The polymine spermine regulates osteogenic differentiation in adipose stem cells†

G.S. Tjabringa a, †, B. Zandieh-Doulabi a, †, * , M.N. Helder a, b, M. Knippenberg a, b, P.I.J.M. Wuisman b, J. Klein-Nulend a, *

a Department of Oral Cell Biology, Academic Center of Dentistry Amsterdam (ACTA), Universiteit van Amsterdam and Vrije Universiteit, Research Institute MOVE, Amsterdam, The Netherlands
b Department of Orthopaedic Surgery, VU University Medical Center, Research Institute MOVE, Amsterdam, The Netherlands

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Abstract

For bone tissue engineering, it is important that mesenchymal stem cells (MSCs) differentiate into osteoblasts. To develop a method for differentiation of adipose tissue-derived mesenchymal stem cells (AT-MSCs) along the osteogenic lineage, we studied the effect of polyamines, which are organic cations implicated in bone growth and development, on differentiation of AT-MSCs. Treatment of goat-derived AT-MSCs with 1,25-dihydroxyvitamin-D3 (1,25(OH)2D3), which stimulates osteogenic differentiation, for 7 days induced gene expression of the polyamine-modulated transcription factor-1 (PMF-1) and spermidine/spermine N(1)-acetyltransferase (SSAT), which are both involved in polyamine metabolism, suggesting that polyamines are involved in osteogenic differentiation of AT-MSCs. Furthermore, treatment of AT-MSCs with the polyamine spermine-regulated gene expression of runx-2, a transcription factor involved in early stages of osteogenic differentiation, and that of osteopontin, a bone matrix protein expressed in later stages of osteogenic differentiation. Runx-2 gene expression was increased 4 and 14 days after a short 30 min. treatment with spermine, while osteopontin gene expression was only increased 4 days after spermine treatment. Finally, alkaline phosphatase activity, which is intimately involved in the formation of extracellular matrix of bone, was increased 4 weeks after the 30 min.-spermine treatment of AT-MSCs. In conclusion, this study shows for the first time that the polyamine spermine regulates differentiation of AT-MSCs along the osteogenic lineage, which can be used as a new method for differentiation of AT-MSCs along the osteogenic lineage. Therefore, polyamines may constitute a promising tool for bone tissue engineering approaches using AT-MSCs, such as a one-step surgical procedure for spinal interbody fusion.

Keywords: polyamines • adipose tissue-derived mesenchymal stem cells • 1,25-dihydroxyvitamin-d3 • spermidine/spermine N(1)-acetyltransferase • polyamine-modulated transcription factor-1 • runx-2, osteopontin • alkaline phosphatase • bone cells

Introduction

Mesenchymal stem cells (MSCs) are currently evaluated for use in tissue engineering approaches. These stem cells are characterized by self renewal capacity, long life-span and the potential to differentiate into several lineages including bone, cartilage, muscle and fat [1]. While bone marrow is a frequently used source for MSCs, relatively low cell numbers can be isolated from bone marrow. Recent studies have reported on the presence of 100–1000-fold higher numbers of MSCs per volume in adipose tissue [2, 3]. Adipose tissue can be harvested from patients by minimally invasive methods and is in general abundantly available [4]. Therefore, adipose tissue-derived mesenchymal stem cells (AT-MSCs) constitute a promising tool for tissue engineering approaches targeting skeletal defects [4, 5]. One of these approaches is a one-step surgical procedure to facilitate spinal interbody fusion [6]. This one-step procedure consists of the
harvesting and processing of AT-MSCs from adipose tissue, osteogenic stimulation of the cells, by, for example, growth factors for a short period of time (30 min.), seeding of stimulated cells on a scaffold, and implantation of the tissue-engineered construct between two vertebrae. The integrated steps can be accomplished within one surgical procedure [6, 7].

For bone tissue engineering, it is important that stem cells differentiate into osteoblast-like cells, and display a bone cell-like response to mechanical loading [6]. Polyamines, which are organic cations derived from amino acids, may be involved in both processes. Polyamines have been associated with a variety of biological processes such as proliferation, differentiation, and apoptosis [8–10]. Furthermore, polyamines were suggested to be involved in growth and development of a range of mammalian tissues, and in re-modelling processes associated with tissue repair [8–10]. In addition to normal growth and development, polyamines have also been associated with tumour growth and metastasis, and enhanced polyamine levels and high activity of enzymes involved in polyamine biosynthesis were observed in tumour cells [11]. Cellular polyamine levels are tightly regulated by a complex network of factors that are involved in polyamine biosynthesis and catabolism [12]. Biosynthesis includes the conversion of the amino acid ornithine to the polyamine putrescine, and subsequently to the polyamines spermidine and spermine. Catabolism of polyamines is mediated via acetylation of spermidine and spermine by the spermidine/spermine N(1)-acetyltransferase (SSAT), which targets them for cellular excretion, or results in their conversion into putrescine [12]. Gene expression of SSAT is regulated by the polyamine-modulated transcription factor-1 (PMF-1) [13, 14]. Polyamines are intimately involved in their own biosynthesis and degradation, since both SSAT and PMF-1 have polyamine-responsive elements in their promotor [13, 14].

Several studies have implicated polyamines in bone and cartilage growth and development [15–17]. Polyamines were shown to be abundantly present in the ossifying area as compared to the rest region in epiphyseal cartilage from calf scapulas [15], and alkaline phosphatase activity, which is a marker for bone cell differentiation, was enhanced by polyamines [15]. This suggests that polyamines may play a role in calcification of pre-ossusseous cartilage. Furthermore, the activity of enzymes involved in polyamine metabolism and polyamine levels in rabbit costal chondrocytes are regulated by parathyroid hormone (PTH), which is involved in bone re-modelling [16]. Finally, polyamines have been demonstrated to regulate cyclooxygenase-2 (COX-2) expression [17], which is intimately involved in bone adaptation to mechanical loading [18, 19]. Together, these studies implicate an important role for polyamines in bone growth and development. Recent studies in our laboratory have implicated polyamines in the response of AT-MSCs to mechanical loading [20]. The polyamine spermine was shown to inhibit nitric oxide production and COX-2 gene expression induced by mechanical loading as applied by pulsating fluid flow (PFF), suggesting that polyamines modulate the response of human AT-MSCs to mechanical loading [20]. Whether polyamines are involved in the differentiation of AT-MSCs along the osteogenic lineage is currently unknown.

We first hypothesized that expression of the polyamine-related genes SSAT and PMF-1 is regulated during 1,25(OH)2D3-stimulated osteogenic differentiation of AT-MSCs. 1,25(OH)2D3 increased gene expression of SSAT and PMF-1 by AT-MSCs, suggesting that polyamine levels are modulated during osteogenic differentiation. Therefore we further hypothesized that polyamines themselves may stimulate osteogenic differentiation of AT-MSCs after a short treatment period of only 30 min. We investigated whether the polyamine spermine affects gene expression of runx-2, which is a transcription factor critically involved in early stages of osteogenic differentiation, and osteopontin, which is a bone matrix protein expressed during later stages of osteogenic differentiation. We also studied whether spermine affects alkaline phosphatase (ALP) activity, which is intimately involved in bone extracellular matrix formation.

This study may support the use of polyamines as a novel approach for differentiation of AT-MSCs towards osteoblasts. This new method may be used in tissue engineering approaches targeting skeletal defects, such as the one-step surgical procedure for spinal interbody fusion.

Materials and methods

Isolation of AT-MSCs

AT-MSCs were isolated as described by Zuk et al. [2, 3] with minor modifications. Briefly, goat adipose tissue was resected from the renal region of 4 adolescent female goats. The animal care and use committee of the Vrije Universiteit Amsterdam approved the use of goats in these experiments. The obtained tissue was washed with phosphate buffered saline (PBS) to remove red blood cells, chopped into small pieces of about 50 mm3, and the extracellular matrix was digested for 90 min. at 37°C with 0.05% collagenase (type 1, Sigma, St Louis, MO, USA) in PBS. A single cell suspension was obtained by filtering the digested material through a 100 µm mesh filter (Stokvis & Smith B.V., Umiinden, The Netherlands) to remove tissue debris. The AT-MSC-containing cell suspension was centrifuged at 600 g, and the pellet was re-suspended in culture medium, which was composed of Dulbecco’s modified Eagle’s medium (D-MEM, Gibco, Paisley, UK) supplemented with 500 µg/ml streptomycin sulphate (Sigma), 600 µg/ml penicillin (Sigma), 50 µg/ml gentamycin (Gibco), 2.5 µg/ml fungizone (Gibco) and 10% foetal bovine serum (Hyclone, Logan, UT, USA). The re-suspended cells were incubated for 15 min. at 37°C with 160 mM NH4Cl (Merck, Darmstadt, Germany) to destroy remaining erythrocytes. AT-MSCs were then washed three times with culture medium, and immediately used for experiments. The polyamine spermine was added, and cells were post-incubated as described below.

Approximately 0.5–2% of the isolated cells do form colony-forming units (CFUs) [4]. Furthermore, goat-AT-MSCs were characterized as described by Knippenberg et al. [5]. Briefly, approximately 35% of freshly isolated AT-MSCs was positive for the stem cell marker CD105/endoglin, while 30% was positive for the stem cell marker CD166/ALCAM. In addition, cultured cells were shown to express the intermediate filament vimentin, which is predominantly found in MSCs [5].
Table 1 Primers used for real time PCR

| Target gene  | Oligonucleotide sequence | Product size (bp), and acc.nr. GenBank | Annealing temperature (°C) |
|--------------|--------------------------|---------------------------------------|---------------------------|
| 18s -FW      | 5’-gtaacccttggaaccccatt- 3’ | 151, gi|76363890| 56 |
| 18s -REV     | 5’-cataacagtctcggtcgc- 3’   |                                       |                           |
| Runx2 -FW    | 5’-atgtcattcctgctc- 3’     | 156, gi|76363891| 56 |
| Runx2 -REV   | 5’-actgcttgccagcttcaaat- 3’|                                       |                           |
| OPN -FW      | 5’-ttcagaagctcgaag- 3’     | 151, gi|76363893| 56 |
| OPN -REV     | 5’-gtaccagttcatagttcatc- 3’|                                       |                           |
| SSAT-FW      | 5’-gacatacgtcggtcgtc- 3’   | 225, gi|76363895| 56 |
| SSAT -REV    | 5’-caaatccagggctcaggttaa- 3’|                                       |                           |
| PMF-1 -FW    | 5’-ttctcaagcttgctgc- 3’    | 219, gi|76363897| 56 |
| PMF-1 -RE    | 5’-gcagtttgcctctcc- 3’     |                                       |                           |

AT-MSC culture and stimulation

To induce osteogenic differentiation, goat-derived AT-ASCs (passage 2) were seeded at 2 x 10^3 cells/well in 6-well plates (Corning Incorporated, Corning, NY, USA), and osteogenic differentiation was induced by culturing the cells in culture medium supplemented with 0.1 mg/ml ascorbic acid (Merck), 10 mM β-glycerophosphate (Sigma), and 10 nM 1,25(OH)2D3 (Sigma) for 7 or 14 days. Treatment is determined by real time PCR.

For stimulation of cells with spermine, freshly isolated AT-MSCs were incubated for 30 min. in culture medium alone (control), or in culture medium containing 0.1 mg/ml ascorbic acid (Merck) and 10 mM β-glycerophosphate (Sigma) with or without various concentrations (1, 3, 10 and 30 µM) of the polyamine spermine (Sigma). Thereafter, cells were seeded in 6-well culture dishes (Corning Incorporated, Corning, NY, USA) at 18 x 10^4 cells per well, and cultured without spermine for 4–28 days in culture medium alone, or in medium containing 0.1 mg/ml ascorbic acid (Merck) and 10 mM β-glycerophosphate (Sigma). Medium was refreshed twice a week. After 4 and 14 days, mRNA was isolated, and gene expression of SSAT, runx-2, osteopontin, osteopontin, and osteopontin was determined by real time PCR. After 2, 3 and 4 weeks, ALP activity was determined as described below.

Real time PCR

Total RNA was extracted using Trizol® Reagent (Invitrogen) according to the manufacturer’s instructions. To increase RNA yield, 5 µg glycogen (Roche Diagnostics, Mannheim, Germany) was added to RNA in isopropanol prior to centrifugation. Total RNA (500–750 ng) was reverse transcribed using 250 U/ml Transcripter Reverse Transcriptase (Roche Diagnostics, Mannheim, Germany), 0.08 A260 units random primers (Roche Diagnostics, Mannheim, Germany), and 1 mM of each dNTP (Invitrogen) in Transcripter reverse transcriptase (RT) reaction buffer for 30 min. at 55°C followed by 5 min. inactivation of reverse transcriptase at 85°C. The cDNA was diluted (5–10x) and 2 µl of cDNA was used per reaction for real-time PCR using a SYBRGreen reaction kit for 18S (3 mM MgCl2) or Fast start plus SYBRGreen kit (both from Roche Diagnostics) for SSAT, runx-2 and osteopontin in a LightCycler (Roche Diagnostics). PCR conditions were as follows. For 18S: 1 min. pre-incubation at 95°C, followed by 30 cycles of amplification at 95°C for 5 sec., 56°C for 10 sec., 72°C for 15 sec., and 82°C for 5 sec., followed by melting curve analysis; for SSAT: 10 min. pre-incubation at 95°C, followed by 35 cycles of amplification at 95°C for 5 sec., 57°C for 10 sec., 72°C for 15 sec., and 82°C for 5 sec., followed by melting curve analysis; for osteopontin: 10 min. pre-incubation at 95°C, followed by 35 cycles of amplification at 95°C for 5 sec., 57°C for 10 sec., 72°C for 15 sec., and 82°C for 5 sec., followed by melting curve analysis, for PMF-1: 10 min pre-incubation at 95°C, followed by 35 cycles of amplification at 95°C for 5 sec., 57°C for 15 sec., 72°C for 15 sec., followed by melting curve analysis; for runx-2: 10 min pre-incubation at 95°C, followed by 35 cycles of amplification at 95°C for 5 sec., 57°C for 15 sec., 72°C for 15 sec., and 82°C for 5 sec., followed by melting curve analysis; for osteopontin: 10 min. pre-incubation at 95°C, followed by 35 cycles of amplification at 95°C for 5 sec., 57°C for 15 sec., 72°C for 15 sec., followed by melting curve analysis.

With the Light Cycler software (version 1.2), the crossing points were assessed and plotted versus the serial dilution of known concentrations of the standards derived from each gene. PCR efficiency (E) was obtained by using the formula $E = 10^{-1/slope}$. Data were used only if the PCR efficiency calculated was between 1.85 and 2.0.

To avoid contamination with genomic DNA, intron-spanning primers (except for 18S) were designed (Table 1) using Clone manager suite software program version 6 (Scientific & Educational Software, Care, NC, USA), and used at a concentration of 1 µM. Data analysis was performed using the LightCycler software (version 2). After normalization for 18S housekeeping gene expression, relative target gene expression was determined.

ALP activity

Goat-derived AT-MSCs were seeded at a density of 10^4 cells/cm^2, either or not stimulated with spermine for 30 min., and cultured for 1, 3 or 4 weeks. The cells were washed with PBS, and fixed in 4% paraformaldehyde (Sigma) for 15 min. at 4°C. The cells were washed again with PBS, and incubated with 0.1 M Tris buffer at pH 9.5 for 10 min. at room temperature.
Staining of the cells was performed using 0.1 M Tris buffer containing 0.1 M NaCl (Sigma), 0.05 M MgCl₂ (Sigma) and 50x diluted with 4-Nitro blue tetrazolium chloride/ 5-Bromo-4-chloro-3-indolyl-phosphate, disodium salt (NBT/BCIP; Roche Diagnostics GmbH, Mannheim, Germany) for 30 min. Thereafter, the cells were washed with water and incubated for 5 min. in 100% methanol (Sigma) to remove non-specific background staining, and cells staining positive for ALP activity were counted.

**Statistical analysis**

Data on gene expression were obtained from six separate experiments using six different goats, and data on ALP activity were obtained from four separate experiments using four different goats. When indicated, real-time PCR data were log-transformed in order to obtain normally distributed data. Treatment-over-control (T/C) values were not log-transformed. Data are presented as mean ± SEM, and statistical analysis was performed using the Student’s t test.

**Results**

To study whether expression of genes involved in polyamine metabolism is modulated during osteogenic stimulation in AT-MSCs, goat-derived AT-MSCs were stimulated for 7 or 14 days in osteogenic medium containing 10 nM 1,25(OH)₂D₃, and gene expression of the polyamine-related genes SSAT and PMF-1 was determined. 1,25(OH)₂D₃ stimulated osteogenic differentiation of AT-MSCs as shown by increased gene expression of both runx-2 (Fig. 1A), which is a transcription factor involved in early stages of osteogenic differentiation, and osteopontin (Fig. 1B), which is a bone matrix molecule expressed during later stages of osteogenic differentiation, after 14 days. Gene expression of both SSAT (Fig. 1C) and PMF-1 (Fig. 1D) was increased after 7 days stimulation in osteogenic medium, suggesting involvement of polyamines in osteogenic differentiation of AT-MSCs.

To study whether polyamines may be used in the one-step surgical procedure for spinal interbody fusion, we investigated whether stimulation of AT-MSCs with the polyamine spermine for a short period of time (30 min.) stimulated expression of genes involved in osteogenic differentiation. Therefore, we first examined whether goat-derived AT-MSCs are responsive to polyamines, by measuring the effect of the polyamine spermine on gene expression of SSAT, which regulates polyamine catabolism. Cells were treated with spermine for 30 min., and 4 days after treatment, gene expression of SSAT was determined by real time PCR. Treatment of AT-MSCs with spermine at concentrations of
3–30 µM significantly increased SSAT gene expression, with a maximum increase of 2.6-fold at 3 µM spermine, suggesting that AT-MSCs are responsive to spermine (Fig. 2). Fourteen days after a 30 min. treatment with spermine, SSAT gene expression in cells treated with spermine was similar to that in control cells (data not shown).

Four days after treatment of AT-MSCs with 3–30 µM spermine, runx-2 gene expression was significantly increased, reaching a 2.4-fold increase at 3 µM spermine (Fig. 3A). In addition, 14 days after treatment with 10-µM spermine, runx-2 gene expression was increased by 3.8-fold (Fig. 3B). Furthermore, four days after treatment of AT-MSCs with 30-µM spermine, osteopontin gene expression was significantly increased by 9.8-fold (Fig. 4A), while no significant effect of spermine on osteopontin gene expression was observed 14 days after treatment (Fig. 4B).

In the present study, we present a new method for differentiation of AT-MSCs along the osteogenic lineage. We demonstrate that polyamines are involved in 1,25(OH)2D3-induced osteogenic differentiation of AT-MSCs. Furthermore, treatment of AT-MSCs with the polyamine spermine for 30 min. was shown to regulate gene expression of runx-2 and osteopontin, which are involved in early and later stages of osteogenic differentiation, respectively. In addition, spermine treatment was shown to increase the number of cells staining positive for ALP activity, which is important for the formation of extracellular bone matrix. Our findings demonstrate that stimulation of AT-MSCs with the polyamine spermine can be used as a new procedure for differentiation of AT-MSCs along the osteogenic lineage.

To further evaluate the effect of spermine on differentiation of AT-MSCs along the osteogenic lineage, the effect of spermine treatment on ALP activity was studied. Cells were treated for 30 min. with different concentrations spermine (1–30 µM), and after 1, 3 and 4 weeks ALP activity was visualized using NBT/BCIP. The number of AT-MSCs showing positive staining for ALP activity was significantly increased by 1.4-fold, 4 weeks after treatment with 3 µM spermine (Fig. 5). The total number of cells was not changed by spermine (data not shown).

Intracellular polyamine levels are tightly regulated by a group of interrelated factors that control both biosynthesis and catabolism via different enzymatic steps [12]. Disregulation of cellular polyamine homeostasis has been associated with tumour formation and metastasis [11]. Continuous high levels of polyamines and enzymes involved in polyamine metabolism, have been demonstrated in different forms of cancer, and enzymes involved in polyamine metabolism are currently evaluated as targets for the development of anti-cancer drugs [23]. We show that 30 min. treatment of AT-MSCs with the polyamine spermine increases gene expression of SSAT, which targets polyamines for degradation, suggesting that polyamine homeostasis will be restored shortly after polyamine treatment. This is supported by the observation that 14 days after spermine treatment, SSAT gene expression has returned to basal levels (data not shown). Restoring polyamine homeostasis may be important, since continued stimulation of cells with polyamines may result in pathological conditions, such as tumour formation and metastases.

Discussion

In the present study, we present a new method for differentiation of AT-MSCs along the osteogenic lineage. We demonstrate that polyamines are involved in 1,25(OH)2D3-induced osteogenic differentiation of AT-MSCs. Furthermore, treatment of AT-MSCs with the polyamine spermine for 30 min. was shown to regulate gene expression of runx-2 and osteopontin, which are involved in early and later stages of osteogenic differentiation, respectively. In addition, spermine treatment was shown to increase the number of cells staining positive for ALP activity, which is important for the formation of extracellular bone matrix. Our findings demonstrate that stimulation of AT-MSCs with the polyamine spermine can be used as a new procedure for differentiation of AT-MSCs along the osteogenic lineage.

For bone tissue engineering it is important that stem cells display a bone cell-like response to mechanical loading, and differentiate along the osteogenic lineage. Earlier studies in our laboratory have shown that polyamines inhibit PFF-induced NO production and COX-2 gene expression [20], suggesting that polyamines regulate the response of AT-MSCs to mechanical loading. In the present study, we investigated whether polyamines are involved in 1,25(OH)2D3-induced osteogenic differentiation of AT-MSCs, and whether they regulate differentiation of AT-MSCs towards the osteogenic lineage. We found that spermine increases gene expression of both runx-2 and osteopontin, and the number of cells exhibiting activated ALP, suggesting that spermine regulates osteogenic differentiation of AT-MSCs. Interestingly, treatment of AT-MSCs with spermine for only 30 min. resulted in changes in ALP activity for up to 4 weeks after treatment, indicating that spermine may be an interesting tool for treatment of disc degeneration by spinal fusion using the one-step procedure, which requires osteogenic stimulation of AT-MSCs for a short period of time to direct differentiation of AT-MSCs towards the osteogenic lineage.

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The polyamines putrescine, spermidine and spermine affect various cellular processes [8–10]. While polyamines are derived from the same precursor, the various polyamines have been demonstrated to activate different intracellular signalling pathways [24]. Both polyamines putrescine and spermidine have been shown to stimulate tyrosine phosphorylation by tyrosine kinases [24]. However, while putrescine increases the expression of the transcription factors c-fos and c-jun, spermidine activates mitogen-activated protein kinases (MAPKs) and c-myc [24]. In contrast to putrescine and spermidine, the signal transduction pathways activated by spermine are still poorly described [24]. Activation of different intracellular signalling pathways by the various polyamines indicates that they display different effects on cellular activities. In the present study, we
show that the polyamine spermine affects differentiation of AT-MSCs. Since putrescine and spermidine may display different effects on cellular activities as compared to spermine, the effects of these polyamines on the differentiation of AT-MSCs needs further study.

In conclusion, this study presents a new way for differentiation of AT-MSCs along the osteogenic lineage by stimulation of AT-MSCs with the polyamine spermine. This new method may be used in tissue engineering approaches targeting skeletal defects, such as the one-step surgical procedure for spinal interbody fusion.

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