 μM dexamethasone (Sigma D4902). AMM was prepared from DMEM, 10% FBS, antibiotic/antimycotic as for AIM, with the addition of 10 μg/ml insulin.

2.2. Treatment media and experimental conditions

SrRN was prepared, consisting of 1:100 molar ratio of Sr^{2+} derived from $SrCL_2$ (Sigma cat# 255521) and ranelate from sodium ranelate (generously donated by Prof Rebecca S Mason, Department of Physiology, University of Sydney, NSW, Australia). Concentrations of SrRN used in this study are expressed as mM Sr^{2+} (added to AIM and AMM) and reflect the relative concentrations of strontium and ranelic acid in the serum of patients treated with 2 g per day SrRN for 3 years as previously described [9]. All experiments were performed three times.

2.3. Oil red O (ORO) staining and measurement

ORO staining was used to assess adipocyte differentiation as an indicator of intracellular lipid accumulation. On day 21, culture medium was removed from tissue culture well and cells were rinsed with phosphate buffered saline (PBS) once, followed by fixation using 10% formaldehyde in PBS for at least 1 h. The fixative was then aspirated and cells were washed with 60% isopropanol before being allowed to dry completely. Cells were stained for 10 min at room temperature with a diluted solution of ORO (66.6%) prepared from a 0.5% w/v ORO dissolved in isopropanol. Cells were then washed 4 times with running tap water to remove excess stain. ORO was eluted with 1 ml 100% isopropanol for 10 min and absorbance measured at 500 nm.

2.4. Effect of SrRN treatment on cell viability

MSC were seeded at a density of 4×10^3 cells/well in four 96 well plates and after 24 h were treated with two different doses of SrRN as described above. At timed intervals starting from the day of induction (day 0), an MTS cell viability assay was performed following manufacturer's instructions (Promega Australia, Alexandria, NSW, Australia; cat# G3582). Briefly, 20 μl of MTS tetrazolium reagent were added into each well containing cells in 100 μl of medium. Incubation was carried out at 37 °C in 5% CO_2 for 2 h. The amount of formazan produced by metabolically active cells was determined by reading absorbance at 490 nm using a plate-reader. The percent survival was defined as [(experimental absorbance − blank absorbance)/(control absorbance − blank absorbance)] × 100. The control absorbance is the optical density obtained by cells while the blank absorbance is the optical density determined in wells containing medium and MTS only.

2.5. Gene expression profiling by real-time polymerase chain reaction (RT-PCR)

Total RNA was extracted on the 21st day of differentiation from differentiating adipocytes treated or untreated with SrRN using a QIAGEN RNeasy Mini extraction kit following manufacturer's instructions (QIAGEN Pty, Doncaster, VIC, Australia; cat# 74104). First strand complementary DNA (cDNA) synthesis was performed using 200 ng of total RNA, 50 ng random hexamers and 50 units reverse transcriptase at 42 °C for 1 h, as described by manufacturer (Bioline Australia Pty, Alexandria, NSW, Australia; cat# BIO-65025). Real-time PCR for expressed genes as markers for adipogenesis was performed in duplicate in a total reaction volume of 25 μl, 10% of which was cDNA (or water for non-template control), 3 mM $MgCl_2$ and 250 nM of each forward and reverse specific primer for target genes and normalizer (Table 1). All PCRs were performed in a Corbett Rotor-Gene™ 3000 (QIAGEN Pty) using SYBR green with no-ROX reaction mix and a standard thermal profile as described by supplier (Bioline Australia Pty, Alexandria, NSW, Australia; cat# QT6750-02). Quantitative RT-PCR data was defined by threshold

Table 1
Oligonucleotide primers used for real-time PCR.

	Primer sequences 5′–3′ (forward & reverse)	Accession number
PPARγ2	TCCATGCTGTTATGGGTGAA TCAAAGGAGTGGGAGTGGTC	NM_015869
aP2	AAAGAAGTAGGAGTGGGCTTTGC CCCCATTCACACTGATGATCAT	NM_001442
Adiponectin	GGCCGTGATGGCAGAGAT TTTCACCGATGTCTCCCTTAGG	NM_004797
C/EBPα	AAGAAGTCGGTGGACAAGAACAG TGCGCACCGCGATGT	NM_004364
Leptin	GGTTGCAAGGCCCAAGAA ACATAGAAAAGATAGGGCCAAAGC	NM_000230
LPL	TCCGCGTGATTGCAGAGA CGCTCGTGGGAGCACTTC	NM_000237
TIMP-1	AATTCCGACCTCGTCATCAG GGAACCCTTTATACATCTTGCT	NM_003254
Angiogenin	GCCAACCCCACCTAGATG CAGCACGAAGACCAACAAC	NM_001145
GAPDH	GAAATCCCATCACCATCTTCC AAATGAGCCCCAGCCTTCTC	NM_002046

Thermal profile for all PCRs: 40 cycles; denaturation 95 °C/15 s; annealing 60 °C/15 s; extension 72 °C/30 s.