Lactoferrin Protects Human Mesenchymal Stem Cells from Oxidative Stress-Induced Senescence and Apoptosis

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Mesenchymal stem cells (MSCs) have been suggested as a primary candidate for cell therapy applications because they have self-renewal and differentiation capabilities. Although they can be expanded in ex vivo system, clinical application of these cells is still limited because they survive poorly and undergo senescence or apoptosis when transplanted and exposed to environmental factors such as oxidative stress. Thus, reducing oxidative stress is expected to improve the efficacy of MSC therapy. The milk protein lactoferrin is a multifunctional iron-binding glycoprotein that plays various roles, including reduction of oxidative stress. Thus, we explored the effect of lactoferrin on oxidative stress-induced senescence and apoptosis of human MSCs (hMSCs). Measurement of reactive oxygen species (ROS) revealed that lactoferrin inhibited the production of hydrogen peroxide-induced intracellular ROS, suggesting lactoferrin as a good candidate as an antioxidant in hMSCs. Pretreatment of lactoferrin suppressed hydrogen peroxide-induced senescence of hMSCs. In addition, lactoferrin reduced hydrogen peroxide-induced apoptosis via inhibition of caspase-3 and Akt activation. These results demonstrate that lactoferrin can be a promising factor to protect hMSCs from oxidative stress-induced senescence and apoptosis, thus increasing the efficacy of MSC therapy.

Keywords: Apoptosis, lactoferrin, mesenchymal stem cells, oxidative stress, senescence

Introduction

Mesenchymal stem cells (MSCs) are heterogeneous subsets of stromal stem cells characterized by self-renewal and pluripotent differentiation capabilities toward the mesodermal lineage cells, such as chondrocytes, adipocytes, myocytes, and osteocytes [1]. MSCs have been suggested as a primary candidate for cell therapy because of their easy isolation, expansion, and preservation [2–6]. Currently, clinical trials of MSC therapy are being performed in several diseases, including cardiovascular and neurological diseases [7–9]. For the clinical use of MSCs, prolonged ex vivo expansion should be achieved because of the low prevalence of MSCs in the primary tissue [9]. However, when the expanded

MSCs are transplanted to the target tissue, they are exposed to several environmental factors, such as oxidative stress, leading to senescence and apoptosis of MSCs and hindering their clinical application [10–14]. Thus, reducing oxidative stress is expected to improve the efficacy of MSC therapy. Lactoferrin, a protein present mainly in mammalian milk, is a pleiotrophic glycoprotein and a member of the transferrin family [15]. It is synthesized by glandular epithelial cells and plays a role in iron homeostasis, anti-inflammation, and host defense against microbial infection [16]. Interestingly, previous studies showed that lactoferrin reduced the level of reactive oxygen species (ROS), suggesting that lactoferrin may have great potential in MSC therapy as an antioxidant [17–19]. Thus, in this study, we investigated

the effect of lactoferrin on the suppression of oxidative stress-induced senescence and apoptosis of human MSCs (hMSCs). Hydrogen peroxide is one of the main sources of intracellular ROS, and thus we treated hMSCs with hydrogen peroxide to induce oxidative stress, and examined the levels of senescence and apoptosis by measuring senescence-associated β -galactosidase (SA- β -galactosidase) activity and the amount of Annexin-V-positive apoptotic cells, respectively, with or without pretreatment with lactoferrin. Our results demonstrate that lactoferrin efficiently lowered the level of intracellular ROS, and suppressed senescence and apoptosis of hMSCs via inhibition of caspase-3 and Akt activation. These results suggest that lactoferrin may be an efficient factor that can increase the efficacy of MSC therapy.

Materials and Methods

Cell Culture and Reagents

Cryopreserved hMSCs were obtained from Lonza (USA). The cells were grown in Minimum Essential Medium- α (MEM- α ; Invitrogen, USA) supplemented with 10% fetal bovine serum (Hyclone, USA) and penicillin/streptomycin (100 U/ml; HyClone). Cells were used at passages 2 to 10 and grown at 37°C in a humidified incubator with 5% CO₂. Hydrogen peroxide and lactoferrin were purchased from Sigma (USA). Pan-caspase inhibitor, Z-VAD-FMK, was purchased from R&D Systems (USA).

Viable Cell Number Counting

Cells were seeded into 24-well cell culture plates at the density of 2×10^4 cells/well. The cells were stimulated with hydrogen peroxide for 2 h to induce oxidative stress, washed with PBS, and incubated in complete medium for 24 h. Then, the cells were trypsinized and the viable cell numbers were counted using a hemocytometer under an optical microscope. In some experiments, cells were incubated with lactoferrin for 24 h prior to stimulation with hydrogen peroxide.

Senescence-Associated β -Galactosidase Staining

Cells were stimulated with hydrogen peroxide for 2 h to induce oxidative stress, washed with PBS, and incubated in complete medium for 72 h. Then, the cells were washed with PBS and fixed in 0.2% glutaraldehyde for 15 min at room temperature. The cells were washed with PBS and incubated with SA- β -galactosidase staining solution (BioVision, USA) at 37°C for 16 h. The development of a blue color in cells was observed under a microscope. In some experiments, cells were incubated with lactoferrin for 24 h prior to stimulation with hydrogen peroxide.

Measurement of Intracellular Reactive Oxygen Species

Cells were treated with lactoferrin for 24 h and stimulated with hydrogen peroxide for 2 h. ROS-sensitive DCF-DA (2',7'-dichlorofluorescein diacetate; Sigma Aldrich; 20 μ M) was added

for the last 30 min and the samples were then immediately analyzed with a fluorescence microscope. For quantification of ROS generation, spectrofluorimetry was performed with a Fluostar Optima spectrofluorimeter (BMG Technologies, USA) following the manufacturer's instructions. Briefly, 3×10^6 cells were suspended in 1 ml of serum-free MEM- α and labeled with 10 μ M of DCF-DA for 10 min at 37°C. The cells are washed twice with serum-free medium and resuspended in 750 μ l of medium. Then, 100 μ l of the suspension was pipetted in each well of the 96-well microplate supplied by the manufacturer (BMG Technologies). The fluorescence was measured with the Fluostar Optima spectrofluorimeter. The filters used for fluorescence measurement were for an absorption spectrum of 485 nm and emission spectrum of 520 nm.

Apoptosis Assay

Cells were pretreated with lactoferrin for 24 h or Z-VAD-FMK for 1 h and stimulated with hydrogen peroxide for 2 h. Then, the cells were washed with PBS and further incubated in complete medium for 24 h. The apoptotic percentage was determined with a Muse Annexin V & Dead cell kit (Millipore, USA) using a Muse Cell analyzer according to the manufacturers' instructions. The activity of caspase-3 was determined with a Muse Caspase-3/7 kit (Millipore) according to the manufacturers' instructions.

Western Blot Analysis

Cells were lysed in lysis buffer (20 mM Tris-HCl (pH 6.8), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% TritonX-100) containing a protease inhibitor (complete-Mini; Roche) for 20 min on ice, and then centrifuged at 13,000 $\times$ g for 20 min at 4°C. Twenty milligrams of the proteins was resolved on 12% sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The membranes were incubated sequentially with primary antibodies and HRP-conjugated secondary antibodies. Immunoreactivity was detected with Enhanced Peroxidase Detection (ELPIS Biotec, Inc) on X-ray film (Sigma-Aldrich).

Statistical Analysis

All experiments were repeated at least three times. Data are presented as the mean $\pm$ standard deviation. Analyses were performed with the Student's *t*-test and values of $p < 0.05$ were considered significant.

Results

Effect of Hydrogen Peroxide on hMSC Senescence and Viability

Since hydrogen peroxide has been frequently used to induce oxidative stress in various cell types, including MSCs [20], we first examined the optimal concentration of hydrogen peroxide that induced senescence and reduced the cell viability. We treated hMSCs with increasing concentrations of hydrogen peroxide and performed SA- β -

Fig. 1. Hydrogen peroxide induces the senescence and decreases the viability of hMSCs.

(A) Effects of hydrogen peroxide on the senescence of hMSCs. hMSCs were stimulated with the indicated concentrations of hydrogen peroxide for 2 h, and senescence-associated β -galactosidase (SA-associated β -galactosidase) staining was performed as described in Materials and Methods. (B) SA- β -galactosidase stain-positive cells were enumerated and expressed as a percentage of total cell counts. * $p < 0.05$ versus no hydrogen peroxide treatment. (C) Dose-dependent effects of hydrogen peroxide on viability of hMSCs. hMSCs (2×10^4 cells/well) were treated with indicated concentrations of hydrogen peroxide for 2 h, and the number of viable cells was counted after 24 h incubation. * $p < 0.05$ versus no hydrogen peroxide treatment. (D) Time-dependent effects of hydrogen peroxide on the viability of hMSCs. Cells (2×10^4 cells/well) were treated with 250 μ M hydrogen peroxide, and further incubated in complete medium for indicated times after washing with PBS. Then, the number of viable cells was counted. The results are averages of three independent experiments (* $p < 0.05$).

galactosidase staining. As shown in Figs. 1A–1B, hydrogen peroxide significantly increased the SA- β -galactosidase stain-positive cells with a peak at 150 μ M. Next, we seeded the same number of hMSCs and treated them with increasing concentrations of hydrogen peroxide. Although the low concentrations of hydrogen peroxide (100, 150, and 200 μ M) had little effect on the viability of hMSCs, the higher concentrations of hydrogen peroxide (250 and 300 μ M) reduced the viability by more than 50% (Fig. 1C). In addition, we treated hMSCs with 250 μ M hydrogen peroxide for the increasing time and found that hydrogen peroxide reduced the hMSC viability in a time-dependent manner (Fig. 1D). These results demonstrated that hydrogen peroxide induces both cell death and senescence in hMSCs depending on the concentrations, which is consistent with a previous report showing that lethal concentrations of hydrogen peroxide

induce apoptosis whereas sublethal concentrations of hydrogen peroxide induce senescence in human diploid fibroblasts [21].

Lactoferrin Decreases the Level of Intracellular ROS

Previous studies showed that lactoferrin played an important role in reducing ROS generation in dexamethasone-induced hypertension and pollen antigen-induced airway inflammation [18, 19]. Thus, we hypothesized that lactoferrin could be a good candidate to reduce the level of ROS and maintain the therapeutic potential of hMSCs. To examine whether lactoferrin decreased the level of ROS in hMSCs, we incubated the cells with lactoferrin for 24 h prior to stimulation with 150 μ M hydrogen peroxide. DCF-DA staining assays showed that the 150 μ M hydrogen peroxide treatment increased the level of ROS in hMSCs, as expected.

Fig. 2. Lactoferrin suppresses hydrogen peroxide-induced intracellular ROS production and cellular senescence in hMSCs.

(A) Microscopic analysis of the inhibitory effect of lactoferrin (LF) on ROS production. (B) Quantification of ROS production. The intensity of DCF fluorescence, reflecting the level of intracellular ROS, was analyzed using ImageJ software, and relative intensity to the control (no treatment) is shown. The results are averages of three independent experiments (* $p < 0.05$). (C) Microscopic analysis of the inhibitory effect of LF on senescence of hMSCs. hMSCs were stimulated with 150 µM hydrogen peroxide for 2 h after pretreatment with the indicated concentration of LF for 24 h. After 72 h incubation in complete medium, senescence-associated β -galactosidase (SA-associated β -galactosidase) staining was performed as described in Materials and Methods. (D) Quantification of SA- β -galactosidase stain-positive cells. SA- β -galactosidase stain-positive cells were enumerated and expressed as a percentage of total cell counts. The results are averages of three independent experiments (* $p < 0.05$ versus no treatment; ** $p < 0.05$ versus hydrogen peroxide treatment).

However, pretreatment with lactoferrin decreased the level of ROS (Figs. 2A and 2B), demonstrating that lactoferrin played a role as an antioxidant in hMSCs.

Lactoferrin Decreases the Hydrogen Peroxide-Induced Senescence of hMSCs

Senescence is irreversible growth arrest and makes hMSCs lose their self-renewal capability, limiting their applications to clinical therapy [13]. One of the important factors that induce hMSC senescence is oxidative stress [12]. Thus, we examined whether lactoferrin could inhibit oxidative stress-induced senescence. Because we found that 150 µM hydrogen peroxide most efficiently induced the senescence of hMSCs (Figs. 1A and 1B), we treated cells with 150 µM hydrogen peroxide after pretreatment with increasing concentrations of lactoferrin. SA- β -galactosidase staining

revealed that lactoferrin suppressed the hydrogen peroxide-induced senescence of hMSCs (Figs. 2C and 2D).

Lactoferrin Decreases the Hydrogen Peroxide-Induced Apoptosis of hMSCs

Besides senescence, apoptosis of MSCs during expansion or after transplantation is the bottleneck to be overcome for efficient clinical use of MSCs [1, 9]. Although apoptosis of MSCs is not fully understood, previous studies suggested that oxidative stress is the main cause [22]. Because we found that 250 µM hydrogen peroxide efficiently decreased the viability of hMSCs (Fig. 1C), we treated cells with 250 µM hydrogen peroxide after pretreatment with increasing concentrations of lactoferrin, and examined the hMSC viability. As shown in Fig. 3A, lactoferrin suppressed the hydrogen peroxide-induced cell death of hMSCs in a

Fig. 3. Lactoferrin suppresses hydrogen peroxide-induced apoptosis of hMSCs.

(A) hMSCs were seeded into 24-well cell culture plates at the density of 2×10^4 cells/well. Next day, cells were treated with the indicated concentrations of lactoferrin (LF) for 24 h. The cells were then stimulated with hydrogen peroxide (250 μ M) for 2 h, washed with PBS, and further incubated in complete medium for 24 h. Then, the cells were trypsinized and the number of viable cells was counted; * $p < 0.05$ versus no treatment. ** $p < 0.05$ versus hydrogen peroxide treatment. (B) hMSCs were treated with LF (100 μ g/ml) for 24 h. Then, the cells were stimulated with the hydrogen peroxide (250 μ M) for 2 h, washed with PBS, and further incubated in complete medium for 24 h. The apoptotic cells were analyzed using Muse Annexin V & Dead cell kit as described in Materials and Methods. (C) hMSCs were treated with hydrogen peroxide (250 μ M) for 2 h after pretreatment with LF (100 μ g/ml) for 24 h or Z-VAD-FMK (10 μ M) for 1 h. The cells were washed with PBS and further incubated in complete medium for 24 h. Then, the activity of caspase-3 was analyzed using the Muse Caspase-3/7 kit as described in Materials and Methods. The results are averages of three independent experiments (* $p < 0.05$ versus no treatment; ** $p < 0.05$ versus hydrogen peroxide treatment).

dose-dependent manner. To examine whether the reduced cell viability was caused by apoptosis, we performed Annexin V staining after treatment of hydrogen peroxide. As shown in Fig. 3B, hydrogen peroxide induced the apoptosis of hMSCs, and pretreatment with lactoferrin suppressed the hydrogen peroxide-induced apoptosis. Furthermore, we measured the hydrogen peroxide-induced activation of caspase-3 after pretreatment with lactoferrin or a pan-caspase inhibitor, Z-VAD-FMK. Caspase-3 activity assay revealed that lactoferrin suppressed the hydrogen peroxide-induced activation of caspase-3 as comparable with the effect of Z-VAD-FMK (Fig. 3C), demonstrating lactoferrin can be a good candidate to reduce oxidative stress-induced apoptosis of hMSCs.

Lactoferrin Inhibits ROS Generation Via AKT Activation

Recent reports showed that inhibition of AKT induces cellular senescence and ROS generation in certain cell types [23–25]. Furthermore, a previous study showed that lactoferrin induces the phosphorylation of Akt in osteoblasts [26]. Because Akt is a key survival factor in various cell types [27], we examined the involvement of the Akt pathway in lactoferrin-induced hMSC survival. Our results showed that pretreatment with lactoferrin increased the hydrogen peroxide-induced phosphorylation of Akt compared with only hydrogen peroxide treatment (Fig. 4A). In addition, treatment of a PI3K/Akt inhibitor, Wortmannin, decreased the lactoferrin-induced inhibition of ROS generation (Figs. 4B and 4C) and cell survival (Fig. 4D) in hydrogen

Fig. 4. Involvement of Akt in the lactoferrin-mediated protection from oxidative stress in hMSCs.

(A) Lactoferrin (LF)-induced Akt activation in H₂O₂-treated hMSCs was determined by western blotting. The image is a representative of three independent experiments. (B) hMSCs were pre-incubated with Wortmannin (Wort) (100 nM) for 2 h prior to incubation with LF for 24 h, and then treated with H₂O₂. The cells were incubated with 20 μM of ROS-sensitive dye DCF-DA and visualized using a fluorescent microscope. The image is a representative of three independent experiments (Magnification, 400×). (C) For quantification of ROS production, the intensity of DCF fluorescence was analyzed using ImageJ software. The results are averages of three independent experiments (**p* < 0.05). (D) Effect of Akt inhibitor (Wortmannin) on LF-mediated protection of H₂O₂-induced cell apoptosis of hMSCs. hMSCs were stimulated with 250 μM hydrogen peroxide for an indicated time after treatment with lactoferrin (100 μg/ml) for 24 h with or without pretreatment of Wortmannin (100 nM) for 2 h. The results are averages of three independent experiments (**p* < 0.05).

peroxide-treated hMSCs. These results suggest that Akt is a downstream target of lactoferrin in hMSCs for senescence or cell survival.

Discussion

ROS, a natural byproduct of normal metabolism, plays important roles of various cellular physiology, including cell signaling and homeostasis [28]. However, excessive ROS may cause oxidative stress, leading to significant damage to cell structures and functions. Thus, regulation of the cellular level of ROS is often a critical target for maintenance of cellular functions.

It has been reported that Akt activation may inhibit or induce ROS generation depending on the physiological conditions or cell types. For example, inactivation of Akt promotes ROS production and thus induces cellular

senescence in leiomyoma cells [25], whereas knock-down of Akt in fibroblasts causes a decrease in ROS production but hyperactivation of Akt results in higher levels of ROS generation [29]. In this study, we found that lactoferrin inhibits ROS generation via Akt activation in hMSCs. Lactoferrin was first discovered in bovine milk as an iron-containing protein, and later research revealed that the lactoferrin is also one of the major proteins in human milk [30]. Specifically, colostrum contains high levels of lactoferrin, and bovine colostrum itself has been known to be effective in treatment of certain infectious diseases [31, 32]. Interestingly, lactoferrin has also been reported to prevent several infections by boosting inflammatory responses in immune systems [33]. Since lactoferrin is a major protein in colostrum, the function of lactoferrin may play a main role in the therapeutic potentials of colostrum. In fact, lactoferrin has previously been reported to suppress

the level of ROS both in vitro and in vivo in various cell types and animal models [17–19, 34]. However, there is no study investigating its antioxidant function in hMSCs. Thus, this study is the first to show that lactoferrin plays an antioxidant role and reduces the oxidative stress-induced senescence and apoptosis of hMSCs, providing a therapeutic value of lactoferrin as a food reagent that increases the efficacy of MSC therapy.

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