Molecular and Cellular Endocrinology 323 (2010) 282–291

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

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

Effect of adiponectin on ATDC5 proliferation, differentiation and signaling pathways

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ARTICLE INFO

Article history:
Received 17 January 2010
Received in revised form 28 March 2010
Accepted 30 March 2010

Keywords:
Adipocytokine
Adiponectin
ATDC5
Chondrocyte
Bone development

ABSTRACT

Adiponectin, an adipose-secreted adipocytokine, exhibits various metabolic functions but has no known effect on bone development through the growth plate and specifically, in chondrocytes. Using the mouse ATDC5 cell line, a widely used *in vitro* model of chondrogenesis, we demonstrated the expression of adiponectin and its receptors during chondrogenic differentiation. Adiponectin at 0.5 μg/ml increased chondrocyte proliferation, proteoglycan synthesis and matrix mineralization, as reflected by upregulation of the expression of type II collagen, aggrecan, Runx2 and type X collagen, and of alkaline phosphatase activity. Quantitative RT-PCR and gelatin zymography showed a significant increase in the matrix metalloproteinase MMP9's expression and activity following adiponectin treatment. We therefore concluded that adiponectin can directly stimulate chondrocyte proliferation and differentiation. To evaluate the underlying mechanisms, we examined the effect of adiponectin on the expression of chondrogenic signaling molecules: Ihh, PTHrP, Ptc1, FGF18, BMP7, IGF1 and p21 were all upregulated while FGF9 was downregulated. This study reveals novel and direct activity of adiponectin in chondrocytes, suggesting its positive effects on bone development.

1. Introduction

The traditional view of the adipocytes as fat-storing cells has been revolutionized in recent years: they are now recognized as an endocrine tissue modulating the physiological functions of other tissues via adipose-derived hormones—the adipocytokines (Mohamed-Ali et al., 1998). Among these hormones, adiponectin is a 30-kDa protein secreted predominantly by differentiated adipocytes and involved in energy homeostasis (Lee and Karsenty, 2008). In contrast to other adipocytokines, such as leptin, TNF-α, resistin, and Il-6 (Ukkola and Santaniemi, 2002), adiponectin mRNA is reduced in adipose tissue of obese and diabetic mice and humans but restored to normal levels following weight loss (Yang et al., 2001). This reduction of adiponectin levels in obesity, conditions of insulin resistance and diabetes, and cardiovascular disease with increasing severity, appears to precede metabolic disorders and leads to metabolic syndrome. Adiponectin is found in human blood (at 5–10 μg/ml), accounting for 0.01% of the total serum (Koerner et al., 2005), and its level is inversely correlated with the percentage of body fat in adults (Ukkola and Santaniemi, 2002).

Adiponectin-transgenic mice, probably the world's fattest mice, can overeat without developing insulin resistance or diabetes, probably since adiponectin enables the mice to store excess calories in fat tissue instead of in the liver, heart or muscle tissue—places where excess fat can lead to inflammation, diabetes and heart disease. This suggests a protective role for adiponectin against the development of obesity, type II diabetes and cardiovascular disease, and makes it a promising hormone in the treatment of metabolic syndrome (Berg and Scherer, 2005).

In addition to its effects on metabolic homeostasis, adiponectin has anti-inflammatory properties in the cardiovascular system (Yokota et al., 2000) and pro-inflammatory effect in articular cartilage and chondrocytes through the induction of nitric oxide (Lago et al., 2008; Gomez et al., 2009). There is some evidence connecting adiponectin to skeletal metabolism: adiponectin receptors are expressed by bone cells and serum levels of adiponectin are positively associated with bone mineral density (Lenchik et al., 2003). *In vitro* studies have shown that adiponectin enhances mRNA expression of alkaline phosphatase (ALP) and mineralization of MC3T3-E1 osteoblasts (Oshima et al., 2005) increases proliferation of primary human osteoblasts and inhibits M-CSF and RANKL-induced differentiation of human CD14-positive mononuclear cells into osteoclasts (Williams et al., 2009). Chen et al. (2006) demonstrated that adiponectin is present in osteoarthritic synovial fluid, and that its receptors, AdipoR1 and AdipoR2 are expressed in cartilage, bone and synovial tissues. They further suggested that adiponectin is involved in the joint cartilage's metabolism and

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doi:10.1016/j.mce.2010.03.025

inflammation by altering the balance of matrix metalloproteinases (MMPs) and their inhibitors TIMPs (Chen et al., 2006). Lago et al. (2008) showed that AdipoR1 and AdipoR2 are expressed and functioning in chondrocytes and induce pro-inflammatory cytokines. Nevertheless, adiponectin's effect on bone development through the growth plate, and specifically its role in the proliferation and differentiation of cartilage cells – the chondrocytes – have never been elucidated.

Longitudinal bone growth occurs via the process of endochondral ossification in which mesenchymal cells differentiate into chondrocytes, which are then replaced by osteoblasts in the growth plate. The chondrocytes are essential for cartilage formation and properties: they synthesize various elements of the extracellular matrix (ECM), which undergoes mineralization and degradation in a precisely regulated manner (Johnstone et al., 2000; Karsenty, 2003; Karsenty and Wagner, 2002; Burdan et al., 2009). Based on chondrocyte differentiation and matrix production and distribution, the growth plate can be divided into different zones: reserve, proliferative, prehypertrophic and hypertrophic (Burdan et al., 2009). In proliferative chondrocytes (i.e., during early chondrogenesis), the ECM proteins are mainly type II collagen (Col II) and aggrecan (Agc1), while in hypertrophic chondrocytes (i.e., during late chondrogenesis), type X collagen (Col X), Indian hedgehog (Ihh) and Runx2 are highly expressed (Lefebvre and Smits, 2005).

The process of longitudinal bone growth is regulated by a multitude of genetic, environmental and nutritional factors (van der Eerden et al., 2003). In this study we focused on the possible role of adiponectin in the regulation of bone growth, using an *in vitro* model of ATDC5 chondrocyte proliferation and differentiation. ATDC5 cells, a chondrogenic cell line derived from a differentiating culture of AT805 teratocarcinoma cells (Atsumi et al., 1990), are capable of differentiating into chondrocytes. The proliferation and differentiation of ATDC5 cells *in vitro* can be mimicked *in vivo* by molecular analysis of early- and late-phase differentiation markers of chondrocytes. In this system, we found that adiponectin and its receptors are expressed and that recombinant adiponectin stimulates proliferation and differentiation of ATDC5 chondrocytes, suggesting adiponectin as a novel regulator of bone development.

2. Materials and methods

2.1. Materials

Insulin transferrin sodium selenite (ITS) was obtained from Sigma–Aldrich, and Dulbecco's modified Eagle's medium and Ham's F-12 (DMEM/F12), trypsin–EDTA solution, fetal bovine serum (FBS), L-glutamine, adiponectin was from Biochemical Industries, Israel.

2.2. Cell culture and differentiation conditions

ATDC5 cells were cultured in a 1:1 mixture (v/v) of DMEM/F12 supplemented with 5% (w/v) FBS and ITS. Cells were subcultured at 80–90% confluency using trypsin/EDTA and incubating at 37 °C in a humidified atmosphere of 95% air, 5% CO_2. The medium was changed every other day throughout the ATDC5 cell differentiation period.

2.3. Cell proliferation

ATDC5 cells were cultured in 96-well plates at a density of 25,000 cell/well in DMEM/F12 containing 1% FBS and treated with recombinant mouse adiponectin for 24 h. After 24 h of incubation, the cells were detached from the wells using trypsin/EDTA and counted directly in a hemocytometer chamber.

2.4. Alcian blue and Alizarin red staining

Cells were cultured in 12-well plates at a density of 50,000 cell/well for 4, 7, 14, and 21 days, with the indicated concentrations of adiponectin. Cells were then fixed in 4% (v/v) formalin for 15 min and stained with Alcian blue 8GX for 30 min or with 1% (w/v) Alizarin red for 10 min (Shukunami et al., 1996). The stained cells were washed three times with 1× PBS and photographed with a scanning camera (Epson Perfection 4490 Photo).

Table 1
Primers used for relative quantification real-time PCR (all primers suitable for mouse cDNA, F = forward, R = reverse).

Gene		Sequence	Accession number
AdipoQ	F	TGTTGCTGGGAGCTGTTCTACTG	NM_004797
	R	ATGTCTCCCTTAGGACCAATAAG	
AdipoR1	F	TCTGGACACATCTGCTTGGTTT	NM_028320
	R	CTGGGCATTGCAGCCATTAT	
AdipoR2	F	CTGGGCATTGCAGCCATTAT	NM_197985
	R	GAACACTCCTGCTCTGACCCC	
Agc1	F	TCTTTGCCACCGGAGA	L07049
	R	TTTTTACACGTGAA	
Col II	F	GAACAGCATCGCCTACCTGG	NM_001844
	R	TGTTTCGTGCAGCCATCCT	
Col X	F	CTCCTACCACGTGCATGTGAA	NM_000493
	R	ACTCCCTGAAGCCTGATCCA	
Runx2	F	GGTTGTAGCCCTCGGAGAGG	NM_001145920
	R	GCCATGACGGTAACCACAGTC	
MMP2	F	ATTGACGCTGTGTATGAGGCC	NM_008610
	R	ACTCATTCCCTGCGAAGAACA	
MMP9	F	AGCCCCTGCTCCTGGCTCTC	NM_013599
	R	CTGCCAGCTGGGTGTCCGTG	
Ihh	F	TGGACTCATTGCCTCCCAGA	BC046984
	R	CAAAGGCTCAGGAGGCTGGA	
Ptc1	F	GATGGGCCTCATTGGGATC	XR_01728
	R	AAAGGCCAAAGCCACGTG	
PTHrP	F	CGGTTTGGGTCAGACGA	NR_002824
	R	TTCCCGGGTGTCTTGAGTG	
P21	F	GGCCCGGAACATCTCAGG	NM_007669
	R	AAATCTGTCAGGCTGGTCTGC	
BMP7	F	AGCCACTTCCTCACTGACGC	NM_007557
	R	TCCCTAGGTTGACGAAGCTCA	
IGF1	F	ACTTCAACAAGCCCACAGGC	NM_001111276
	R	TCTGAGGTGCCCTCCGAAT	
FGFR3	F	TATCCAGGACCCGGCTGAC	NM_001163213
	R	GGCAGTACGGTCCTTG	
FGF9	F	GGGAACCAGGAAAGACCACA	NM_002010
	R	TCATGCCGAGGTAGAGT	
FGF18	F	TGATGTGAGTCGGAAGCAGCT	NM_003862
	R	CATACTTGTCCCCGTCCTCG	
GAPDH	F	TGACGTGCCGCGTGGAGAA	NM_001102369
	R	AGTGTAGCCAACATGCCCTTCAG	

2.5. Alkaline phosphatase (ALP) assay

ATDC5 cells were seeded in 6-well plates at a density of 100,000 cell/well and treated with the indicated amounts of adiponectin. Harvested cells were suspended in lysis buffer and ALP activity was assessed in a reaction mixture of 0.5 mM *p*-nitrophenyl phosphate (Sigma–Aldrich) as the substrate and 0.5 mM $MgCl_2$ for 15 min at 37 °C. *p*-Nitrophenol concentration was measured at 405 nm. Protein content of cell lysates was determined using the BCA protein reagent kit (Pierce Biotechnology)

2.6. RNA isolation, reverse transcription (RT)

Total RNA was extracted from ATDC5 cells using TRIzol reagent (Sigma–Aldrich) according to the manufacturer's protocol. Total RNA (2 μg) was reverse-transcribed in a final volume of 20 μl using a high-capacity cDNA reverse-transcription kit (Applied Biosystems, Foster City, CA) as described in Reich et al. (2008, 2010).

2.7. Quantitative real-time PCR

Relative quantification real-time PCR was performed using platinum SYBR Green (Invitrogen Life Science, Israel) according to the manufacturer's protocols, 2 μL of cDNA template, and the gene specific primer sets (Table 1). PCR was carried out in the ABI Prism 7300 system (Applied Biosystems). Relative quantification of the interest gene was normalized to GAPDH housekeeping in the comparative C_T method