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# Adiponectin is partially associated with exosomes in mouse serum

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ABSTRACT

Exosomes are membrane vesicles 30–120 nm in diameter that are released by many cell types and carry a cargo of proteins, lipids, mRNA, and microRNA. Cultured adipocytes reportedly release exosomes that may play a role in cell-to-cell communication during the development of metabolic diseases. However, the characteristics and function of exosomes released from adipocytes *in vivo* remain to be elucidated. Clearly, adipocyte-derived exosomes could exist in the circulation and may be associated with adipocyte-specific proteins such as adipocytokines. We isolated exosomes from serum of mice by differential centrifugation and analyzed adiponectin, leptin, and resistin in the exosome fraction. Western blotting detected adiponectin but no leptin and only trace amounts of resistin in the exosome fraction. The adiponectin signal in the exosome fraction was decreased by proteinase K treatment and completely quenched by a combination of proteinase K and Triton X-100. Quantitative ELISA showed that the exosome fraction contains considerable amounts of adiponectin, but not leptin or resistin. The concentration of adiponectin in the serum and the ratio of adiponectin to total protein in the exosome fraction were lower in obese mice than in lean mice. These results suggest that a portion of adiponectin exists as a transmembrane protein in the exosomes in mouse serum. We propose adiponectin as a marker of exosomes released from adipocytes *in vivo*.

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## 1. Introduction

Exosomes are membrane vesicles with a size of 30–120 nm that are released by many different cell types under physiological and pathological conditions [1]. They have been found in many biological fluids including blood, bronchoalveolar lavage fluid, urine, bile, and breast milk [1]. Exosomes harbor a wide variety of proteins, lipids, mRNA, and microRNA, which can be transferred to another cell, and are implicated in intercellular communication by transferring molecules [2–4]. Indeed, there is increasing evidence that exosomes play an important role in normal physiological processes, development, viral infections and other human diseases [5–8]. In addition, recent studies have highlighted important applications of exosomes as a source of disease biomarkers [4,9,10] and as drug delivery vehicles [11,12]. Thus, exosomes have gained much attention, both with regard to their physiological and pathological functions and more practical applications.

Previous studies have identified exosomes in the culture supernatant of mouse adipose tissue [13], rat primary adipocytes [14–16], and mouse adipocyte cell line 3T3-L1 [17]. Deng et al. isolated exosomes from the culture supernatant of visceral adipose tissue excised from mice and showed that injection of the exosomes derived from diet-induced or genetically (leptin-deficient *ob*/*ob*) obese mice into wild-type lean mice results in macrophage activation and insulin resistance [13]. Müller et al. found that rat primary adipocytes release exosomes, which may transfer lipogenic and/or lipolytic information between large and small adipocytes [14–16]. More recently, Sano et al. isolated exosomes from the culture supernatant of differentiated 3T3-L1 cells and demonstrated that exosomes isolated from cells cultured under hypoxic conditions are enriched in enzymes related to lipogenesis and promote lipid accumulation in recipient 3T3-L1 adipocytes [17]. These findings suggest that adipocyte-derived exosomes play a role in cell-to-cell communication during the development of metabolic diseases. However, the characteristics and function of exosomes released from adipocytes *in vivo* remain to be elucidated.

Clearly, exosomes released from adipocytes could exist in the circulation. In addition, because the composition of exosomes is heterogenic, depending on the cellular origin of the exosome, adipocyte-derived exosomes could be accompanied by molecules produced specifically in adipocytes. In this context, we postulated that such molecules associated with exosomes in the serum could be

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markers for adipocyte-derived exosomes *in vivo*. In the present study, we particularly focused on secretory proteins produced specifically in adipocytes, namely adipocytokines including adiponectin, leptin, and resistin.

## 2. Materials and methods

### 2.1. Animal care

All study protocols were approved by the Animal Use Committee of Hokkaido University (approval No. 08-0139). Animals were maintained in accordance with the Hokkaido University guidelines for the care and use of laboratory animals. Male C57BL/6J JmsSlc (B6), C57BL/6J HamSlc-+/+ (+/+), and C57BL/6J HamSlc-*ob*/*ob* (*ob*/*ob*) mice (age, 5 weeks) were purchased from Japan SLC and housed in standard plastic cages in a temperature-controlled (23 ± 2 °C) room under a 12-h light–dark cycle. They were allowed free access to water and standard chow diet (MR stock; Nosan Corporation) for 2 weeks. In a separate experiment, male B6 mice (age, 5 weeks) were fed either normal-fat diet (NFD) (D12450B; 11.1%kcal from fat, Research Diets) or high-fat diet (HFD) (D12492; 62.7%kcal from fat, Research Diets) for 4 months.

### 2.2. Exosome isolation

Mice were deprived of food overnight, and total blood was obtained from the carotid artery under diethyl ether anesthesia. Exosomes were isolated by differential centrifugation according to Théry et al. [18]. The post-ultracentrifuge pellet was suspended in the same volume of PBS as the original serum samples. This suspension was referred to as the exosome fraction. The protein concentration in the serum and exosome fraction was determined by a BCA protein assay (Thermo Scientific) according to the manufacturer's instructions. Samples were stored at −80 °C until use.

### 2.3. Treatment of exosomes with proteinase K

The exosome fraction was treated with either 30 μg/mL proteinase K, 0.5% Triton X-100, or a combination of both proteinase K and Triton X-100 at 37 °C for 5, 15, or 30 min, after which 3× SDS–PAGE loading buffer composed of 195 mM Tris–HCl, pH 6.8, 10% (w/v) SDS, 30% (w/v) glycerol, 15% (v/v) β-mercaptoethanol, and 0.01% (w/v) bromophenol blue was added. After boiling for 3 min, the samples were Western blotted.

### 2.4. Discontinuous OptiPrep gradient ultracentrifugation

Exosomes were further separated by discontinuous OptiPrep gradient ultracentrifugation according to Mathivanan et al. [19] with slight modification. In brief, the exosome fraction (500 μL) was overlaid on a discontinuous OptiPrep-sucrose gradient (50, 25, 12.5, and 6% iodixanol concentration from OptiPrep solution (Axis-Shield) in 0.25 M sucrose, 10 mM Tris–HCl, pH 7.4) and ultracentrifuged for 18 h at 100,000*g*, 4 °C in a Beckman SW40Ti rotor. Fractions (1 mL) were collected from the top of the gradient and diluted with 2 mL 20 mM Tris–HCl, pH 7.4, followed by ultracentrifugation for 3 h at 100,000*g*, 4 °C. The pellet in each fraction was resuspended in 100 μL PBS and then Western blotted. The density of each fraction was determined by absorbance at 340 nm using a duplicate parallel discontinuous OptiPrep gradient overlaid with 0.25 M sucrose, 10 mM Tris, pH 7.4, according to the manufacturer's application sheet (Axis-Shield). The absorbance was measured using a Synergy Mx 96-well microplate reader (BioTek Instruments).

### 2.5. Western blot analysis

Samples were separated by SDS–PAGE under reducing and nonreducing conditions. For reducing conditions, samples were added to 3× SDS–PAGE loading buffer, described above, and then boiled for 3 min. For nonreducing conditions, β-mercaptoethanol was excluded from the loading buffer, and the samples were not boiled. Electrophoresed proteins were electrophoretically transferred to Hybond C extra nitrocellulose membrane (Amersham International). Membranes were then probed with mouse anti-adiponectin (Enzo Life Sciences), rat anti-CD63 (BioLegend), rabbit anti-leptin (BioVendor), and rat anti-resistin (Enzo Life Sciences) overnight at 4 °C. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at RT. The blots were visualized by ECL chemiluminescence (GE Healthcare) according to the manufacturer's instructions.

### 2.6. ELISA

The concentration of adiponectin, leptin, and resistin in serum and exosome fraction was determined using ELISA kits (Mouse Adiponectin/Acrp30 DuoSet, R&D Systems; Mouse Leptin ELISA Kit, Morinaga Institute of Biological Science; Mouse Resistin Quantikine ELISA Kit, R&D Systems) according to the manufacturers' instructions.

### 2.7. Statistical analysis

Results are presented as means ± SEM. Student's *t*-test was used to compare mean values. Data analysis was performed using GraphPad Prism for Macintosh (version 6; GraphPad Software). *P* values of <0.05 were considered to be statistically significant.

## 3. Results

### 3.1. Serum adiponectin is partially associated with exosomes

When samples containing the same amount of protein in the serum, exosome fraction, and supernatant from the initial ultracentrifugation of serum were separated by SDS–PAGE and Western blotted with a probe to an antibody against CD63, a typical marker protein of exosomes [1], the probe signal was observed in all three samples, and the intensity was higher in the exosome fraction than in the serum or supernatant (Fig. 1A). Next, we separated samples having the same volume of serum, exosome fraction, and supernatant. On Western blotting with anti-adiponectin antibody, a probe signal was observed in all three fractions, with a lower intensity in the exosome fraction (Fig. 1B). In addition, a leptin probe signal was observed in the serum and supernatant, but not the exosome fraction. Furthermore, a resistin probe signal was observed in the serum and supernatant, but only a faint signal was detected in the exosome fraction. Although the signal intensity for both CD63 and adiponectin was unchanged by Triton X-100 treatment, proteinase K treatment quenched the CD63 signal (Fig. 2A). Similarly, the signal intensity for adiponectin was lowered by proteinase K treatment, though a faint signal remained. However, the combination of proteinase K and Triton X-100 completely quenched the adiponectin signal. On Western blots of fractions from the ultracentrifugation on a discontinuous gradient, signals for adiponectin and CD63 were detected at a density of 1.17 g/mL (fraction 10) (Fig. 2B). In addition, weaker signals for adiponectin and CD63 were also observed at a density of 1.07–1.08 g/mL (fractions 4 and 5).

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