Biochemical and Biophysical Research Communications 446 (2014) 30–36

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Reversine increases the plasticity of lineage-committed preadipocytes to osteogenesis by inhibiting adipogenesis through induction of TGF-β pathway *in vitro*

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ARTICLE INFO

Article history:
Received 30 January 2014
Available online 15 February 2014

Keywords:
Reversine
3T3-L1
Preadipocytes
Adipogenesis
Osteogenesis
TGF-β

ABSTRACT

Reversine has been reported to reverse differentiation of lineage-committed cells to mesenchymal stem cells (MSCs), which then enables them to be differentiated into other various lineages. Both adipocytes and osteoblasts are known to originate from common MSCs, and the balance between adipogenesis and osteogenesis in MSCs is reported to modulate the progression of various human diseases, such as obesity and osteoporosis. However, the role of reversine in modulating the adipogenic potential of lineage-committed preadipocytes and their plasticity to osteogenesis is unclear. Here we report that reversine has an anti-adipogenic function in 3T3-L1 preadipocytes *in vitro* and alters cell morphology and viability. The transforming growth factor-β (TGF-β) pathway appears to be required for the anti-adipogenic effect of reversine, due to reversine-induced expression of genes involved in TGF-β pathway and reversal of reversine-inhibited adipogenesis by inhibition of TGF-β pathway. We show that treatment with reversine transformed 3T3-L1 preadipocytes into MSC-like cells, as evidenced by the expression of MSCs marker genes. This, in turn, allowed differentiation of lineage-committed 3T3-L1 preadipocytes to osteoblasts under the osteogenic condition *in vitro*. Collectively, these findings reveal a new function of reversine in reversing lineage-committed preadipocytes to osteogenesis *in vitro*, and provide new insights into adipose tissue-based regeneration of osteoblasts.

1. Introduction

Adipose tissue plays a key role in the development of various chronic diseases and excess adipose tissue is inversely correlated with bone and muscle generation [1]. Adipocytes, myocytes and osteoblasts are known to originate from common mesenchymal stem cells (MSCs) [2,3]. While the transcription programs and cellular signaling pathways that determine the progenitor cell fate to a specific lineage have been extensively studied, the molecular mechanisms of transdifferentiation of lineage-committed cells to other cell types have not yet been fully explored.

Previous studies have reported the transdifferentiation potential of preadipocytes to osteoblasts and myoblasts. Ectopic expression of runt-related transcription factor 2 (Runx2), a transcription factor essential for osteoblast differentiation and mitogen-activated protein kinase phosphatase-1 (MKP-1) in mouse 3T3-L1 preadipocytes have been reported to promote transdifferentiation of preadipocytes to bone-forming osteoblasts *in vitro* [4]. In addition, activated morphogenetic protein-2 (BMP-2) and BMP-2/BMP receptor axis in differentiated human adipocytes [5] and mouse 3T3-F442A preadipocytes [6] have been shown to promote osteoblast differentiation. On the other hand, overexpression of aortic carboxypeptidase-like protein (ACLP) has been reported to transdifferentiate preadipocytes and adipocyte-derived multipotent progenitor cells into smooth muscle-like cells [7] and skeletal myocytes [8], respectively.

Reversine, a synthetic 2, 6-disubstituted purine analog, has been reported to induce dedifferentiation of lineage-committed mouse myoblasts to multipotent progenitor cells that can differentiate into either osteoblasts or adipocytes under the proper condition [9,10]. Reversine was also found to transform primary murine and human dermal fibroblasts into myogenic-competent cells [11]. Reversine has also been shown to promote differentiation of porcine muscle derived stem cells to female germ-like cells [12],

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http://dx.doi.org/10.1016/j.bbrc.2014.02.036
0006-291X/

and dedifferentiation of mouse macrophages to mesenchymal progenitor-like cells [13]. Despite the knowledge of its potential role in regenerative medicine, the role of reversine in reprogramming of lineage-committed preadipocytes is unclear.

In the present study, we examine the role of reversine in modulating the differentiation ability of lineage-committed preadipocytes to adipocytes and osteoblasts *in vitro*. We present the evidence that reversine-treated 3T3-L1 preadipocytes exhibit impaired adipogenesis, at least in part, through induction of transforming growth factor-β1 (TGF-β1) pathway. Furthermore, we show that reversine redirects the differentiation ability of 3T3-L1 preadipocytes toward osteogenesis under osteogenic condition. Collectively, our data suggest that reversine reciprocally regulates adipogenesis and osteogenesis in 3T3-L1 preadipocytes by upregulating the TGF-β1 pathway, thus indicating a novel function of reversine in reprogramming lineage-committed preadipocytes to osteoblasts.

2. Materials and methods

2.1. 3T3-L1 cell culture and adipogenesis

The 3T3-L1 preadipocytes were differentiated as described [14]. Briefly, preadipocytes were grown until confluence, then 2 days post-confluence (designated as Day 0), differentiation was induced with 10% fetal bovine serum (FBS)–DMEM supplemented with the standard adipogenic cocktail [167 nM insulin, 0.25 μM dexamethasone (Dex), and 0.5 mM isobutylmethylxanthine (IBMX)]. After 2 days of adipogenesis the medium was changed to 10% FBS–DMEM containing 167 nM insulin. On Day 4, the medium was changed to 10% FBS–DMEM with no additional supplements. For Oil Red O (ORO) staining, differentiated cells were fixed with 3% (v/v) formaldehyde for 1 h at room temperature and stained with ORO solution as previously described [14]. ORO-stained intracellular lipids in differentiated cells were extracted using isopropyl alcohol and then quantified using a spectrophotometric analysis at 490 nm using a microplate reader (Bio-Rad). To examine the effect of various cell signaling inhibitors on reversine-inhibited adipogenesis, 3T3-L1 preadipocytes were pretreated with 2.5 μM reversine in the absence or presence of 50 μM SB431542, SP600125, U0126, or LY204002 for 2 days (*i.e.*, Day −2 to Day 0). Adipogenesis was then initiated using the aforementioned method.

2.2. Electron microscopy

3T3-L1 preadipocytes treated with or without 2.5 μM reversine for 24 h were treated with a primary fixative 2.0% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4. After the initial fixation the cells were further treated with 1% osmium tetroxide. Cells were then embedded in 1.5% agarose and then dehydrated by ethanol wash. The cells were further processed by resin infiltration, embedded in beam capsule, and polymerized for 48 h at 60 °C. Samples were imaged using a Philips CM-100 Transmission Electron Microscope (TEM) (FEI company) operated at 100 kv, 200 μm condenser aperture and 70 μm objective aperture.

2.3. Cell viability and apoptosis assays

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Alfa Aesar) assay was performed as previously described [15], with modifications. Proliferating preadipocytes at 80% confluence were treated with various concentrations of reversine for 48 h. Cells were then incubated with fresh 10% FCS–DMEM containing 0.5 mg/ml MTT solution for 1 h at 37 °C. The medium was then aspirated and the remaining purple formazan crystals were solubilized using dimethoxyl sulfoxide (DMSO) for spectrophotometric analysis. Apoptosis in these cells was assessed using the GloMax-Multi Detection System (Promega) according to the manufacturer's instructions. 2×10^5 3T3-L1 preadipocytes per well were seeded on a 96-well plate and treated with various concentration of reversine (0–5 μM). After the 24 h treatment, Caspase-Glo 3/7 substrate was added to the wells and incubated for 1 h at room temperature, followed by measurement of luminescence by a Multi-well Plate Reader (Promega).

2.4. Osteogenesis

3T3-L1 preadipocytes were treated with 2.5 μM reversine for 3 days. Control cells were incubated with 0.1% DMSO. To induce osteogenic transdifferentiation, reversine or DMSO-treated 3T3-L1 preadipocytes were cultured in 10% FBS–DMEM supplemented with 0.1 μM Dex, 50 μg/ml ascobate-2-phosphate, 10 mM β-glycerophosphate for 14 days. Cells were then fixed with 3% formaldehyde at room temperature for 1 h. Osteogenesis of these cells was assessed by incubation with naphthol-ASMX–phosphatate (Sigma) and Fast Red TR salt (Sigma).

2.5. Real-time PCR assay

Total RNA from cultured cells was extracted using Reliaprep™ RNA cell miniprep system (Promega). First-strand cDNA was synthesized using ImPromII reverse transcriptase system (Promega). Real-time PCR was performed using iQ™SYGn Green supermix (Bio-Rad) in the iCycler iQ real-time PCR detection system (Bio-Rad). The results of the real-time PCR for gene transcripts were analyzed by the $2^{-\Delta\Delta Ct}$ calculation. The specific primers used for real-time PCR analyses are listed in Supplementary Table 1.

2.6. Statistical analysis

The data were analyzed using the one-way ANOVA of Statistical Analysis System 9.0. Dunnett's multiple comparison was performed by a Student's *t*-test procedure to compare the treatment group to a control group. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Reversine modulates cellular morphology, viability and apoptosis of 3T3-L1 preadipocytes

Previously, reversine has been shown to alter cell cycle and apoptosis in various cell types through inhibition of multiple cellular signaling pathways [10,16–20]. Thus, we first examined the effect of reversine on viability, apoptosis and cell morphology of 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were cultured in DMEM in the presence or absence of 2.5 μM reversine for 24 h. Reversine treatment resulted in a dramatic morphological change in 3T3-L1 preadipocytes, in that many were enlarged and flattened compared with those of non-treated control cells (Fig. 1A). Electron microscopy further showed multiple nuclei in reversine treated 3T3-L1 preadipocytes (Fig. 1B). Next, the effect of reversine on cellular viability and apoptosis in 3T3-L1 preadipocytes was determined using an MTT assay and a caspase 3/7-based luminescence assay, respectively. Reversine treatment resulted in a dose-dependent inhibition of 3T3-L1 preadipocyte viability, with approximately 30% decrease in cell viability at 5 μM reversine (Fig. 1C). In addition, reversine treated cells exhibited elevated levels of caspase 3/7 activity (Fig. 1D). These results indicate that reversine alters cell morphology, and modulates cell cycle progression and viability of preadipocytes.