Adiponectin Gene Cloning and Its Expression in Insect Cell Expression System

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ABSTRACT

This study was to examine expression of the recombinant full-length adiponectin (recombinant adiponectin) in insect ovarian cell culture system and to characterize structural properties of the recombinant adiponectin secreted in medium. Gene construct encoding the recombinant adiponectin contained N-terminal collagen-like domain (110 Amino Acids, AAs), C-terminal globular domain (137 AAs) and C-terminal peptides for detection with V5 antibody (26 AAs included adaptor peptide) and purification using the 6xHis tag (6 AAs). The approximate molecular weight of the product (monomer) was 35 kDa. Molecular mass species of the expressed recombinant adiponectin were monomer (∼35 kDa), dimer (∼70 kDa), trimer (∼105 kDa) and hexamer (∼210 kDa). The major secreted species were the LMW forms, such as monomer, dimer, and trimer. There was MMW of hexamer as minor form. HMW multimers (∼300 kDa) were shown as a tracer or not detected on the SDS-PAGE in several experiments (data not shown). The multimer forms in this study were not compatible to those in animal or human serum and adipose tissue by other researcher's study in which the major multimer forms were HMW. By protein denaturing experiments with reducing reagent (β-MeOH), anionic detergent (SDS) and heat (95℃) on the SDS-PAGE, not all adiponectin multimers seemed to have disulfide bond linked structure to form multimers. The recombinant adiponectin which expressed in insect ovarian cell culture system seemed to have the limitation as full physiological regulator for the application to animal and human study.

(Key words : Recombinant adiponectin, Multimer, Insect ovarian cell, SDS-PAGE)

INTRODUCTION

Adipose tissue plays an active role on regulating whole-body metabolism by secreting a variety of bioactive molecules termed adipokines (Wong *et al*., 2003; Meier and Gressner, 2004; Kadowaki and Yamauchi, 2005). Adiponectin known one of adipokines is an adipocyte-specific secretory protein and induced during adipocytes differentiation. It also has been known as adipocyte complement-related protein of 30 kDa (acrp30), adipoQ (Scherer *et al*., 1995; Hu *et al*., 1996; Maeda *et al*., 1996; Nakano *et al*., 1996).

Adiponectin contains a modular structure that includes an N-terminal collagen-like domain and a C-terminal globular domain with significant sequence and structural similarities to the complement factor C1q. Adiponectins in human and animal sera and those expressed in NIH-3T3 or bacteria form various types of multimers from low molecular weight monomer to high molecular multimers (Waki *et al*., 2003). The disulfide bond through an animo-terminal cysteine is required for the formation of multimers larger than a trimer (Waki *et al*., 2003). Different oligomers of adiponectin activate different signal transduction pathways, suggesting different physiological regulation via changes of its oligomerization state (Tsao *et al*., 2003). Recent studies showed that adiponectin plays significant role in metabolic effects, such as inhibition of gluconeogenesis, inhibition of glycogenesis, increased fatty acids oxidation, increased glucose uptake, and increased insulin sensitivity. These effects were mediated through distinct adiponectin receptors, AdipoR1 and AdipoR2 (Yamauch *et al*., 2003). AdipoR1 is abundantly produced in skeletal muscle, whereas AdipoR2 is mostly expressed in the liver. Adiponectin receptors are also expressed in human macrophages and their expression levels may be regulated by agonists of the nuclear receptors PPARα, PPARγ and liver X receptor (Chinetti *et al*., 2004).

As previously mentioned, adiponectin is secretory and multimeric protein consisting of low molecular weight (LMW), middle molecular weight (MMW) and high molecular weight (HMW) multimers. These multimer forms might be regulated by various posttranscriptional modifications depended upon by species differences, variation of amino acid composition, and modification

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of the conserved region necessary for glycosylation, hydroxylation or disulfide bond formation. The expression of human recombinant adiponectin by the bacterial culture system appears a complete lack of differentially modified isoforms and fails to form large multimers (Waki *et al.*, 2003; Richards *et al.*, 2006). The HMW multimers showes to be the more bioactive forms. In addition, different multimers of adiponectin differentially regulate the physiological response by different signaling pathway (Tsao *et al.*, 2003). The development of the mammalian adiponectin expression and purification system can be a way to full-fill the quantity of adiponectin, and various adiponectin multimeric isoforms required to examine their roles in human and animals.

The purpose of this research was to develop and examine the adiponectin expression system by use of insect ovarian cells as host cells for expression. The recombinant adiponectin was expressed in the insect ovarian cell culture system and the general structure of the expressed protein in the medium was biochemically analyzed.

MATERIALS AND METHODS

Mouse Fibroblasts Differentiation

NIH-3T3 mouse fibroblasts were cultured and differentiated according to the methods described by Engelman *et al.* (1998) and Hu *et al.* (1996). Briefly, the cells were grown in DMEM (Dulbeccos' Modified Eagle's Medium) containing FCS (10%) and penicillin/streptomycin (100 units/ml) and allowed to reach confluence. Upon confluence, the medium was changed to DMEM containing FCS (10%), 5 μg/ml insulin, 25 μM dexamethasone and 0.25 mM 3-isobutyl-1-1-methylxanthine. Two days later, the medium was switched to DMEM containing FBS (10%) and 5 μg/ml insulin. After another 2 days, the cells were switched back to the DMEM containing 10% FCS and cultured for two days. Total RNAs were extracted using Trizol (Life Technologies, Inc.). The cDNA for the full length adiponectin was synthesized from the total RNA and cloned into pIZ-V5-Histidin vector system (Invitrogen, USA).

Cloning of the Full-Length Adiponectin:

The full-length of adiponectin was cloned into the pIZ/V5-His cloning vector (Invitrogen, USA). pIZ/V5-His cloning vector contains the Zeocin™ resistance gene for the selection of transformants in *E · Coli* and selection of stable adiponectin expression line in High Five insect ovarian cells (see Cell Culture and Transfection). The OpIE2 promotor was baculovirus immediate-early promoter and utilized the host cell transcription machinery. For the detection of the expressed recombinant adiponectin and the purification of the recombinant adiponectin, C-terminal peptide containing V5 epitope and 6xHistidine tag were fused into the cloning vector. The DNA sequence encoding the full length adiponectin protein was amplified using primers (forward primer: ACG ACT AGT CAG GAT GCT ACT GTT GC-3', backward primer: 5'-ACG ACT CGA GTT GGT ATC ATG GTA GAG A-3'). For the increase of translation efficiency, Kozak translation sequence was artificially inserted in front of the translation initiation codon, ATG. For the expression of C-terminus V5 epitope and 6xHis tag, native stop codons on full length adiponectin cDNA and on cloning site of XbaI in the vector plasmid were deleted or replaced by another codon. The V5 epitope and 6xHistidine-tagged full-length of adiponectin was transfected into insect High Five™ cells using lipid-mediated transfection.

Cell Culture and Transfection

For the expression of recombinant protein, High Five insect ovarian cells (Invitrogen, USA) were cultured and transfected. The High Five cell line was originated from the ovarian cells of the cabbage looper, *Trichoplusia ni* (Davis *et al.*, 1992). General methods for cell culture and transfection were conducted according to manufacture's introduction. The conditioned media from the transient or stable expression system were collected, and concentrated with the centriprep filter YM10 (Millipore, USA) and kept at −20℃ freezer until assaying expression adiponectin and purifying adiponectin. The fusion proteins were precipitated by polyethylene glycol (PEG, MW 3,350) and then purified by elution through wheat germ agglutination column, Q-sepharose column and/or a nickel-ion agarose (Ni-NTA) column.

Precipitation of the Recombinant Adiponectin by Polyethylene Glycol (PEG):

PEG (MW 3,350) was used for the broad fractionation of proteins in the High Five cell cultured media. The scheme for the protein fractionation was as follows. All fractionation procedures were conducted in the cold chamber room at 4℃. Solid PEG-3350 was added in the collected media to give 5% of PEG (w/w) by stirring for 1 hour, and then the precipitate was fractionated by centrifugation (3,000 g, 1 h). The precipitate was dissolved in sodium phosphate buffer (100 mM, pH). The cold supernatant was added more with PEG (6∼10%) to generate a second precipitate as the same condition of previous PEG (0∼5%) precipitation method. The following percentage scales of PEG for the precipitation were PEG (11∼20%), PEG (21∼30%) or PEG (31∼40%). The precipitate was analyzed by 8% SDS-PAGE in the presence or absence of various protein denature conditions such as β-mercaptoethanol as reducing reagent, sodium dodecyl sulfate (SDS) as anio-

nic detergent and heat with steam heat at 95℃ for 5 min. The adiponectin oligomer complexes from the precipitate were detected with anti-V5 epitope antibody by western blotting method.

Wheat Germ Agglutination (WGA) Purification

Bio-Rad plastic chromatographic column was set in the refrigerator and WGA bound beads were packed into column. The WGA column was washed with WGA wash buffer (50 mM Hepes, 1% Triton X-100, pH 7.6) and the concentrated media was loaded, equilibrated, and passed. After washing the column with wash buffer, sample was eluted with WGA elution buffer (50 mM Hepes, 0.1% Triton X-100, 300 mM N-Acetylglucosamine, pH 7.6)

Ni-NTA Column Chromatography

For the purification of 6xHis-tagged recombinant adiponectin from the culture medium or the sample of WGA column elute, affinity chromatography on metal-chelating resin (Ni-NTA) specifically designed to purify 6xHis-tagged proteins was used. Bio-Rad plastic column was set in the refrigerator and filled with Ni- NTA bead. The column was washed with wash buffer (50 mM Hepes, 10 mM Imidazole, pH 6.3), and sample from WGA column elute was loaded. The column was washed with wash buffer (10 volume of bead volume) and sample was eluted with elution buffer (50 mM Hepes, 200 mM Imidazole, pH 5.9).

Q-Sepharose Column Purification

The anion exchanger Q-sepharose column was washed with wash buffer (20 mM Tris-Cl, pH 8.0), loaded the precipitate by PEG (20%), washed again, and eluted with NaCl at various concentrations (0.1, 0.3, 0.5, 0.7 or 1 M). The elute was dialysed with dialysis bag (MW 8,000 cut off) in buffer (20 mM Tris-Cl, pH 8.0) for overnight.

Testing for Adiponectin Expression

The samples from the conditioned medium or the elutes on various columns were mixed with SDS-PAGE sample buffer from Invitrogen technical manual if the components of SDS-PAGE sample buffer were not specified for the experimental purpose. After electrophoresis, proteins were transferred into nitrocellulose membrane, blotted and probed with V5 epitope antibody. The recombinant adiponectin fused with V5 epitope and 6xHistidine was detected and visualized using chemiluminescence.

RESULTS

Adiponectin in circulating serum presents in commonly three different molecular mass groups, a low molecular weight trimer (LMW, ~67 kDa), middle molecular weight hexamer (MMW, ~140 kDa) and high molecular weight multimers (HMW, ~300 kDa) (Waki *et al.*, 2003).

Gene construct encoding the recombinant adiponectin was shown in Fig. 1. The recombinant adiponectin contained a N-terminal collagen-like domain (110 AAs) and a C-terminal globular domain (137 AAs). In addition, this gene construct included C-terminal peptides for detection with V5 antibody (26 AAs included adaptor peptide) and purification using the 6xHis tag (6 AAs). The approximate molecular weight of the estimated product was 35 kDa.

The expressed recombinant adiponectin was begun to precipitate from PEG 10% (6~10 %), and the most effective concentration of PEG for the precipitation was 20% (11~20%; Fig. 2). Over 20% of PEG did not affect on the precipitation of the recombinant adiponectin. Molecular mass species of the expressed recombinant adiponectin were monomer (~35 kDa), dimer (~70 kDa), trimer (~105 kDa), and hexamer (~210 kDa) in Fig. 2. The major secreted species were the LMW forms such as monomer, dimer, and trimer. There was MMW of hexamer as minor form. HMW multimers (~300 kDa) were shown as a tracer or not detected on the SDS-PAGE in several experiments (data not shown).

To further characterize the recombinant adiponectin expressed in the medium of insect ovarian cell culture system, the recombinant adiponectin was treated under different protein denaturing conditions by reducing reagent (β-mercaptoethanol, β-MeOH), anionic detergent (sodium dodecyl sulfate, SDS) and/or heat treatment (95℃, Fig. 3 and Fig. 4). In Fig. 3, multimer forms of the recombinant adiponectin were clearly separated by non-reducing, non-anionic detergent, and non-heat denaturing condition (lane 6, 7). Both of reducing reagent and anionic detergent without heat denaturation converted the forms of dimer and trimer to the dimer or monomer in 10 or 20% PEG precipitates. Similar results were obtained in Fig. 4. Non-reducing, non-anionic detergent and non-heat-denaturation condition separated the multimer species of recombinant adiponectin (lane 8). In contrast, simultaneous treatment with reducing, anionic detergent and heat at 95℃ completely denatured and converted multimer forms of the recombinant

Full length recombinant adiponectin (collagen-like domain + globular C1q domain)
MW: 35 kDa

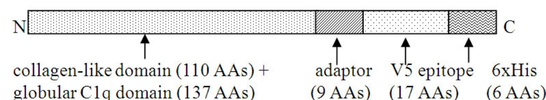


Fig. 1. Constructs encoding the recombinant full-length adiponectin, V5 epitope, and 6xHis tag. This recombinant gene construct includes the C-terminal peptides for detection with V5 antibody and purification using the 6xHis tag.

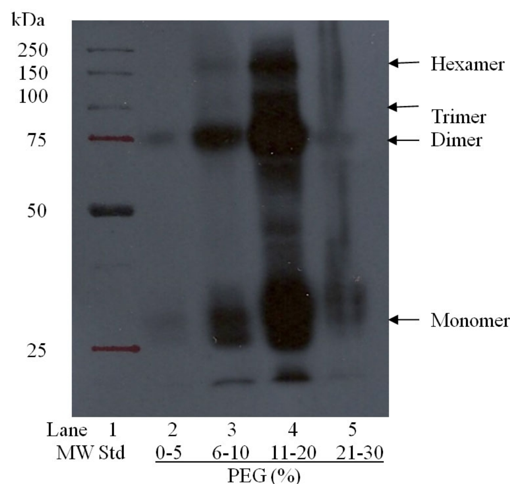
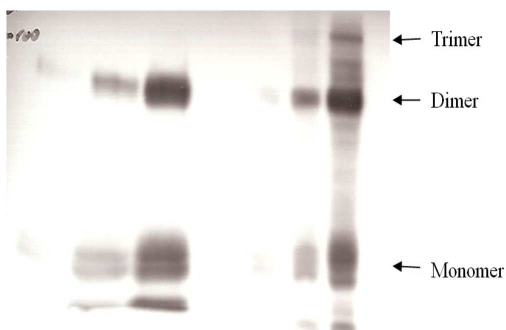
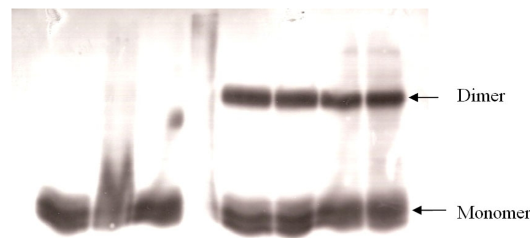


Fig. 2. Precipitation of the recombinant adiponectin from the High Five insect ovarian cell culture medium. Lane: 1, MW Standard; 2, sample from polyethylene glycol (PEG, MW 3,350) precipitation (0~5%); 3, sample from PEG precipitation (6~10%); 4, sample from PEG precipitation (11~20%); 5, sample from PEG precipitation (21~30%). Numbers on the left indicate molecular weight (kDa). The samples was separated by 8% SDS-PAGE and the recombinant adiponectin was detected by western-blot probed with V5 epitope antibody. All samples for the electrophoretic separation were prepared with the non-denaturing sample buffer (without β-mercaptoethanol (5%) and sodium dodecyl sulfate (SDS, 2%) and without heat denature (95℃ at steam bath for 5 min).



Lane	1	2	3	4	5	6	7
PEG (%)	5	10	20		5	10	20
β-MeOH +SDS	+	+	+		–	–	–
Heat	–	–	–		–	–	–

Fig. 3. The electrophoretic patterns of the recombinant adiponectin under various denaturing conditions in 8% SDS-PAGE after WGA column and Ni-NTA column purification. Before running on WGA column and Ni-NTA column, sample was precipitated by PEG (20%) and the PEG precipitate was dialyzed (MW 8000 cut off) for overnight at room temperature. The recombinant adiponectin was detected by western blot probed with V5 epitope antibody. Each lane was denoted with "+" (included denaturing reagents in sample buffer or heat treatment) or "—" (not included denaturing reagents in sample buffer or not heat treatment). β-mercaptoethanol (5% β-MeOH), SDS (2%), and heat denature (95℃ at steam bath for 5 min).



Lane	1	2	3	4	5	6	7	8
β-MeOH	+	+	–	–	+	+	–	–
SDS	+	–	+	–	+	–	+	–
Heat	+	+	+	+	–	–	–	–

Fig. 4. The electrophoretic patterns of the recombinant adiponectin under various denaturing conditions in 8% SDS-PAGE after WGA column and Ni-NTA column purification. The recombinant adiponectin was detected by western blot probed with V5 epitope antibody. Before running on WGA column and Ni-NTA column, sample was precipitated by PEG (20%) and the PEG precipitate was dialyzed (MW 8000 cut off) for overnight at room temperature. The recombinant adiponectin was detected by western blot probed with V5 epitope antibody. Each lane was denoted with "+" (included denaturing reagents in sample buffer or heat treatment) or "—" (not included denaturing reagents in sample buffer or not heat treatment). β-MeOH (5%), SDS (2%), heat denature (95℃ at steam bath for 5 min).

Lane 1 2 3 4 5 6 7

Fig. 5. The purification of the recombinant adiponectin on Q-sepharose and the electrophoretic patterns of the samples eluted at various NaCl concentrations. The purification procedures were described in the MATERIAL and METHODS. In brief, the sample was precipitated with PEG (20%) and then the precipitate was eluted on Q-sepharose at various NaCl concentrations (0.1, 0.3, 0.5, 0.7 or 1 M). Samples were denatured under reducing condition and SDS anionic detergent but not with heat treatment. The samples were separated with 8% SDS-PAGE and the recombinant adiponectin was detected by western-blot probed with V5 epitope antibody. Lane: 1, flow through; 2, wash; 3, 0.1 M; 4, 0.3 M; 5, 0.5 M; 6, 0.7 M; 1.0 M NaCl.

adiponectin to a monomer (lane 1). Neither reducing condition alone nor anionic detergent condition alone under heat treatment completely converted the multimers to monomer by showing trailing spot or dragging patterns in the gel. Heat treatment alone at 95℃ showed dragging pattern of protein migration in the gel (lane

4) suggesting non-denature of the covalently linked disulfide bond and ionic interaction among the adiponectin multimers. There seemed to be the intermediate multimers, such as tetramer, pentamer, on the gel (data not shown). In Fig. 5, the optimum NaCl concentration was 0.7 M for the elution of the expressed recombinant adiponectin through the Q-sepharose anionic exchanger column and the Q-sepharose-bound adiponectin complexes may be effectively eluted by increasing NaCl gradient from 0.5 to 1.0 M.

DISCUSSION

Adponectin has identified to form several different molecular weight multimers by gel filtration and velocity gradient studies (Scherer *et al*., 1995; Tsao *et al*., 2003). The major multimer species of adiponectin in human serum or adipocytes and the recombinant adiponectin expressed in mammalian cells are three different molecular mass species, trimer (~67 kDa), hexamer (~136 kDa), and high molecular weight multimers (~>300 kDa) (Waki *et al*., 2003). In this experiment, molecular mass species of the recombinant adiponectin expressed in insect ovarian cells were monomer (~35 kDa), dimer (~70 kDa), trimer (~105 kDa), and hexamer (~210 kDa). In contrast to those of previously mentioned in human body or those expressed in mammalian cell culture system, the major secretory species in insect cell culture system were LMW forms such as monomer, dimer, and trimer rather than MMW or HMW. The molecular basic structure and multimerization patterns of the recombinant adiponectin expressed in the medium of insect cells were similar to those of circulating serum or adipocytes of human and animals but the majority of multimer forms were clearly different. Furthermore, the quantitative densities of the monomer and dimer of adiponectin in SDS-PAGE were greater than those of other multimers. This result suggests that the expressed major forms of adiponectin should be monomer and dimer in insect ovarian cell expression system. When the recombinant adiponectin in this experiment was denatured in stepwise by treatments of reducing reagent, anionic detergent, and/or heat, the forms of denatured molecular mass species were pretty similar to those of human or animals. In Fig. 3, and Fig. 4, the recombinant full-length adiponectin was detected as dimer and monomer under reducing condition by β-mercaptoethanol treatment. This suggests that not all adiponectin multimers were disulfide bond-linked structure to form multimers.

Adiponectin multimer species can be changed by protein synthesis process of the host cell because of difference of posttranslational modification system and variation of amino acids sequences within eukaryote or even among animal species (Tsao *et al*., 2003; Richards *et al*., 2006). The amino terminal region of globular domain of adiponectin which contains the cysteine residue important for multimer formation has very low homology among animals and might affect the multimerization of adiponectin (Waki *et al*., 2003). The interchains between the collagenous domain are responsible for formation of highly ordered trimer and further stabilize the disulfide bond within trimer (Tsao *et al*., 2003; Waki *et al*., 2003).

In our experiment, multimerization patterns of recombinant adiponectin were more complex than those of bacterial expression system of human adiponectin by Richards *et al*. (2006) but less than those of human serum and adipocytes. Bacterially expressed human adiponectin diaplayed a complete lack of differentially modified isoforms and failed to form trimers and larger multimers. These results indicate that recombinant adiponectin expressed under insect ovarian cell culture system may not represent the native forms of adiponectin in the serum or adipocytes of mammals such as human and animals. The multimerization state such as trimer (LMW), hexamer or HMW adiponectin activated different signal transduction pathway suggesting different physiological roles in body. Thus, the recombinant adiponecin which expressed in insect cell culture system seems to have a limitation to apply in animal and human experiments.

In this experiment, the expressed patterns of recombinant adiponectin were shown by western blot and SDS-PAGE after several purification steps. The amounts of the secreted recombinant adiponectin into the medium and the yield of the expressed recombinant adiponectin from each purification steps were very low by quantitative analysis of recombinant adiponectin (data not shown). We also could not clearly detect it by protein dye method with significant amounts of sample except by western blot analysis after a couple of efficient purification steps. It is not clear whether the low expression level of total recombinant adiponectin and high expression of LMW forms relative to MMW and HMW were due to the low transcription efficiency, the less posttranslational modification for multimerization in the insect host cells or low transfection efficiency of the cloned vector into insect cells or not. It has been known that the adiponectin gene mutants which were translated but not assembled into stable trimers and larger multimers resulted in impaired secretion from the cells (Waki *et al*., 2003).

As summary, molecular mass species of the recombinant adiponectin expressed in insect ovarian cells were monomer (~35 kDa), dimer (~70 kDa), trimer (~105 kDa), and hexamer (~210 kDa). The quantitative densities of the monomer and dimer of adiponectin in SDS-PAGE were greater than those of other multimers. This result indicates the major forms of recombinant

adiponectin expressed in insect ovarian cell expression system were low molecular weight isoforms, dimer, and monomer. Not all adiponectin multimers have disulfide bond-linked structure to form multimers.

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