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Altered expression of CD63 and exosomes in scleroderma dermal fibroblasts

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ABSTRACT

Background: Exosomes are small vesicles shed from various cells. They contain proteins, lipids, and nucleic acids, and are regarded as a tool of cell-cell communication.
Objectives: To reveal the putative role of exosomes in systemic sclerosis (SSc), and to elucidate the effect of exosomes on wound healing.
Methods: The expression of common markers for exosomes (CD63, CD9, and CD81) and type I collagen were examined with real-time PCR, immunohistochemical analysis, ELISA, immunoblotting, and flow cytometry. The effect of serum-derived exosomes on wound healing was tested on full-thickness wounds in the mid-dorsal skin of BALB/c mice.
Results: The expression levels of CD63 as well as CD9 and CD81 tended to be increased in SSc dermal fibroblasts compared to normal fibroblasts. Increased exosomes in a cultured media of SSc fibroblasts stimulated the expression levels of type I collagen in normal fibroblasts. As the mechanism, collagen-related microRNA levels in SSc fibroblast-derived exosomes were dysregulated, indicating that both the amount and the content of exosomes were altered in SSc. On the other hand, SSc sera showed significantly decreased exosome levels compared to normal sera. The frequencies of vascular involvements, including skin ulcers or pitting scars, were significantly increased in patients with decreased serum exosome levels. The healing of mice wounds was accelerated by treatment with serum-derived exosomes.
Conclusions: Vascular abnormalities in SSc may account for the decreased serum exosome levels by the disturbed transfer of exosomes from the skin tissue to the blood stream. Our study suggests the possibility that SSc patients with vascular involvements have decreased serum exosome levels, which causes the delay of wound healing due to down-regulation of collagen, resulting in higher susceptibility to pitting scars and/or ulcers. Exosome research will lead to a detailed understanding of SSc pathogenesis and new therapeutic approaches.

1. Introduction

Systemic sclerosis (SSc, scleroderma) is a chronic multi-system autoimmune disorder, characterized by tissue fibrosis of the skin and internal organs. SSc patients also exhibit vascular abnormalities as well as autoimmune dysfunction. Skin fibrosis is thought to be due to excessive production of extracellular matrix from dermal fibroblasts [1,2]. Although transforming growth factor (TGF)-β1 seems to play a central role in the fibroblast activation and subsequent fibrosis [3], the precise mechanisms have yet to be elucidated.

Exosomes are small vesicles 0.03–0.1 μm in size, which are shed from most cell types into the extracellular space via intracellular endocytosis. Accordingly, exosomes are found abundantly in body fluids such as blood, saliva, urine, and breast milk [4]. They contain proteins, lipids, and nucleic acids such as coding or non-coding RNAs. Although the exact function of exosomes is still unclear, they are thought to have various roles in human body. For example, exosomes protect RNAs from harsh conditions [5]: naked RNAs added into body fluids will be immediately degraded by RNase, while RNAs are found to be stable in body fluids, probably because they are packaged in exosomes. Furthermore, previous studies have demonstrated that exosomes can be incorporated into other

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cells, and can function as a 'message in a bottle' to affect the recipient's cell phenotype [6]. Kosaka et al. suggested that breast milk contain genetic materials including microRNAs (miRNAs) [7]. They can be transferred from a mother to an infant, and may be incorporated into the infant cells, which will affect the immune system development in cooperation with IgA. Similarly, Zhang et al. reported that plant tissues also contain miRNAs, and orally administrated exogenous plant miRNAs were detected in the sera of various animals [8]. Plant miR-168a targeting human low-density lipoprotein (LDL) receptor adapter protein 1 may be able to affect plasma LDL levels in human. There is a possibility that exosomes are involved in these processes by protecting or transferring genetic information.

Several reports have indicated that exosomes play a crucial part in human diseases (e.g. cancer growth or spreading pathogens such as prions and viruses) [9]. However, the roles of exosomes in rheumatic diseases have not been clarified. In the present study, we found that exosome levels were up-regulated in SSc dermal fibroblasts both in vivo and in vitro compared to those in normal fibroblasts. In contrast, serum exosome levels were significantly decreased in SSc patients compared to those in normal subjects (NS). We suggest the possibility that exosome supplementation to skin ulcers have significant therapeutic value.

2. Materials and methods

2.1. Patients

Skin specimens were obtained from involved forearm skins of patients with diffuse cutaneous SSc (dcSSc, n = 7) and limited cutaneous SSc (lcSSc, n = 6). Control skins were derived from routinely discarded skins of 5 NS undergoing skin graft. Serum samples were collected from dcSSc patients (n = 19), lcSSc patients (n = 25) and NS (n = 13).

This study was approved by the Ethics Review Committee in Kumamoto University (No.1452). Written informed consent was obtained according to Declaration of Helsinki.

2.2. Cell cultures

Human dermal fibroblasts were obtained by skin biopsy from affected areas of dcSSc patients (n = 6) and from control skin of NS (n = 6) [10]. Biopsies were performed with institutional review board approval and written informed consent according to Declaration of Helsinki.

Dermal fibroblasts were cultured in MEM (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum (Nichirei, Tokyo, Japan) and Antibiotic-Antimycotic (Invitrogen, Carlsbad, CA, USA). Fibroblasts at the third to fifth subpassages were used for experiments.

2.3. RNA isolation and quantitative real-time polymerase chain reaction (PCR)

Total RNA from paraffin sections was extracted using RNeasy FFPE kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions [11]. Total RNA of cultured fibroblasts was extracted with RNeasy mini kit (Qiagen).

cDNA was synthesized from the total RNA with PrimeScript™ RT reagent kit (Takara, Shiga, Japan). CD63, CD9, CD81, COL1A1, COL1A2, and α-smooth muscle actin primer sets were purchased from Takara, and GAPDH primer was from Qiagen. All of these primers were prevalidated to generate single amplicons. Quantitative real-time PCR was performed on Takara thermal cycler dice (TP800) using SYBR® Premix Ex Taq™ II (Takara) following the recommended protocol. Data of each PCR reaction was analyzed utilizing thermal cycler dice real time system ver. 2.10 (Takara). Specificity of each reaction was determined by melting curve analysis. Transcript levels of each gene were normalized to those of GAPDH.

2.4. miRNA isolation and quantitative real-time PCR

Exosomes were isolated from cell cultured media, and miRNA was extracted from these exosomes using SeraMir exosome RNA amplification kit (SBI, Mountain View, CA, USA) [12]. Primers of U6, miR-142-3p, miR-150a, and miR-196a were purchased from Takara. Quantitative real-time PCR was performed on Takara thermal cycler dice (TP800) using SYBR advantage premix (Clontech, Mountain View, CA, USA). Transcript levels of each gene were normalized to U6 [13].

2.5. Immunohistochemical staining

For the detection of CD63, paraffin-embedded skin sections (4-μm thickness) were deparaffinized with Clear Plus® (Farma, Tokyo, Japan), and hydrated in graded ethanol series [14]. Antigens were retrieved by incubation with citrate buffer pH 6 for 5 min at 121 °C with an autoclave. Endogenous peroxidase activity was inhibited with 0.3% hydrogen peroxide in methanol for 30 min. Sections were incubated with 10% goat serum for 30 min, and reacted with a primary antibody for CD63 (1:100, Santa Cruz, Santa Cruz, CA, USA) overnight at 4 °C. After washing excess antibody with PBS, these sections were incubated with a secondary antibody (1:200, anti-rabbit, Bio-Rad, Richmond, CA, USA) for 60 min at room temperature. The reaction was visualized with DAB-buffer tablets (Merck, Darmstadt, Germany). Slides were counterstained with haematoxylin, and examined under a light microscope (OLYMPUS BX50, Olympus, Tokyo, Japan).

For double staining, skin sections were reacted with primary antibodies for CD63 and CD3 (1:200, Nichirei), CD20 (1:100, Dako, Carpinteria, CA, USA), CD31 (1:200, Dako) or CD34 (1:100, Dako) overnight at 4 °C. The sections were incubated with mach 2 double stain 1 (mouse-AP + rabbit-HRP, Biocare Medical, Concord, CA, USA) for 1 h. After washing with PBS, the reaction was visualized with DAB-buffer tablets and vulcan fast red chromogen kit 2 (Biocare Medical).

2.6. Immunoblotting

Fibroblasts were washed with PBS and dissolved with RIPA buffer (Nakarai, Kyoto, Japan). Samples were homogenized with an electric homogenizer, incubated for 1 h on ice, and centrifuged for 10 min at 10,000*g* at 4 °C [15]. The supernatants were collected, and their protein concentrations were normalized utilizing Pierce BCA protein assay kit (Thermo, Hudson, NH, USA). Equal amounts of protein were electrophoresed on SDS-PAGE gel at 100 V for 2 h. Following electrophoresis, the blot was transferred to 0.2 μm PVDF membrane (Bio-Rad). The membrane was blocked for 1 h with blocking one-P (Nakarai) and incubated with primary antibodies for CD63, CD9, CD81 (1:500, Santa Cruz), or β-actin (1:1000, Santa Cruz) overnight at 4 °C. The membrane was washed in Tris-buffered saline (TBS) and 0.1% Tween 20, incubated with secondary antibodies (Bio-Rad), and washed again. The detection of bands was performed using Pierce western blotting substrate (Thermo) according to the manufacturer's recommendations.

2.7. Enzyme-linked immunosorbent assay (ELISA) for protein concentration of exosome

Exosomes in sera and cultured media were harvested with ExoQuick (SBI) and ExoQuick-TC (SBI), respectively [12,16].