Effects of mesenchymal stem cell-derived exosomes on oxidative stress responses in skin cells

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Abstract
The mechanism by which reactive oxygen species (ROS) produced by oxidative stress promote cellular senescence has been studied in detail. This study aimed to verify the preventive or therapeutic effects of mesenchymal stem cell-derived exosomes (MSC-Ex) on the production of ROS induced by oxidative stress in human skin fibroblasts and clarify the mechanisms that promote cellular senescence. In a system where H2O2 was applied to skin fibroblasts, we assessed the effects of the application of MSC-Ex before and after oxidative stress and measured the fluctuations in several signaling molecules involved in subsequent intracellular stress responses. Exosomes were isolated from MSCs (MSC-Ex) and normal human dermal fibroblasts (NHDFs, NHDF-Ex) before and after exposure to H2O2. NHDFs were treated with exosomes before and after exposure to H2O2. mRNA expression (aquaporin-1 and aquaporin-3) and hyaluronan secretion associated with skin moisturization were reduced by H2O2 treatment, whereas MSC-Ex reversed these effects. The cellular senescence induced by H2O2 was also reproduced in fibroblasts. Specifically, the downregulation of SIRT1 led to increased acetylated p53 expression over time, which induced the expression of p21, a downstream molecule of p53, and arrested the cell cycle, leading to cell senescence. MSC-Ex enhanced these signal transduction systems. MSC-Ex was also effective at blocking the increase of β-galactosidase activity and accumulation of ROS in cells. This effect was stronger than that of NHDF-Ex. MSC-Ex were found to act defensively against epidermal and cellular senescence induced by oxidative stress.

Keywords Exosome · Mesenchymal stem cells · Oxidative stress · Reactive oxygen species · β-Galactosidase · Fibroblasts

Introduction
The free radical theory states cellular damage induced by oxidative stress throughout the body is one of the main causes of various senile diseases and that this damage changes biological structures and reduces function [1]. In particular, damage to DNA, proteins, and lipids by reactive oxygen species (ROS) generated following exposure to hydrogen peroxide plays an important role in accelerating aging and the development and severity of senile diseases [2, 3]. The mechanism by which ROS generated by oxidative stress accelerates cellular senescence has been examined in detail, and various phenomena in cells and the roles of various molecules are being clarified. DNA fragmentation [4], shortening of telomeric regions [5], and poly Adenosine diphosphate-ribose polymerase activation are induced; NAD+ is consumed [6]; and SIRT1 activity is reduced [7]. In addition, experiments using H2O2 as an oxidative stress in human embryonic lung fibroblasts strongly suggested that decreased SIRT1 activity leads to increased acetylated p53 expression over time and induces the expression of p21, a downstream molecule of p53, leading to cell senescence because of cell cycle arrest [8].

Aquaporin (AQP)-1 and AQP-3 were recently implicated in the activation of skin fibroblasts, and oxidative stress has
also been demonstrated to reduce their expression [9]. H₂O₂ is also transported into cells by AQP-3, regulating cell signaling as a second messenger, and AQP-3 dysregulation is essential for various skin conditions [10].

As regenerative medicine advances, it has been reported that autologous mesenchymal stem cells (MSCs) can be cultured in vitro and injected into the body to promote the regeneration of damaged tissues [11]. There is also accumulating evidence that exosomes, in addition to stem cells, have therapeutic effects in a variety of disease models [12]. Specifically, exosomes derived from progenitor adipocytes stimulated cell proliferation in vitro in wound-healing models [13]. In addition, exosomes have exhibited beneficial effects in models of kidney [14–18], heart [19–21], brain [22–24], and lung diseases [25–27] such as growth promotion of target cells or suppression of apoptosis, but the mechanism has not been elucidated. Although autologous stem cells are used in the cosmetic surgery field as an anti-aging modality, there is little evidence supporting their moisturizing and anti-aging effects on skin cells.

This study aimed to verify the preventive or therapeutic effects of mesenchymal stem cell-derived exosomes (MSC-Ex) on the production of ROS induced by oxidative stress in human skin fibroblasts and clarify the mechanisms that promote cellular senescence. In a system in which H₂O₂ was applied to skin fibroblasts, we assessed the effects of the application of MSC-Ex before and after oxidative stress and measured the fluctuations in several signaling molecules involved in subsequent intracellular stress responses. In this study, we found that MSC-Ex protect against oxidative stress-induced ROS production and cellular senescence.

Materials and methods

Cell cultures

Human MSCs from adipose tissue (C-12977) and dedicated medium (C-28009), normal human dermal fibroblasts (NHDFs) from juvenile foreskin (C-12300) and dedicated medium (C-23020), and normal human epidermal keratinocytes from juvenile foreskin (single donor, C-12001) and dedicated medium (C-20111) were all purchased from Promo Cell. All media were supplemented with 10% fetal bovine serum (Carson, CA, USA), penicillin (100 U ml⁻¹), streptomycin (100 µg ml⁻¹), and kanamycin (10 µg ml⁻¹). Cells were cultured in an incubator at 37 °C under 5% CO₂ and used in the experiments.

Biochemical reagents

H₂O₂ and other reagents were purchased from Wako Pure Chemical Industry Co., Ltd.

Extracting, quantifying, and adding exosomes

MSCs and fibroblasts were cultured for 7 days in culture flasks, and culture supernatant was collected from the cells when they reached approximately 80% confluency, with cellular debris removed via centrifugation (3000×g, 15 min). The supernatant was stored at 4 °C until exosome isolation. A MiRURY Exosome Isolation Kit (Product #: 300102; manufactured by EXIQON) was used to isolate exosomes present in the culture medium.

Pellet of recovered Exosome was suspended in 1 ml of resuspension buffer contained in the kits. Isolated exosome levels were measured relatively as particles coexpressing CD9/CD63, a Exosome surface antigen, using CD9/CD63 enzyme-linked immunosorbent assay (ELISA) kits (for human exosome assays). The yield was 2.9 µg/ml for MSCs and 2.3 µg/ml for fibroblast respectively. Isolated exosomes were added to the culture medium at a 1/100 volume (final level, 100 pg ml⁻¹) and incubated for 4 h. These EX-containing media were named MSC-derived exosomes (MSC-Ex) and NHDF-derived exosomes (NHDF-Ex).

Oxidative stress treatment and exosome exposure in cells

NHDFs were incubated for 2 h in medium supplemented with 0.2 mM H₂O₂, washed with PBS, and then incubated in conventional medium [28]. Conversely, the preventive effects of exosomes were investigated by incubating NHDFs with MSC-Ex for 6 h, washing the cells with PBS, and then treating them with 0.2 mM H₂O₂ for 2 h in the same manner. Cells or culture medium was collected at various times after incubation. Total RNA was extracted from cells after exosome and H₂O₂ treatment, and culture supernatants were used for hyaluronan measurements. In addition, measurements of p21 mRNA expression were also performed after 16 h of exosome exposure and 2 h of H₂O₂ stress.

Wound-healing effects

Wound-healing effects were examined using a CytoSelect 24-well Wound-Healing Assay Kit (Cell Biolabs, Inc. San Diego, CA, USA). In the assay, a wound field was created near the center of NHDF cultures for 48 h using an insert placed in the 24-well plate. Thereafter, the cells were cultured for 48 h in culture medium containing various exosomes and then stained with DAPI to observe their growth via fluorescence microscopy, and images were acquired. The effect was measured as the percent closure (migrated cell surface area/total surface area × 100).
Cell growth-promoting effect

NHDFs were cultured in the presence of exosomes and oxidative stress molecules, and the cells and culture medium were recovered at various times. The total number of cells and the number of surviving cells were counted using a Countess cell counting chamber slides (Thermo Fisher Scientific K. K. Tokyo, JAPAN) and a CytoTox-ONE Assay Kit (Promega, Madison, WI, USA), respectively (n = 3/group).

Quantitation of mRNA by quantitative RT-PCR (qRT-PCR)

The mRNA levels of AQP-1, AQP-3 [29], SIRT-1 [30], p53 [31], and p21 [32] in NHDFs were measured using the one-step qRT-PCR method. The mRNA level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also measured as an internal control [33].

qRT-PCR was performed in the final volume of 20 μl of solution containing 10 μl of 2 × Luna Universal One-Step Reaction Mix, 1 μl of Luna WarmStart® RT Enzyme Mix, 2 μl of total RNA solution, 1.6 μl of the primer pair mix (0.4 μM of each primer), and 7.3 μl of H2O under the following conditions: 55 °C for 10 min for reverse transcription and 95 °C for 1 min for initial denaturation, followed by 45 cycles of 95 °C for 10 s and 60 °C for 30 s. The relative gene expression was calculated using the ΔΔCt method [34], and GAPDH gene expression was used for normalization. The primers used in qRT-PCR are shown in Table 1. Significant differences were judged when ΔΔCt > 2.

Table 1  Primers for PCR

| Gene    | Primer (5′→3′) | References |
|---------|----------------|------------|
| AQP-1   | Forward TCT GTA GCC CTT GGA CAC CT | [29] |
|         | Reverse CAA AGG ACC GAG CAG GTG TA | |
| AQP-3   | Forward TGC TAC CTA CCC CTC TGG AC | [29] |
|         | Reverse GCC AGC ACA CAC AGC ATA AG | |
| SIRT-1  | Forward GAG TTG TTG GTA GGT TAG GTG | [30] |
|         | Reverse AAA TAT GAA GAG GTG TTG GTG G | |
| p53     | Forward GCT CAA GAC TGG CGC TAA AA | [31] |
|         | Reverse GTG ACT CAG AGA GGA CTC AT | |
| p21     | Forward CCC GGG CTT GTG TTT TGT T | [32] |
|         | Reverse GAG TGG GTG GTG GTG CAC TCT TCT G | |
| GAPDH   | Forward AGG GCT GCT TTT AAC TCT TGT G | [33] |
|         | Reverse CCC CAC TCG ATT TTG GAG GGA | |

AQP aquaporin, GAPDH glyceraldehyde-3-phosphate dehydrogenase

Measurement of intracellular ROS production

Intracellular ROS levels were measured using the fluorescent probe CM-H2DCFDA (Molecular Probes Inc., Eugene, OR, USA). NHDFs were washed with PBS and then incubated with 1 μM fluorescent probe at 37 °C for 60 min. Intracellular ROS levels were assessed according to the fluorescence intensity using a microplate reader (SYNERGY/HT, BioTek, Japan).

Statistical analysis

All results are presented as the mean ± SD. Statistical significance was determined using one-way analysis of variance, and differences were considered statistically significant when P < 0.05.

Results

Cell proliferation-promoting and wound-healing effects of exosomes

The effects of exosomes on the growth of cultured NHDFs were studied. MSC-Ex tended to more strongly induce cell proliferation than NHDF-Ex during the observations (Fig. 1a). We investigated whether MSC-Ex has wound-healing effects. As shown in Fig. 1b (mean of five samples per group), wound closure tended to be improved by exposure to NHDF-Ex (18 ± 7%) and MSC-Ex (21 ± 8%) compared with the findings in untreated NHDFs (15 ± 4%), but the differences were not significant.
Effects of oxidative stress on cell survival

Treatment of NHDFs with exosomes had no particular effect on survival, but the survival of cells treated with 0.2 mM H$_2$O$_2$ was reduced by 88%. Exposure to exosomes following oxidative stress had no effect on viability, whereas cells treated with MSC-Ex prior to exposure to oxidative stress exhibited significantly improved survival ($P < 0.001$). In addition, MSC-Ex had a significantly stronger effect on cell survival following exposure to oxidative stress than NHDF-Ex ($P < 0.01$) (Fig. 2).

Secretion-stimulating effects of AQP5s and involvement of hyaluronan in NHDF activation

Regarding factors involved in skin moisturization, the mRNA expression of AQP-1 and AQP-3 as determined using qRT-PCR are shown in Fig. 3.

The results illustrated that AQP-1 and AQP-3 mRNA levels were decreased by oxidative stress in NHDFs. Meanwhile, exosomes prevented these effects of oxidative stress when used before or after exposure to H$_2$O$_2$, and MSC-Ex exerted stronger effects than NHDF-Ex (Fig. 3).

The amounts of hyaluronan secreted in the culture supernatant by fibroblasts as determined using ELISA are shown in Fig. 4. The amount of hyaluronan secreted by fibroblasts was also decreased by oxidative stress, but its secretion tended to be more strongly restored by MSC-Ex treatment than by NHDF-Ex treatment (Fig. 4).

Effect on cellular senescence due to oxidative stress

The mRNA levels of molecules involved in intracellular signaling were quantified. Oxidative stress reduced SIRT1 expression ($\Delta \Delta Ct = -4.8$), whereas MSC-Ex treatment restored its expression ($\Delta \Delta Ct = 2.5$; $P < 0.001$ vs. H$_2$O$_2$). The effects of MSC-Ex were stronger when administered prior to oxidative stress ($\Delta \Delta Ct = -0.3$). The decreased activity of SIRT1 resulted in increased p53 expression ($\Delta \Delta Ct = 19.9$), whereas the expression of p21, a downstream molecule of p53, was not changed at 6 h ($\Delta \Delta Ct = 1.3$, data not shown), although induction was observed at 16 h ($\Delta \Delta Ct = 17.0$). The effect of exosomes was not strongly inhibited after oxidative stress, but the effect was strongly resistant to oxidative stress when MSC-Ex were administered prior to exposure to the stress.
(\(p_{53}, \Delta \Delta C_t = 14.1, P < 0.05; p21, \Delta \Delta C_t = 8.5, P < 0.001;\) Fig. 5). β-Galactosidase activity was also measured using the SPiDER-βGal probe as an indicator of cellular senescence (Fig. 6). Oxidative stress increased the fluorescence intensity of the probe to 4730 arbitrary units (AU), compared with 613 AU in unstressed cells. Treatment with NHDF-Ex before and after exposure oxidative stress did not significantly reduce the fluorescence of the probe (pretreatment, 4381 AU; after treatment, 4567 AU), whereas MSC-Ex exerted a significant inhibitory effect both before and after exposure to oxidative stress (pretreatment, 3403 AU; after treatment, 3730 AU).

**Discussion**

In this study, we examined at first the effects of exosomes secreted by MSCs on the growth and viability of NHDFs. MSC-Ex did not exert any distinct effects on NHDFs alone such as growth promotion or wound-healing (Fig. 1). In an experimental system in which oxidative stress reduced
cell viability by 10% or more, the effect was significantly improved when cells were exposed to MSC-Ex prior to exposure to oxidative stress, and the effect was significantly stronger than that of NHDF-Ex (Fig. 2).

It is known that the fibroblasts moisturize the epidermis, and the existence of AQPs, which are associated with water molecule influx into cells, is noticed. In particular, AQP-1 and AQP-3 are expressed in fibroblasts. It was previously reported that hyaluronan secreted by cells is effective for moisturizing the skin [35]. Indeed, oxidative stress reduced the mRNA expression of AQP-1 and AQP-3 and hyaluronan secretion by the cells, and these effects were reversed by exposure to MSC-Ex. The effects of MSC-Ex were stronger than those of NHDF-Ex, particularly in the presence of hyaluronan.

Initially, when examined 6 h after stress exposure, SIRT1 and p53 expression fluctuated as expected, whereas p21 levels were unchanged. Meanwhile, the 16-h study revealed that p21 expression was elevated, and these effects were suppressed by MSC-Ex. Furukawa et al. clarified the mechanisms by which acetylated p53 expression is increased over time following the downregulation of SIRT1, thereby inducing the expression of p21 and leading to cellular senescence via cell cycle arrest [8]. p53 acetylation was not examined in this study, but the findings appeared to support the free radical theory in part.

β-Galactosidase activity and intracellular ROS production were measured as indicators of cellular senescence. MSC-Ex reversed the effects of oxidative stress on both of these variables.

The results demonstrated that exosomes reversed the changes in signal transduction that were induced by H₂O₂ and prevented oxidative stress-induced cellular senescence. Moreover, the observations indicate that the exosomes released by NHDFs themselves have some effect; whereas, those secreted by stem cells may be a part of the defense mechanisms that mediate effects on fibroblasts at distant sites.

Although the mechanism responsible for difference in effects between NHDF-Ex and MSC-Ex is unclear, it was
clarified that exosomes act defensively against epidermal and cellular senescence. In the future, novel anti-aging therapies for the skin could be developed from the molecular findings in this study.

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Author contributions All authors contributed to this work. TM, KD, KY, KT, and TS designed the research. TM, KD, and KT performed the experiments and analyzed the results. All authors interpreted the results and designed the research strategy. TM, KD, and KT prepared the manuscript.

Declarations

Conflict of interest The authors have no financial interest or conflicts of interest.
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