ORIGINAL ARTICLE

Acceleratory Effect of Novel Synthesized Collagen-like Peptide from Adiponectin on Osteoblastic Differentiation

Jun-ichi TANABE, Aiko KAMADA and Takashi IKEO

Department of Biochemistry, Osaka Dental University, Osaka, Japan

SYNOPSIS

Adiponectin, an adipocyte-derived biologically active molecule, is a ~30-kDa polypeptide containing an N-terminal signal sequence, variable domain, collagen-like domain, and C-terminal globular domain. Previously we demonstrated that full-length adiponectin up-regulated the expression of genes related to osteoblastic differentiation, although the free globular domain down-regulated them. It was hypothesized that the collagen-like domain may be involved in osteoblastic differentiation, so we investigated the effect of novel synthesized collagen-like peptide on osteoblastic differentiation of murine pro-osteoblastic cells.

Microarray analysis showed that the collagen-like peptide up-regulated the genes related to various signaling pathways involved in osteoblastic differentiation. In contrast, the globular domain suppressed the above genes and induced the expression of genes related to cell morphogenesis and neurological system processes. Real-time RT-PCR for osteocalcin clarified the microarray observation. Therefore, the novel synthesized collagen-like peptide had positive action on osteoblastic differentiation.

Key words*: adiponectin, collagen-like peptide, osteoblast, microarray*

INTRODUCTION

Adiponectin / Acrp30 (adipocyte complement-related protein of 30 kDa), an adipose-derived hormone, exhibits various biological functions, such as regulating energy homeostasis, increasing insulin sensitivity in the liver and skeletal muscle, and protecting vascular walls from atherosclerosis [1, 2]. Furthermore, recent studies suggest that adiponectin also plays important roles in bone metabolism. Adiponectin might act on bone formation through the autocrine / paracrine and endocrine pathways, and the indirect pathway by circulating adiponectin via enhancement of the insulin signaling [3]. It could also increase bone mass by suppressing osteoclast differentiation and activity [4-8]. We previously demonstrated the gene expression of adipocytokine receptors in murine pro-osteoblastic cells during osteoblastic differentiation, and indicated that adiponectin rather than leptin may be involved in osteoblastic differentiation [9].

Adiponectin contains three easily recognizable domains[2,10], an N-terminal signal sequence, variable domain, collagen-like domain comprising 22 Gly-X-Y repeats, and a C-terminal globular domain (Fig. 1a). Circulating adiponectin includes both a full-length type and a globular type (gAN), which is a proteolytic cleavage product of full-length adiponectin in its C-terminal globular domain. Some studies have shown that full-length adiponectin synergizes with insulin to inhibit hepatic glucose production; however, gAN simulates fatty acid oxidation in skeletal muscle [1,11]. Shinoda *et al*.[3] have demonstrated three distinct adiponectin actions on bone formation; a positive action through the autocrine/paracrine pathway by locally produced adiponectin, a negative action through the direct pathway by circulating adiponectin, and a positive action through the indirect pathway by circulating adiponectin via enhancement of the insulin signaling. It was thought that these different actions might be attributed to different forms of adiponectin; locally produced adiponectin has full length, though circulating adiponectin includes gAN. We previously analyzed gene expression profiles in osteoblasts stimulated by full-length adiponectin or gAN using a microarray method, and showed that full-length adiponectin up-regulated the expression of genes related to various signaling pathways and osteoblastic differentiation, although the free globular domain, gAN, down-regulated them[12]. This indicates that the collagen-like domain could be involved in osteoblastic differentiation. Accordingly, we tested the hypothesis that the novel synthesized collagen-like peptide promoted the gene expression related to the differentiation of a murine pro-osteoblastic cell line, MC3T3-E1 cells.

MATERIALS AND METHODS

Materials

A murine pro-osteoblastic cell line, MC3T3-E1, was purchased from the American Type Culture Collection (CRL-2594; Manassas, VA, USA). Cells were maintained in α-modified Eagle's medium (α-MEM; Nacalai Tesque, Inc., Kyoto) containing 10% fetal bovine serum (FBS; Lot No 915150; Biological Industries, Ashrat, Israel), penicillin (100 U/mL) and streptomycin sulfate (100 μg/mL) (GibcoBRL, Grand Island, NY, USA) at 37°C under humidified 5% CO_2 conditions. Recombinant murine gAN (gAcrp30) was purchased from Pepro Tech EC (London, UK). The collagen-like peptide, cAN, was designed based on the database of murine adiponectin (GI:1051268), as shown in Fig. 1a, prepared using a solid-phase peptide synthesizing system by Sigma- Aldrich Co. (St. Louis, MO, USA), and confirmed as a ~6-kDa single peptide by SDS-PAGE (Fig. 1b).

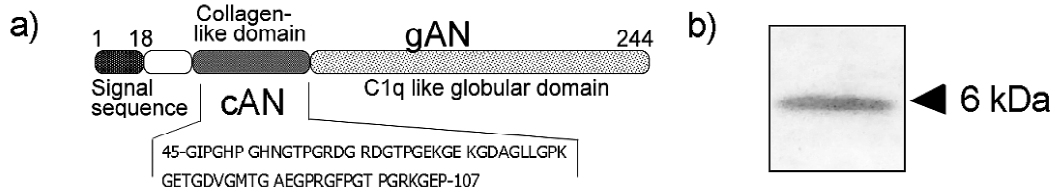

Fig. 1 Characterization of adiponectin, collagen-like peptide (cAN) and globular adiponectin (gAN).
(a) Full-length adiponectin is an ~30-kDa polypeptide containing an N-terminal signal sequence, a variable domain, a collagen-like domain, and a C-terminal globular domain (gAN). Collagen-like peptide (cAN) was synthesized based on the amino acid sequence of the collagen-like domain. (b) SDS-PAGE for newly synthesized cAN showed a ~6-kDa band.

Cell culture

MC3T3-E1 cells were plated in 24-well (2×10^4 cells/well) tissue culture plates and grown to subconfluency in α-MEM supplemented with 10% FBS. Confluent monolayers were precultured in control medium (α-MEM containing 0.1% FBS) for 24 h, and then cultured in differential medium (DM, control medium supplemented with 50 μg / mL ascorbic acid (VC) and 10 mM β–glycerophosphate (GP))[13] with or without gAN or cAN for 1 to 4 days.

Total RNA was isolated using the MagExtractor RNA purification kit (Toyobo, Osaka, Japan) from cultured cells. The quantity and purity of extracted total RNA were determined spectrophotometrically at 260 and 280 nm. Total RNA was reverse-transcribed into cDNA using the High Capacity RNA-to-cDNA Master Mix, including random primers and oligo(dT) primers (Applied Biosystems, Foster City, CA, USA).

Microarray analysis

Microarray analysis was performed with cDNA from cultured cells 3 days after the addition of a differential medium.

Two micrograms of the cDNA products were labeled with Cys3 using an Agilent Genomic DNA Enzymatic Labeling kit, and the labeled targets were hybridized to the Agilent Whole Genome Array (4 x 44K) according to manufacturer's instructions (Agilent Technologies, Santa Clara, CA, USA). Gene expression data were considered to be up- or down-regulated if the fold change in the log ratio was more than 1.5 in any comparisons among the three experimental groups; the log ratio of differential medium to control medium (DM vs Control), differential medium with gAN to differential medium (DM+ gAN vs DM), and differential medium with cAN to differential medium (DM+ cAN vs DM). All significant expression genes were hierarchically clustered with a similar expression pattern using Cluster3.0 [14] and visualized as a heatmap using Java Treeview [15,16].

Quantitative real-time PCR

Relative levels of mRNA expression were measured by quantitative real-time PCR with the cDNA products from cultured cells 1 or 4 days after the addition of a differential medium.

Specific primers and the FAM™-labeled probe for osteocalcin were obtained commercially and are proprietary; thus, the sequences are not available (TaqMan Gene Expression Assays; Applied Biosystems). Analysis was performed using TaqMan Fast Universal PCR Master Mix with a Step One Plus Real-Time PCR System (Applied Biosystems) as described in the manufacturer's protocol. Gene expression in a multiplex reaction was quantified relatively using the comparative C_T method, normalizing the amount of the target (FAM) to endogenous GAPDH (VIC) expression.

The results are presented as the mean ± S.D. values of five experiments and were analyzed by nonpaired Student's t test. Values between $p < 0.01$ and 0.05 were considered significant, as specified in the figure legends.

RESULTS

Effect of collagen-like peptide cAN on the gene expression profiles in osteoblasts

All significant expression genes detected by microarray analysis were hierarchically clustered with a similar expression pattern and visualized as a heatmap, shown in Fig. 2. Up- and down-regulated expressions are represented as red and green, respectively, and black represents no change of expression.

The heatmap indicated that gAN and cAN affected different osteoblast genes. Then, the characteristic subsets for DM, gAN or cAN addition were selected as Cluster 1 - 5.

Functional classification of specific osteoblast genes in response to cAN

Functional cluster annotations for the selected clusters were generated based on the significant GO terms (Table 1). GO terms were provided by the Gene

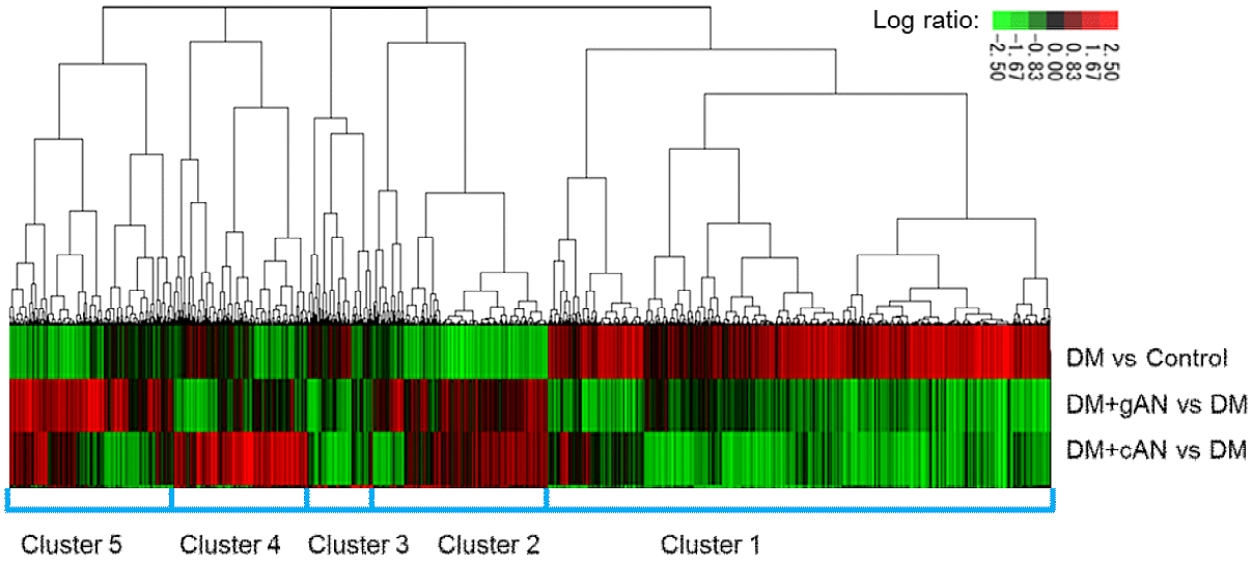

Fig. 2 Effects of adiponectin-derived peptides on gene expression in osteoblasts.
All significant expression genes are expressed as a heatmap. Characteristic gene expressions of treatment with gAN or c AN were clustered into Cluster 1 - 5. DM, addition of differential medium; DM+gAN, addition of differential medium with globular adiponectin; DM+cAN, addition of differential medium with collagen-like peptide.

Table 1 GO analysis of genes included in Clusters.

	Function *	Gene
Cluster 1	cell development	Pparg, Fgfr2, Fgfr3, Adar, Wnt5a, Igf1, Igf2, Kras, Akt1 etc.
	transcription factor, gene expression	Runx1, Isgf3, Atf1, Atf2, Pdx2, Tcfe3, Cebpa, Tep, Nrob2 etc.
	intercellular signaling	Adipor2, Aoc3, Axl, Cd248, Hla-a, Il4r, Itga9, Lama2ltb, Egfr etc.
	PI3K/Akt signaling pathway	Akt1,Bad,BCL2,Cdkn1a,Ctnnb1,ITGB1,Jak1,Mapk3,Mdm2 etc.
Cluster 2	intercellular signaling, immune response	Ccl21, MUC4, Cd8A, Cdh9, Lta, Cxcl9, Ifna7, Itgb1, Mmp7 etc.
	cell motility, cell adhesion	Cdh2, Cdh3, Cdh9, Cdh17, Alcam, Mllt4, Mmp7, Itgb1, Mfap5 etc.
	lipid and carbohydrate metabolism	Ppara, Apoa5, Cpt1, Rarb, Pygl, Inadl ,Gcg, Gmr5, Gip etc.
Cluster 3	G-protein signaling pathway	Adora3, Gpr174, Galr3, Vlrc1, Oprm1, Olfr611, Olfr1459 etc.
Cluster 4	MAPK signaling pathway	Prok1, Dusp19, Map2k6, Map3k5, Map4k4, Jun etc.
	JUN signaling pathway	Jun, Atf2, Fosl2, Alox12, Pglyrp2, Crem, Creb5, Trip4, Il2r etc.
	WNT signaling pathway	Wnt9a, Wnt10b, Fzd4 ,Dkk4, Rspo3 ,Lrrfip2, Csnk1g1 etc.
	lipid metabolism	Tnf, Il17c, Fabp9, Fabp4, Il17c, Il1, 1rl1, Pax8, Lipc etc.
Cluster 5	cell surface receptor	Adrb1, Insr, Cubn ,Ccr2, Nr3c2, Fgf22, Il11, Sfrp4, Fgfr4 etc.
	neurological system process	Nav1, Nav3, Ngf, Wnt8, Ncam, Cspg5, Rims2, Rims3, Klk2 etc.
	cell morphogenesis, cell motility	Cxcl13, Cx3xr1, Ccr2, Arrb2, Cdh2, Cdh8, Cdh10, Cdh4, Vamp2 etc.
	signaling pathway	Adipor1, Olfr640, Hif3a, Pak7, Adrbk2, Dlc1, Wnt8a, Adam8, Itgae etc.

* Functions contain some GO terms.

Ontology project, which is a major bioinformatics initiative with the aim of standardizing the representation of gene and gene product attributes across species and databases (http://www.geneontology.org/). Gene ontology organizes information for molecular functions, biological processes, and cellular components for a number of different organisms. Therefore, the identifications of co-expression gene groups can lead to the identification of common regulatory motifs, inference of signal pathways and genetic networks in the context of GO.

The significant genes up-regulated by DM were included in Cluster 1 related to cell development, transcription factor, intercellular signaling and the PI3K/Akt signaling pathway. Adiponectin receptor 2 (Adipor2) and osteocalcin (Bglap, bone gamma carboxyglutamate protein) were included in this cluster. Cluster 2 included the genes which were suppressed by DM and improved both by gAN and cAN. Lipid and carbohydrate metabolism, and cell adhesion protein as a cadherin family were contained in this cluster. The collagen-like peptide cAN significantly up-regulated the genes in Cluster 4 related to several signaling pathways, such as Wnt, Jun, Mapk. Cluster 5, in which genes were up-regulated by gAN, included genes related to cell morphogenesis and neurological system processes. This cluster indicated that gAN may be involved in neuronal differentiation.

Effect of collagen-like peptide cAN on the osteocalcin expression in osteoblasts

Real-time RT-PCR for osteocalcin gene expression clarified the microarray observation, as shown in Fig. 3. The collagen-like peptide cAN improved the increase of osteocalcin expression by differential medium, although gAN suppressed it.

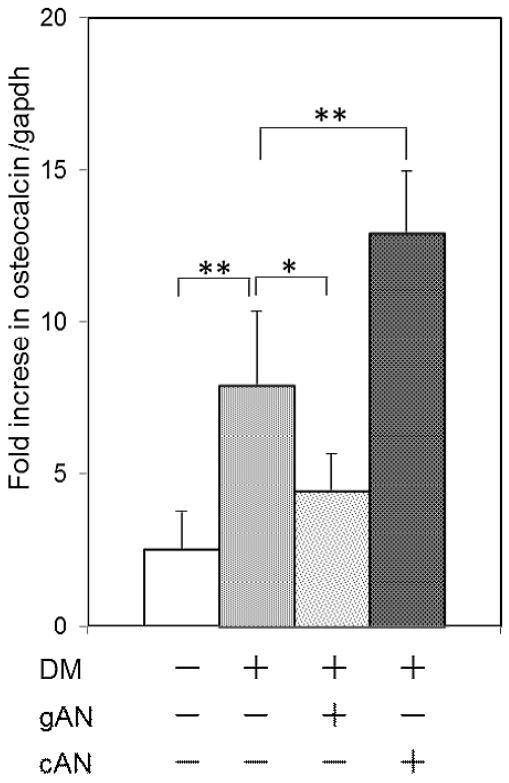

Fig. 3 Effects of adiponectin-derived peptide on the osteocalcin gene expression in osteoblasts.
Confluent monolayers of MC3T3-E1 cells were cultured for 4 days. Quantitative real-time RT-PCR results were normalized to GAPDH expression and then reported relative to the day one control. Results are expressed as the mean and range. Significant differences are shown as * $p<0.05$; ** $p<0.01$.

DISCUSSION

Microarray analysis showed that the collagen-like peptide induced the expression of genes related to various signaling pathways (Mapk, Wnt, Jun) involved in osteoblastic differentiation. In contrast, the globular domain suppressed the above genes and induced the expression of genes related to cell morphogenesis and neurological system processes.

The Wnt signaling pathway is well known to induce osteoblastic differentiation of mesenchymal stem cells and to lead to bone formation [17]. The canon-

ical Wnt ligands were found to up-regulate the expression of osteogenic transcription factors, such as Runx2 and osterix, while down-regulating PPAR- gamma2 and C/EBP- alpha [18-20]. Therefore, Wnt signaling is involved in several aspects of osteoblast differentiation and function, while concomitantly inhibiting adipogenesis. The Mapk signaling pathway is also required for osteoblast differentiation[21], and adiponectin induces the activation of MAPK in cultured osteoblasts [22]. Recently, a study on the differentiation of human mesenchymal stem cells (MSCs) from bone marrow demonstrated that contractile cells promoted osteogenesis by enhancing c-Jun N-terminal kinase (JNK) and extracellular related kinase (ERK1/2) activation in conjunction with elevated signaling [23]. Cluster 4, which was up-regulated by cAN, included many genes related to these signaling pathways, therefore indicating that not the gAN region but the cAN region in adiponectin molecules may be involved in osteoblast differentiation.

On the other hand, gAN seemed to induce neurological characteristics in pro-osteoblastic cells, since microarray analysis showed significant gene expressions related to neurological system processes in Cluster 5. The functions of the genes up-regulated by gAN in this study are similar to those in our previous study comparing gAN to full-length adiponectin [12]. Adiponectin has been shown to be a neuroendocrine hormone [24], acting directly on the brain to regulate autonomic function [25] and sympathetic nerve activity [26]. Interestingly, the three- dimensional structure of the C-terminal globular domain of adiponectin has homology to that of TNF-α [10]. It was demonstrated that globular adiponectin (gAN) strongly inhibited TNF-α/RANKL- induced differentiation of osteoclasts by interfering with TNF receptor-associated factor production and calcium signaling [6]. These characteristic effects of gAN may be attributed to its structural resemblance to TNF-α. Microarray analysis in our studies also proposed the globular domain of adiponectin as neuroendocrine hormone on osteoblasts.

Our previous studies using a microarray [12] or siRNA for adiponectin receptors [27], AdipoR1 and AdipoR2, demonstrated that AdipoR2 rather than AdipoR1 may be involved in osteoblastic differentiation. Also in this study, the gene for AdipoR2 included Cluster 1, which is involved in osteoblastic differentiation and cell growth.

Adiponectin is modified at the post-translational level during its secretion from adipocytes, *in vivo*. Several conserved lysine residues (positions 68, 71, 80, and 104) within the collagenous domain of murine adiponectin are hydroxylated and glycosylated in hepatocytes [28]. Some studies have demonstrated that hydroxylation and glycosylation of the lysine residues within the collagenous domain of adiponectin are critically involved in regulating the formation of its high molecular weight oligomeric complex and consequently contribute to the insulin-sensitizing activity of adiponectin in hepatocytes [29-31]. The higher-order structure of adiponectin is considered to be involved with bone metabolism[32, 33]. In this study, although real-time RT-PCR for osteocalcin gene expression clarified the microarray observation, the synthesized collagen-like peptide had less marked effects on osteoblastic differentiation than expected. One reason for such results may be related to modification at the post- translational level of the collagenous domain of adiponectin, *in vivo*. Therefore, investigation of hydroxylated and glycosylated collagen-like peptide is necessary for further acceleration of osteoblastic differentiation. The novel

synthesized collagen-like peptide from adiponectin had a positive action on osteoblastic differentiation.

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Corresponding author:
Aiko Kamada, Ph.,D.
Department of Biochemistry
Osaka Dental University
8-1 Kuzuhahanazonocho, Hirakata-shi,
Osaka, 573-1121, Japan
Tel; +81-72-864-3055
Fax; +81-72-864-3155
E-mail: kamada-a@cc.osaka-dent.ac.jp