Altered expression of CD63 and exosomes in scleroderma dermal fibroblasts

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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's 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).

dNA 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 (Merk, 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 10,000g 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 100V 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].
Fig. 1. In vivo expression and localization of exosome-related markers in normal skin and systemic sclerosis skin.
(A) Total RNA was extracted from involved skin tissues of diffuse cutaneous systemic sclerosis (dcSSc) patients (n=7) and limited cutaneous systemic sclerosis (lcSSc) patients (n=6) as well as control skin of normal subjects (NS, n=5). mRNA levels of CD63, CD9, and CD81 were analyzed by real-time PCR. Bars show the means. The mean value in NS was set at 1. *P < 0.05. **P < 0.01.
(B) Paraffin sections were subjected to immunohistochemical analysis for CD63. (left) NS skin, (right) SSc skin. Original magnification x400.
(C) Paraffin sections of SSc skin were subjected to double staining for CD63 (brown) and CD3, CD20, CD31, or CD34 (red). Original magnification x400.
Fig. 2. Expression of exosome-related markers in SSc dermal fibroblasts in vitro.
(A) Cultured NS or SSc fibroblasts were incubated in the presence or absence of TGF-β1 for 48 h. Cell lysates were extracted and subjected to immunoblotting using antibody for CD63, CD9, or CD81. β-actin was used as the loading control. The 2 representative results from 5 samples of each cell type are shown.
(B) Cultured NS or SSc fibroblasts were incubated in the presence or absence of TGF-β1 for 6 h. Total RNA was extracted, and mRNA levels of CD63, CD9, and CD81 were analyzed by real-time PCR (n=6). Bars show the means. The mean value in NS was set at 1. *P < 0.05.
(C) NS (n=4) or SSc fibroblasts (n=7) were cultured in media supplemented with exosome-depleted FBS in the presence or absence of TGF-β1 for 72 h. Exosomes were isolated from the cultured media, and exosome levels were analyzed with ELISA. The values were normalized with the cell number. Bars show the means. *P < 0.05.
(D) NS fibroblasts were treated with 1 μM 5-Aza-deoxycytidine (5-AdC) or vehicle for 96 h. Relative transcript levels of CD63 were determined by real-time PCR.
(E) NS fibroblasts were treated with or without 1 μM trichostatin A (TSA) for 96 h. Relative transcript levels of CD63 were determined by real-time PCR.
Amounts of exosomes were analyzed with Pierce BCA protein assay kit (Thermo) or with ExoELISA kit (SBI) following the manufacturer’s instructions [17]. Briefly, each pellet of exosomes was added with 200 µl exosome binding buffer, and incubated at 37 °C for 20 min. Each sample was added to the wells of the micro-titer plate, and incubated at 37 °C for overnight. After washing, the samples were incubated with diluted CD63 antibody (1:100, SBI), and subsequently with exosome validated secondary antibody (1:5000, SBI). After adding super-sensitive TMB ELISA, CD63-positive exosome levels were quantified with a spectrometric plated reader at 450 nm absorbance.

2.8. Exosome removal from cultured media

The cultured media of fibroblasts were collected and centrifuged at 2000 rpm for 10 min, and then filtered through a 0.45 µm filter and a 0.20 µm filter, to remove cell debris and small membrane vesicles, respectively [18]. To remove exosomes (0.03–0.1 µm), the media were re-filtered through a 0.02 µm filter.

2.9. Exosome purification from mice blood

All animal experimental protocols in this study were approved by the Committee on the Animal Research at Kumamoto University (A27-091). Eight-week-old BALB/c male mice (Charles River Laboratories, Boston, MA, USA) were used for experiments. Prior to euthanasia, blood samples of mice were collected and centrifuged for serum separation. Exosomes were isolated by PureExo isolation kit (101Bio, Palo Alto, CA, USA) [19].

To confirm the purity of the isolated exosomes, flow cytometric analysis was performed [20]. The dilute samples were blocked with FcR blocking reagent mouse (Miltenyi Biotec GmbH, Germany) for 10 min at 4 °C. Then, the samples were incubated for staining with isotype-matched control antibody or anti-CD63 mouse-mono (SPM524)-PE antibody (Novus, Littleton, CO, USA) for 20 min at room temperature in the dark. Flow cytometric analysis was performed by EasyCyte mini flow cytometry system (Guava Technologies, Hayward, CA, USA).

2.10. Mice wound healing experiment

Under local anesthesia, one full-thickness wound (10 mm excisional in size) was created on the mid-dorsal skin in mice [21]. The wounds were treated with exosome-containing buffer (n = 3) or control buffer (PBS, n = 3) every day.

2.11. Statistical analysis

Data presented as bar graphs was the means ± standard deviation (SD) of at least three experiments. The statistical analysis was carried out with Mann-Whitney U test for the comparison of medians, and with Fisher’s exact probability test for the analysis of frequency. P values <0.05 were considered to be significant.

3. Results

3.1. Expression and localization of exosome markers in SSc skin in vivo

We first examined the expression of exosome markers, CD63, CD9, and CD81, in the skin of SSc and NS. Real-time PCR utilizing skin samples of dcSSc (n = 7), lcSSc (n = 6) and NS (n = 5) showed that skin mRNA levels of these exosome markers tended to be up-regulated in both dcSSc and lcSSc compared to those in NS (Fig. 1A), and the differences were statistically significant except for difference of CD81 levels between NS and dcSSc.

To identify the localization of overexpressed CD63 in SSc skin, immunohistochemical staining for CD63 was performed using skin specimens of dcSSc and NS (Fig. 1B). We found that CD63 expression was evident in spindle-shaped cells of the dermis in SSc skin, but not in NS skin. Because the CD63-positive cells were not double stained with CD3 (T cells), CD20 (B cells), CD31 or CD34 (blood cells and endothelial cells) (Fig. 1C), we concluded that the CD63-positive cells are likely to be dermal fibroblasts.

3.2. Expression of exosome markers in SSc fibroblasts in vitro

We next examined expression of exosome markers in cultured SSc and NS fibroblasts in vitro. Total protein and RNA samples were extracted from SSc fibroblasts, NS fibroblasts and TGF-β1-stimulated NS fibroblasts. The protein levels of CD63, CD9, and CD81 were increased in the cell lysates of SSc fibroblasts compared to NS fibroblasts (Fig. 2A). Consistently, SSc fibroblasts showed significantly up-regulated CD63 mRNA levels in compared to NS fibroblasts (Fig. 2B). We found that the mRNA levels of CD9 and CD81 were also increased in SSc fibroblasts, albeit statistically insignificant. Furthermore, we determined exosome secretion

Fig. 3. Effects of exosome on type I collagen expression.

NS or Sc fibroblasts were cultured in media supplemented with exosome-depleted FBS for 48 h. Exosomes were then removed from the cultured media as described in the Methods section. NS fibroblasts were stimulated with the exosome-removed media (−) or exosome-containing media (+) either from NS fibroblasts or Sc fibroblasts for 6 h. Total RNA was extracted, and mRNA levels of COL1A1 and COL1A2 were determined by real-time PCR. “*” <0.05.
levels from SSc and NS fibroblasts. Exosomes were isolated from cultured media of SSc fibroblasts, NS fibroblasts and TGF-β1-stimulated NS fibroblasts using polymer-based precipitation methods. Consistent with the increased exosome levels in the lysates of SSc fibroblasts, ELISA analysis indicated that exosome secretion levels were significantly elevated in cultured media of SSc fibroblasts compared to NS fibroblasts (Fig. 2C).

On the other hand, there were no significant differences in the levels of intracellular CD63 or extracellular exosomes between NS fibroblasts and TGF-β1-stimulated NS fibroblasts (Fig. 2A–C). Accordingly, both the synthesis and the secretion of exosomes seemed to be up-regulated in SSc fibroblasts, which may be mediated by other factors than TGF-β1 activation seen in these cells. For example, the treatment of NS fibroblasts with 5-Aza-

![Graph A](image)

![Graph B](image)

**Fig. 4.** Serum exosome levels in SSc patients. (A) NS (n=3) or SSc fibroblasts (n=7) were cultured in the media supplemented with exosome-depleted FBS for 72 h. Exosomes were then isolated from the cultured media, and microRNA (miRNA) was extracted from the exosomes. Expression levels of indicated miRNAs were analyzed by real-time PCR. Bars show the means. The mean values in NS were set at 1. *P < 0.05. (B) Exosome levels in sera of dcSSc patients (n=19), lcSSc patients (n=25) and NS (n=13) were analyzed with ELISA. Bars show the means. *P < 0.05.
deoxycytidine (5-AdC), which is used to relieve the inhibitory effects by DNA methylation in situ [22], led to the significant up-regulation of CD63 (Fig. 2D) in NS fibroblasts, whereas histone deacetylase inhibitors (trichostatin A) did not (Fig. 2E). Thus, exosome levels in fibroblasts can be controlled by DNA methylation, but not by histone acetylation. Given that dysregulation of DNA methylation in SSc fibroblasts has been reported by several studies [23,24], it may be involved in the mechanism of exosome overexpression in these cells.

3.3. Function of exosomes in SSc fibroblasts

We determined the direct effects of exosomes on type I collagen expression using exosome-containing culture media and exosome-removed media: SSc and NS fibroblasts were cultured in exosome-depleted FBS, and cultured media were collected and treated with (exosome-removed media) or without filter membranes (exosome-containing media) according to a previous study [18]. NS fibroblasts were stimulated with each medium.

The mRNA levels of COL1A1 and COL1A2 were significantly induced by the exosome-containing media derived from SSc fibroblasts compared to SS exosome-removed media, but not by those from NS fibroblasts (Fig. 3). This inducible effect of SSc exosome-containing media on type I collagen levels may be caused by the increased amounts of exosomes included (see Fig. 2C).

3.4. Decreased serum exosome levels in SSc patients

We then determined the difference in the contents of exosomes between SSc and NS fibroblasts. Small RNA was extracted from exosomes of their cultured media. The levels of several miRNAs such as miR-142-3p, miR-150a, and miR-196a were measured by real-time PCR (Fig. 4A): these miRNAs have already been reported to be dysregulated in SSc patients [25–27]. Consistent with the previous reports, the expression levels of miR-142-3p were significantly up-regulated while those of miR-150a and miR-196a were significantly down-regulated in SSc exosomes compared to NS exosomes. Taken together, these changes in exosome may contribute to the reported abnormalities of miRNA expression in SSc fibroblasts. Furthermore, the changes of exosomes both in the quantity and quality may cause type I collagen induction by the SSc exosome-containing media.

Next, exosomes were isolated from serum samples of dcSSc (n = 19), lcSSc (n = 25), or NS (n = 13), and exosome levels were determined with specific ELISA. Contrary to our expectation, the serum exosome levels were significantly decreased in both dcSSc and lcSSc patients compared to those in NS (Fig. 4B). We supposed that this decrease is probably caused by the disturbed transfer of exosomes from the skin tissue to the blood stream due to the vascular abnormalities in SSc. Consistently, when the correlation of serum exosome levels with clinical/serological features of SSc patients was investigated (Table 1), patients with decreased serum

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<th>Table 1 Correlation of serum exosome levels with clinical/serological features in patients with systemic sclerosis (SSc, n=44).</th>
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<tr>
<td>Serum exosome</td>
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<td>Age at the time of serum sampling (mean years)</td>
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<td>Duration of disease (mean months)</td>
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<td>Type (diffuse:limited)</td>
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<td>Clinical features</td>
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Unless indicated, values are percentages. MRSS, modified Rodnan’s total skin thickness score; SF, sublingual frenulum; VC, vital capacity; DLco, diffusion capacity for carbon monooxidase; ANA, antinuclear antibodies.
Fig. 5. Mice wound healing by serum-derived exosomes. 
(A–C) CD63 flow cytometry was performed using mice serum. (A) Before exosome isolation, (B) after exosome isolation, and (C) isolated exosomes.
(D) Representative wound closure in mice ulcers treated with (n = 3) or without (n = 3) exosomes at Day 1, 6, and 10.
(E) Percentage of wound closure at Day 0, 2, 4, 6, 8 and 10 in mice ulcers treated with (solid line) or without exosome (dotted line). Data are expressed as the mean ± SD of 3 independent experiments. *P < 0.05.
(F) Total RNA was extracted from skin specimens of mice ulcers treated with or without exosomes (n = 3). mRNA levels of COL1A1, COL1A2, and αSMA were evaluated by real-time PCR. Bars show the means. The mean values in NS were set at 1.
exosome levels tended to have vascular involvements, including skin ulcers and pitting scars, at statistically higher frequency than those without. Our results suggest the possibility that SSc patients with vascular involvements have decreased serum exosome levels due to the disturbed transfer, which in turn causes delay of wound healing by down-regulation of type I collagen as the vicious circle, resulting in higher susceptibility to pitting scars and/or skin ulcers.

3.5. The effect of topical serum-derived exosome treatment on wound healing in mice

Above results prompted us to examine the effect of exosomes derived from serum on wound healing in vivo. A full thickness 10 mm excisional wound was created in the mid-dorsal skin of BALB/c mice. Serum samples were collected from some of these mice after euthanasia. Flow cytometric analysis showed the presence of CD63-positive particles in mice serum (Fig. 5A). The CD63-positive exosomes were successfully purified by our method using PureExo isolation kit (Fig. 5B and C).

We found that the healing of the cutaneous wounds treated with exosome-containing buffer was accelerated compared to that treated with control buffer (Fig. 5D): The diameter of wounds was significantly smaller in mice treated with exosome-containing buffer than those with control buffer at Day 4, 6, and 8 (Fig. 5E).

Almost insignificant, the mRNA levels of COL1A1 and COL1A2 were up-regulated in the tissues of exosome-treated mice wounds compared to those in control mice (Fig. 5F), which is consistent with in vitro results. The α-smooth muscle actin mRNA levels were not altered by the exosome treatment.

4. Discussion

Originally, exosomes were thought to be a mechanism that discards waste from each cell. However, they are recently regarded as a tool of cell–cell communication by shuttling the information and properties of donor cells, which are translated by the receiving cells. This is the first report investigating the expression and function of exosomes in SSc. ‘Exosome’ is also known as a corresponding antigen of PM-Scl antibody seen in SSc patients overlapped with myositis, but this ‘exosome’ is a multi-protein complex which plays a role in diverse RNA processing and degradation pathway [28], and is completely different from the exosome referred to in this paper.

Our results indicated up-regulation of exosome levels in SSc skin. We demonstrated that increased exosomes in cultured media of SSc fibroblasts stimulated type I collagen expression in NS fibroblasts. As the mechanism, we showed that collagen-related miRNAs in SSc exosomes were also dysregulated, indicating that both the amount and content of exosomes were altered in SSc. As the limitations of this study, we purified exosomes using commercially available kits, but the purity of exosomes was not evaluated. Thus, the results of exosome levels in the skin should be confirmed using electron microscopy. In addition, further studies should be conducted for clarifying the mechanism of exosome up-regulation in SSc (e.g. DNA methylation).

On the other hand, exosome levels in SSc sera were significantly decreased compared to those in NS. There have been no previous reports demonstrating up- or down-regulation of exosomes in SSc sera. Vascular abnormalities seen in SSc, especially microangiopathy, may account for the decreased serum exosomes by the disturbed transfer of exosomes from the skin tissue to the blood stream. This hypothesis is supported by the notion that frequencies of skin ulcers and pitting scars were significantly increased in patients with decreased serum exosome levels. Vascular abnormalities and decreased type I collagen expression levels, which were caused by the decreased serum exosomes, may cooperatively mediate delayed wound healing in this disease. However, we did not show the direct evidences: although we compared exosome levels in the sera and skin of patients with skin ulcers, significant correlation was not found. Furthermore, there was no apparent difference in the levels of endothelial cell exosome expression between SSc and NS skin. Further studies are needed to prove our hypothesis.

Skin ulcers are one of the frequent complications in SSc patients, which severely affect their quality of life. Recently, new therapeutic strategies including topical negative pressure therapy and platelet-rich plasma have been developed [29,30]. In the present study, we investigated the effect of serum-derived exosome supplementation on cutaneous wound healing in mice. This paper is the first to demonstrate the therapeutic values of serum-derived exosomes. The evaluation of adverse effects, comparison of the effect of systemic and local exosome supplementation, or the identification of cellular sources of serum-derived exosomes will be necessary. Furthermore, more pathogenic/mechanistic data need to be clarified in the future: For example, although our results indicated that exosomes may induce wound healing by stimulating collagen expression, other factors in exosomes may also affect wound healing via angiogenesis.

Taken together, our study suggests that the exosome researches lead to a detailed understanding of SSc pathogenesis. Furthermore, exosomes also have a great potential as new therapeutic tools.

Competing interests

This study was supported in part by grants for scientific research from the Japanese Ministry of Education, Science, Sports and Culture, and by project research on intractable diseases from the Japanese Ministry of Health, Labour and Welfare. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. In addition, the funders do not alter our adherence to all the journal’s policies on sharing data and materials.

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Conflict of interest

None.

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