Therapeutic Effect of Genistein-Stimulated Human Mesenchymal Stem Cells in Myocardial Infarction

Abstract
Stem cells offer significant therapeutic promise for the treatment of ischemic diseases. However, stem cells transplanted into ischemic tissue exhibit limited therapeutic efficacy because of poor engraftment in vivo. Several strategies aimed at improving the survival and engraftment of stem cells in ischemic myocardium have been developed, including cell transplantation in combination with growth factor delivery, genetic modification of stem cells, and cell therapy by using scaffolds. In this study, we examined the effects of genistein on therapeutic efficacy in an acute myocardial ischemia model. We found that treatment with genistein induced enhanced human mesenchymal stem cell (hMSC) proliferation. These responses were accompanied by increases in the phosphorylation of extracellular signal-regulated kinase (ERK)1/2 signaling. Blockade of each signal pathway abrogated the genistein-induced promotion of hMSC proliferation, suggesting that genistein affects both of these essential signaling pathways. Injection of genistein-stimulated-hMSCs (geni + hMSCs) into myocardial ischemic sites in vivo induced cellular proliferation and survival of cells at the ischemic sites and thereby enhanced angiogenic cytokine secretion. These results show that genistein-stimulate-hMSCs exhibits markedly enhanced anti-apoptotic capabilities compared to those exhibited by hMSCs alone; thus, they enhance the repair of ischemic myocardial injury through cell survival and angiogenic cytokine production.

Introduction
Stem cell therapy holds great promise for therapeutic angiogenesis and the treatment of ischemic diseases. Endothelial progenitor cells derived from embryonic stem cells or mobilized from bone marrow contribute to postnatal neovascularization by directly participating in blood vessel formation [1,2]. Mesenchymal stem cells (MSCs) isolated from either bone marrow or adipose tissue induce angiogenesis mainly through paracrine secretion of angiogenic growth factors [3,4]. Furthermore, clinical trials involving MSC transplantation for ischemic myocardium have confirmed this possibility [5,6]. However, despite several potential advantages, stem cells have low therapeutic efficacy after engraftment into ischemic myocardium because of poor cell survival, which is one of the most important hurdles in improving the efficacy of cell therapy [7]. Thus, we propose a new method of augmenting neoangiogenesis by overcoming the poor engraftment of MSCs into ischemic tissue and enhancing their survival.

Genistein, an isoflavone derived from soybeans, has a weak affinity for estrogen receptor-α, which is present in reproductive organs. In contrast, the affinity of genistein for estrogen receptor-β, which is present in the vasculature, is similar to that of estrogen. Therefore, it can be administered to both sexes [8]. In vitro and in vivo studies have shown that genistein promotes proliferation of estrogen-dependent breast and thyroid cancer cells [9,10], protects against myocardial ischemia-reperfusion injury in a rat model [11], and improves endothelium-dependent vasodilation in ovariectomized rats after 4 weeks of therapy [12] and in postmenopausal women after 6 months of therapy [13]. These reports suggest a therapeutic application of genistein for vascular repair and that the therapeutic applications of genistein for vascular repair are similar to those of estrogen. Here, we investigate the role of genistein (a plant-derived estrogen) on the bioactivity of MSCs in order to define its potential therapeutic impact on myocardial regeneration after infarction, which may provide a new method for improved engraftment of MSCs into ischemic tissues by augmenting neoangiogenesis and enhancing MSC survival.

Methods and Materials
Human adipose-derived mesenchymal stem cell culture
Human adipose-derived MSCs (hMSCs) were obtained from American Type Culture Collection (ATCC, Manassas, VA).
and cultured in Dulbecco’s modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin–streptomycin.

**Animals**

Experiments were performed on male 8 to 10 week old BALB/Ca-nu/nu mice maintained under a 12-h light/dark cycle, in accordance with the regulations of the Soonchunhyang University. Standard laboratory chow and water were available to the mice ad libitum. The protocols were approved by the Institutional Animal Care and Use Committee of the Soonchunhyang University Seoul Hospital, Korea (IACUC2013-5).

**Mouse myocardial ischemia (MI) models**

The mice were subjected to myocardial infarction (MI) by ligation of the left anterior descending coronary artery (LAD) [14-16]. Immediately after LAD ligation, one set of mice received an intramyocardial injection of 2.5 × 10^6 hMSCs in a total volume of 15 μl at 5 different sites (basal anterior, mid anterior, mid lateral, apical anterior, and apical lateral) in the peri-infarct area.

**Western blot analysis**

Cell homogenates (20 μg protein) were separated using 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. After the blots had been washed with TBST (10 mM Tris-HCl [pH 7.6], 150 mM NaCl, 0.05% Tween-20), the membranes were blocked using 5% skim milk for 1 h and incubated with the appropriate primary antibodies at the dilutions recommended by the manufacturers. The membranes were then washed, and the primary antibodies were detected using horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG secondary antibodies. The bands were visualized using enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK).

**Immunohistochemistry**

Three or 28 days after implantation of hMSCs alone or genestin stimulate-hMSCs (geni + hMSCs), the mice were euthanized, and their hearts were removed. The excised hearts were subjected to retrograde perfusion with phosphate-buffered saline (PBS) to wash the coronary vasculature and left ventricle, fixed with 4% paraformaldehyde overnight at 4°C, and then fixed with 15% sucrose overnight at 4°C. Each tissue sample was embedded in paraffin or frozen in optimal cutting temperature compound (Tissue-Tek, Sakura, Torrance, CA, USA). To detect transplanted human cells, the sections were subject to immunofluorescence staining by using an anti-human nuclear antigen (HNA, Chemicon). For viewing the capillaries and arterioles in ischemic regions, the sections were immunofluorescence stained by using an anti-human nuclear antigen (DAPI; Vector Laboratories) and examined using a FluoView 1000 confocal microscope (Olympus) under a 600× objective.

**Fluorescence-activated cell sorter (FACS) analysis**

hMSCs were dissociated in trypsin/ethylenediaminetetraacetic acid (EDTA), pelleted by centrifugation, and resuspended at ~10^6 cells/mL in PBS containing 0.1% bovine serum albumin (BSA). The hMSCs were fixed with 70% ice-cold ethanol for 30 min at 4°C, followed by incubation in a freshly prepared nuclei staining buffer consisting of 250 μg/ml propidium iodide (PI) and 100 μg/mL RNase for 30 min at 37°C. Cell cycle histograms were generated after analyzing the PI-stained cells by FACS (Becton Dickinson). Cell Quest software was used for further analysis.

**Immunofluorescence microscopy**

The hMSCs were plated onto cover slips, serum starved for 24 h, and then stimulated for 60 min and 12 h with genestin (10^{-10} M). Cells were fixed with 3.5% paraformaldehyde in PBS, permeabilized for 10 min with 0.1% (v/v) Triton X-100, and washed three times for 10 min with PBS. Cells were preincubated with 10% BSA (Sigma–Aldrich) in PBS for 20 min to decrease nonspecific antibody binding. Cells were incubated for 60 min with a 1:100 dilution of primary antibody (anti-cyclin D1, anti-cyclin E, and anti-extracellular signal-regulated kinases [ERK]1/2 polyclonal antibody) in a solution containing 1% (v/v) BSA in PBS and washed three times for 10 min with PBS. Cells were incubated with 1% (v/v) BSA for 5 min, incubated for 60 min with fluorescein isothiocyanate (FITC)-conjugated secondary antibody, anti-mouse IgM-FITC (Sigma–Aldrich, green), anti-rabbit IgM-FITC (Sigma–Aldrich, green), anti-rabbit IgG-Alexa Fluor® 594 (Invitrogen Co., red), and anti-goat IgG-Alexa Fluor® 594 (Invitrogen Co., red) in PBS containing 1% (v/v) BSA, and washed three times for 10 min with PBS. Primary antibodies were obtained from Santa Cruz Biotechnology. Samples were mounted on slides and visualized with a FluoView 1000 confocal microscope (Olympus) with a 600× objective.

**Statistical analyses**

The results are expressed as the mean ± standard error. All experiments were analyzed by analysis of variance (ANOVA). In some experiments, this was followed by a comparison of the treatment means with the control using a Bonferroni–Dunn test. A P value less than 0.05 was considered significant.

**Results**

**Effect of genestin on hMSC proliferation**

The hMSCs were incubated with various concentrations (10^{-10} - 10^{-3} M) of genestin. Figure 1A shows that genestin at 10^{-10} M significantly increased cell number. Furthermore, in experiments to elucidate genestin-induced cell proliferation, hMSCs were incubated for various times (0–24 h) with genestin. Figure 1B shows that genestin at 10^{-10} M significantly increased the cell-cycle regulatory protein expression level. Furthermore, genestin increased the localization of cyclin D1 and E (Figure 1C). As shown in Figure 1D, genestin treatment increased ERα/β expression. These results suggest that genestin may induce enhanced proliferation at low concentrations in hMSCs.

**Involvement of the ERK1/2 pathway in genestin-induced hMSC proliferation**

We determined whether ERK1/2 was involved in genestin-
Genistein enhances survival of hMSCs in myocardial ischemic tissue

In the previous in vitro experiments, we demonstrated that culture of hMSCs in genistein activated an ERK1/2 signaling pathway, and increased their proliferative potential. We further tested the hypothesis that genistein pretreatment of MSCs would have beneficial effects by preparing the cells for better survival at the site of ischemic injury and repairing the damaged tissue in the MI injury model. As shown in Figure 3A, transplanted genistein-stimulated-hMSCs (geni+hMSCs) increased the number of PCNA-positive hMSCs compared to transplantation of hMSCs alone, but the ERK1/2 inhibitor U0126 (10^{-6} M) blocked the effect of genistein. Indeed, the transplanted hMSCs (HNA and proliferating cell marker (Ki67) - double positive cells) at 3 days were more abundant in the genistein-stimulate-hMSCs than in the hMSCs alone, but the ERK1/2 inhibitor U0126 (10^{-6} M) again blocked the effect of genistein (Figure 3B). In addition, immunofluorescence staining of caspase-3 and HNA in ischemic muscle 3 days after transplantation showed that apoptotic hMSCs were significantly less abundant with grafting of genistein-stimulate-hMSCs (geni+hMSCs) compared to grafting of hMSCs alone. However, the genistein effect was attenuated by U0126 (10^{-6} M) (Figure 3C).
Genistein enhances hMSC secretion of paracrine cytokines that lead to cell neovascularization

Transplantation of genistein stimulate-hMSCs (geni + hMSCs) into ischemic tissue enhanced paracrine secretion of angiogenic growth factors. As shown in Figure 4A, genistein induced an increase of human angiogenic growth factor (e.g., hVEGF) expression. In order to confirm the geni + hMSCs as an inducer of human angiogenic growth factor expression in ischemic tissue, western blot analysis was used to show that expression of human angiogenic growth factors was more extensive in genistein stimulate-hMSCs (geni + hMSCs) than in hMSCs alone (Figure 4B). In addition, immunofluorescence staining for the human angiogenic growth factor hVEGF indicated that secretion from transplanted genistein stimulate-hMSC (geni + hMSCs) began within 3 days of transplantation, whereas most hMSCs transplanted alone did not secrete angiogenic growth factors until after 3 days (Figure 4C). Therefore, secretion of human growth factors was more extensive in genistein stimulate-hMSCs (geni + hMSCs) than in hMSCs transplanted alone.

The transplantation of genistein stimulate-hMSCs (geni + hMSCs) promoted angiogenesis within ischemic tissue. Immunofluorescence staining for CD31 and quantification of capillary density revealed that transplantation with genistein stimulate-hMSCs (geni + hMSCs) significantly enhanced capillary formation compared with hMSCs transplanted alone (Figure 5A). hMSCs stained with HNA (red) were found in capillaries stained with CD31 (green). When the number of cells incorporated into capillaries was measured, genistein stimulate-hMSCs (geni + hMSCs) showed significantly augmented incorporation into capillaries compared to MSCs alone (Figure 5B). Immunohistological analysis revealed that genistein stimulate-hMSCs (geni + hMSCs) contributed more to the incorporation of hMSCs into vessels than that by hMSCs alone (Figure 5C). These findings demonstrate that genistein stimulate-hMSC (geni + hMSCs) may contribute to incorporation into capillaries and vessels.

Discussion

Previous experimental studies demonstrated that transplantation of stem/progenitor cells such as MSCs, bone marrow stem cells, and
cardiac stem cells reduces ischemia-induced myocardial tissue injury and improves left ventricular function [17-20]. However, stem cells transplanted into the ischemic myocardium are susceptible to a hostile tissue microenvironment with reduced oxygen supply and free radical damage, thereby hindering the full therapeutic benefit. Previous studies have shown that genistein, a phytoestrogen, protects against MI-reperfusion injury in a rat model and promotes proliferation of estrogen-dependent breast and thyroid cancer cells [21,22]. However, it is not clear whether genistein-mediated enhancement of survival in the injured myocardium can also modulate the secretion of angiogenic cytokines and vascularization of transplanted hMSCs. Therefore, we tested whether genistein modulates hMSC biology leading to enhanced survival and function after transplantation in an ischemic myocardium.

Generally, genistein significantly inhibits growth at a high concentration [23] but stimulates growth at low concentrations. In this study, genistein at $10^{-10}$ M significantly increased cell numbers. In addition, genistein at $10^{-10}$ M increased cell-cycle regulatory expression levels. These findings strongly suggest that low concentration genistein plays a pivotal role in stimulating the proliferation of hMSCs. Our study also sheds light on the potential role of ERK1/2 in genistein-induced hMSC proliferation. In this study, genistein increased ERK1/2 activation in MSCs. Other studies, using various cells have demonstrated that ERK1/2 is essential for cell proliferation. Therefore, we examined whether ERK1/2 was involved in genistein-induced proliferation of hMSCs. In this report, we found that genistein-stimulated cell proliferation was dependent on ERK1/2 activation in hMSCs, suggesting that ERK1/2 is a regulator of the cell cycle. Furthermore, the increase in cell proliferation and survival following transplantation of genistein-stimulate-hMSCs (geni + hMSCs) to ischemic myocardium was inhibited by an ERK1/2 inhibitor. To the best of our knowledge, this is the first study to show the effect of genistein on hMSCs.

In vitro expression of hVEGF was higher in genistein-stimulated hMSCs than in non-treated hMSCs. In vivo, we have shown that borderzone myocardium in the genistein stimulate-hMSCs (geni
Figure 4: Enhanced secretions of angiogenic growth factors from human mesenchymal stem cells (hMSCs) stimulated with genistein in the ischemic borderzone. A: The hMSCs were cultured in serum-free medium for 24 h, and then treated with genistein for 12 h. Human-specific vascular endothelial growth factor (hVEGF) determined by Western blotting. hMSCs were cultured in serum-free medium for 24 h and then treated with genistein (10^{-10} M) for 12 h. B: Western blot analysis of hVEGF 3 days after transplantation of hMSCs genistein stimulate-hMSCs (geni+hMSCs) or hMSCs alone. The lower panel depicts the mean ± standard error of 5 independent experiments for each condition, as determined from densitometry relative to β-actin and alpha-tubulin. *P < 0.05 vs. control or hMSCs. C: Immunofluorescence staining of hVEGF in ischemic heart tissue 3 days after grafting of genistein stimulate-hMSCs (geni+hMSCs) or hMSCs alone (n = 8).

Figure 5: Enhancement of neovascularization in the ischemic borderzone by transplantation of human mesenchymal stem cells (hMSCs) stimulated with genistein. A: Immunofluorescence staining of alpha-smooth muscle actin (α-SMA; red) and CD 31 (green) in ischemic heart tissue 28 days after grafting of genistein stimulate-hMSCs (geni+hMSCs) or hMSCs alone. The bar graph shows quantitative analysis of the number of CD31+ capillaries (n = 10). B: HNA+ cells associated CD31+ vasculature and C: HNA+ cells associated α-SMA+ vasculature (n = 10).
References


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