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Adipocyte-derived microvesicles contain RNA that is transported into macrophages and might be secreted into blood circulation

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ABSTRACT

We have recently found that 3T3-L1 adipocytes secrete microvesicles, known as adipocyte-derived microvesicles (ADMs), with angiogenic activity. In this study, we found that ADMs contain RNA without typical 28S and 18S ribosomal RNA inside the vesicles. Microarray analysis revealed that ADMs contain approximately 7000 mRNAs and 140 microRNAs. Most of transcripts for adipocyte-specific and dominant genes were contained in the ADMs, and their abundance was mostly correlated with that in the donor cells. Abundance of adipocyte-related microRNAs was also mostly correlated with that in the donor cells. ADMs mediated transport of adiponectin and resistin gene transcripts into RAW264.7 macrophages. Moreover, adipocyte-specific gene transcripts such as adiponectin, resistin, and PPARγ2 were found in microvesicles isolated from rat serum. Thus, ADM might play a role as a novel intercellular communication tool by transporting RNA in paracrine and possibly endocrine manners.

1. Introduction

Microvesicles including exosomes are secreted from many different types of cells under both normal and pathophysiological conditions and are heterogeneous in size and range from 30 nm to 1 μm. Microvesicles have been reported to be secreted by many types of cells including reticulocytes [1], B cells [2], T cells [3], dendritic cells [4], mast cells [5], epithelial cells [6,7] and tumor cells [8,9]. Microvesicles are now recognized as important mediators of intercellular communication, though they had previously been believed to be inert cell debris. The mechanisms by which microvesicles might mediate intercellular signaling could involve the activation of receptors on the plasma membrane of the recipient cells. In this way, microvesicles would be able to carry membrane-bound ligands. Alternatively, microvesicles might be able to exert signals to target cells by directly transferring bioactive molecules inside the vesicles.

Adipocytes produce a variety of bioactive molecules collectively known as adipocytokines or adipokines, including leptin, adiponectin, resistin, plasminogen activator inhibitor-1 (PAI-1), and TNF-α [10,11]. Dysregulated production of these adipocytokines participates in the pathogenesis of obesity-associated metabolic syndrome. Besides the adipocytokines, we have recently reported that mouse 3T3-L1 and rat primary adipocytes secret microvesicles [12], hereinafter re-named adipocyte-derived microvesicles (ADMs). More recently, we have found that ADMs are associated with multiple angiogenic factors thereby exhibiting angiogenic activity *in vitro* and *in vivo* [13].

It has been demonstrated that microvesicles can transfer some of their components to other cell types. For instance, chemokine receptor CCR5 could be transferred from peripheral blood mononuclear cells to endothelial cells via membrane-derived microvesicles [14]. Further, oncogenic form of epidermal growth factor receptor, known as EGFRvIII, was shown to be transferred between glioma cells via microvesicles, contributing to a horizontal propagation of oncogenes and their associated transforming phenotype [15]. More interestingly, it has recently reported that exosomes secreted by mast cells [16], glioblastoma cells [17] and embryonic stem cells [18] contained mRNA and microRNA (miRNA) inside the vesicles, which could be delivered to other neighboring cells and might be functional in their new location.

In the present study, we showed that RNA was present in the microvesicles secreted by 3T3-L1 adipocytes as revealed by microarray and RT-PCR analyses. We also showed ADM-mediated transfer of mRNAs into macrophages. Finally, we found the presence of adipocyte-dominant transcripts in the microvesicles isolated from rat serum, suggesting that ADMs are circulating and function *in vivo*.

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2. Materials and methods

2.1. Materials

All reagents used were from Sigma (St. Louis, MO) or Wako Pure Chemicals (Osaka, Japan), unless otherwise noted.

2.2. Cell culture

3T3-L1 cells were maintained and induced to adipocytes as described [12]. Fetal calf serum (FCS) was depleted of endogenous membrane vesicles by ultracentrifugation, unless otherwise noted. RAW264.7 cells were maintained in DMEM supplemented with 10% FCS.

2.3. Animal maintenance and blood collection

Animal procedures were performed according to the guidelines for the care and use of experimental animals of Mie University. The animal protocols were approved by the Institutional Animal Care and Use Committee of Mie University. Wistar rats (male, 6–8 week-old) were obtained from Japan SLC (Hamamatsu, Japan) and maintained on a commercial diet (CE-2; CLEA, Tokyo, Japan) by *ad libitum* feeding in a room with controlled temperature (25 ± 2 °C) and lighting (light on from 7:00 to 19:00). Blood was collected from tail vein of rats under anesthesia and centrifuged to yield serum.

2.4. Preparation of ADMs and microvesicles

Cell-conditioned media and rat serum were first centrifuged at 15,000*g* for 15 min to remove cell debris, remnants and aggregates. The supernatant was ultracentrifuged at 100,000*g* for 2 h. Resultant pelleted vesicles were suspended in an original volume of PBS and re-ultracentrifuged under the same conditions for washing, and then used for subsequent RNA isolation and immunoblotting [12].

2.5. RNA isolation

Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA). The RNA was quantified by using NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA) and confirmed by using Bioanalyzer (Agilent Technologies, Santa Clara, CA) or e-spect UV–vis spectrometer (Malcom, Tokyo, Japan) and confirmed by using Bioanalyzer (Agilent Technologies, Santa Clara, CA).

Table 2
PCR primers for miRNAs.

Target miRNA	Accession No.	Sequence
mmu-let-7b	MIMAT0000522	TGAGGTAGTAGGTTGTGTGGTT
mmu-miR-103	MIMAT0000546	AGCAGCATTGTACAGGGCTATGA
mmu-miR-146b	MIMAT0003475	TGAGAACTGAATTCCATAGGCT
mmu-miR-148a	MIMAT0000516	TCAGTGCACTACAGAACTTTGT
mmu-miR-221	MIMAT0000669	AGCTACATTGTCTGCTGGGTTTC

2.6. Microarray analysis

The microarray analyses were done by DNA Chip Research Inc. (Yokohama, Japan) using the Agilent whole mouse genome microarray (4 × 44 K, two-color array) and mouse miRNA microarray (version 2) according to Agilent microarray DNA chip analysis.

2.7. Quantitative RT-PCR

Total RNA was first digested with DNase I (Invitrogen), reverse transcribed using a cDNA synthesis kit (Roche, Basel, Switzerland) with oligo (dT) primer, and subjected to quantitative real-time PCR on a BioFlux LineGene (TOYOBO, Osaka, Japan) using a SYBR Green real-time PCR master mix (TOYOBO) according to the manufacturer's instructions. For amplification of miRNA, DNase I-digested total RNA was reverse transcribed using a Mir-X™ miRNA First Strand Synthesis Kit (Takara Bio Inc., Ohtsu, Japan), and subjected to quantitative real-time PCR as above using a SYBR Advantage qPCR Premix (Takara Bio Inc.) according to the manufacturer's instructions. Primer sets for individual mRNAs and miRNAs are listed in Tables 1 and 2, respectively. For all SYBR Green assays, standard curves were generated for each primer set to determine their efficiency, and dissociation curves were generated to detect non-specific amplified products and primer-dimers. PCR products were also run on an agarose gel to confirm that a single PCR product was generated.

To compare the abundance of different ADM mRNAs and miRNAs, we relied on using equivalent amounts of total RNA in

Table 1
PCR primers for mRNAs.

Gene name	Accession No.	Forward primer	Reverse primer
Adiponectin	NM_009605	ATCCACACGTGTACTCAC	AGCATGGTCTACTTCCAG
Adiponectin-5′	NM_009605	CCAGACTAATGAGACCTG	TTTGGTGTCGTCAGATCC
Adiponectin-3′	NM_009605	ATCCACACGTGTACTCAC	AGCATGGTCTACTTCCAG
Leptin	BC125245	GGTGTGAAAGAACCTGAGCTGAGG	CAGTGGATGCTAATGTGCCCTG
Resistin	NM_022984	CCACGGGATGAAGAACCTTT	AACTGACCGACATCAGGAAG
aP2	K02109	AAGACAGCTCCTCCTCGAAGGTT	TGACCAAATCCCCATTTACGC
PAI-1	M33960	TCAGCCCTTGCTTGCCTCAT	GCATAGCCAGCACCGAGGA
IL-6	NM_031168	CCAGAGATACAAAGAAATGATGG	ACTCCAGAAGACCAGAGGAAAT
MCP-1	BC145869	CCACTCACCTGCTGCTACTCAT	TGGTGATCCTCTTGTAGCTCTCC
MFG-E8	AB025280	TCTGGTGACTTCTGTGACTC	AACCGGTTTCACAGTGGATG
MMP-2	BC070430	ATGGCGAGTACTGCAAGTTC	TCTTATCCCGGTCATAGTCC
MMP-9	BC046991	GAAGTCTCAGAAGGTGGATC	CACGGTTGAAGCAAAGAAGG
PPARγ2	NM_011146	CCAGAGTCTGCTGATCTGCG	GCCACCTCTTTGCTCTGCTC
PPARγ2–5′	NM_011146	TGTTGACCCAGAGCATGG	CAACCATTGGGTCAGCTC
PPARγ2–3′	NM_011146	AGATGACAGTGACTTGGC	ACATGCAGTAGCTGCACG
C/EBPα	BC058161	GTCGGTGGACAAGAACAGC	CAGCTGGCGGAAGATGC
C/EBPβ	NM_009883	ACAAGGCCAAGATGCGCAAC	GCTGCTTGAACAAGTTCCGC
C/EBPδ	NM_007679	TTCCAACCCCTTCCCTGAT	CTGGAGGGTTTGTGTTTTCTGT
36B4	XR_003896	GCTCCAAGCAGATGCAGCA	CCGGATGTGAGGCAGCAG

PAI-1, plasminogen activator inhibitor-1; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1; MFG-E8, milk fat globule-epidermal growth factor 8; MMP, matrix metalloproteinase; PPARγ2, peroxisome proliferator-activated receptory2; C/EBP, CCAAT/enhancer-binding protein.