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

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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 stimulate-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.

Abbreviations

hMSCs: human Mesenchymal Stem Cells; ERK: Extracellular Signal-Regulated Kinase; DMEM: Dulbecco's Modified Eagle's Medium; FBS: Fetal Bovine Serum; MI: Myocardial Ischemia; LAD: Left Anterior Descending Coronary Artery; SDS-PAGE: Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis; PBS: Phosphate-Buffered Saline; HNA: Anti-Human Nuclear Antigen; SM: Smooth Muscle; VEGF: Vascular Endothelial Growth Factor; PCNA: Proliferating Cell Nuclear Antigen; DAPI: 4′,6-Diamidino-2-Phenylindole; FACS: Fluorescence-Activated Cell Sorter; EDTA: Ethylenediaminetetraacetic Acid; BSA: Bovine Serum Albumin; PI: Propidium Iodide; FITC: Fluorescein Isothiocyanate; ANOVA: Analysis Of Variance; CDK: Cyclin-Dependent Kinase

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 neovascularization 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 neovascularization 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)

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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 2.5×10^4 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 genistein 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 using anti-CD31 (Abcam) and anti-smooth muscle (SM) α-actin (Abcam), respectively. A primary antibody against human-specific vascular endothelial growth factor (VEGF; Santa Cruz Biotechnology, Santa Cruz, CA) was used to examine the production of human angiogenic factors in ischemic tissues. Primary antibodies against proliferating cell nuclear antigen (PCNA; Abcam), Ki67 (Abcam), and caspase-3 (Santa Cruz Biotechnology, Santa Cruz, CA) were also used for immunofluorescence staining of ischemic tissue. The sections were counterstained with 4′,6-diamidino-2-phenylindole (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 MSCs 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 genistein (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 genistein on hMSC proliferation

The hMSCs were incubated with various concentrations (10^{-10}, -10^{-5} M) of genistein. Figure 1A shows that genistein at 10^{-10} M significantly increased cell number. Furthermore, in experiments to elucidate genistein-induced cell proliferation, hMSCs were incubated for various times (0–24 h) with genistein. Figure 1B shows that genistein at 10^{-10} M significantly increased the cell-cycle regulatory protein expression level. Furthermore, genistein increased the localization of cyclin D1 and E (Figure 1C). As shown in Figure 1D, genistein treatment increased ERα/β expression. These results suggest that genistein may induce enhanced proliferation at low concentrations in hMSCs.

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

We determined whether ERK1/2 was involved in genistein-

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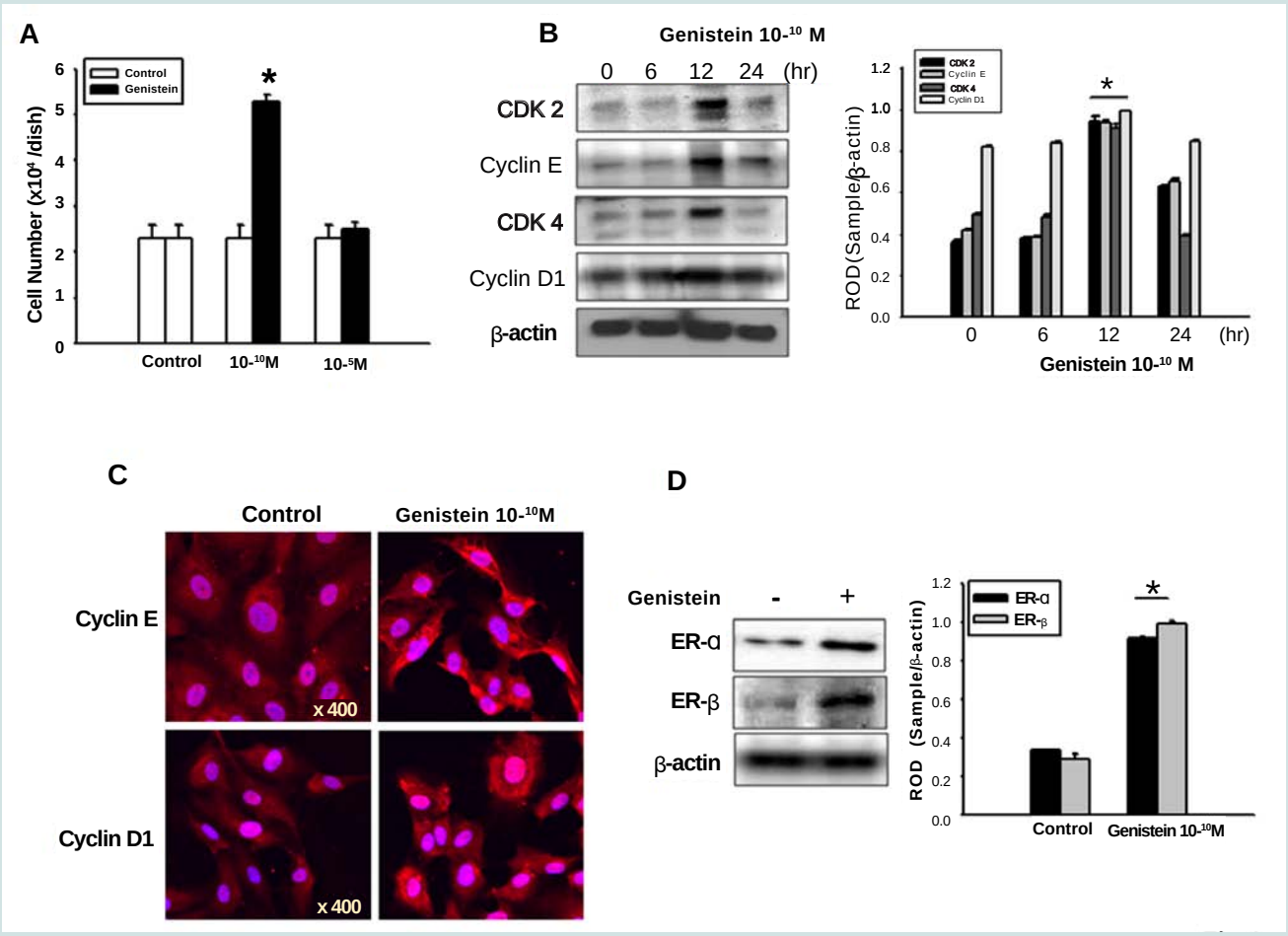

Figure 1: Effect of genistein on cell proliferation. **A:** Human mesenchymal stem cells (hMSCs) were incubated for 12 h with various concentration of genistein (10^{-10}–10^{-5} M) and then counted using a hemocytometer. Values represent the mean ± standard error (SE) of 3 independent experiments, each performed in triplicate. *P < 0.05 vs. control. **B:** The hMSCs were treated with genistein for various times (0–24 h) and the total proteins were then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted with cyclin-dependent kinase (CDK) 2, cyclin E, cyclin-dependent kinase (CDK) 4, cyclin D_1 antibodies. The lower panel depicts the mean ± SE of 5 independent experiments for each condition, as determined from densitometry relative to β-actin. *P < 0.05 vs. 0 time point. **C:** hMSCs were treated with genistein for 12 h. Cyclin D_1 and cyclin E levels were determined using immunofluorescence staining. The figures are representative of 4 independent experiments. **D:** hMSCs were treated with genistein for 12 h and ERα and β was detected by Western blotting. The lower panel depicts the mean ± SE of 5 independent experiments for each condition, as determined from densitometry relative to β-actin. *P < 0.05 vs. control.

induced hMSC proliferation. As shown in Figures 2A and B, genistein initially increased ERK1/2 phosphorylation in a time-dependent manner. Fundamentally, genistein increased the levels of the cell-cycle regulatory proteins cyclin D_1, cyclin E, cyclin-dependent kinase (CDK) 2, and CDK 4 and the localization of cyclin D1 and E (Figure 1B and C). To assess the involvement of ERK1/2 in genistein-induced cell proliferation, as shown in Figure 2C, the genistein-induced increase in the percentage of the cell population in the S phase was significantly blocked by U0126 (an ERK1/2 inhibitor, 10^{-6} M). These results support the suggestion that genistein increases cell proliferation via ERK1/2.

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 stimulate-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 (geni + 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).

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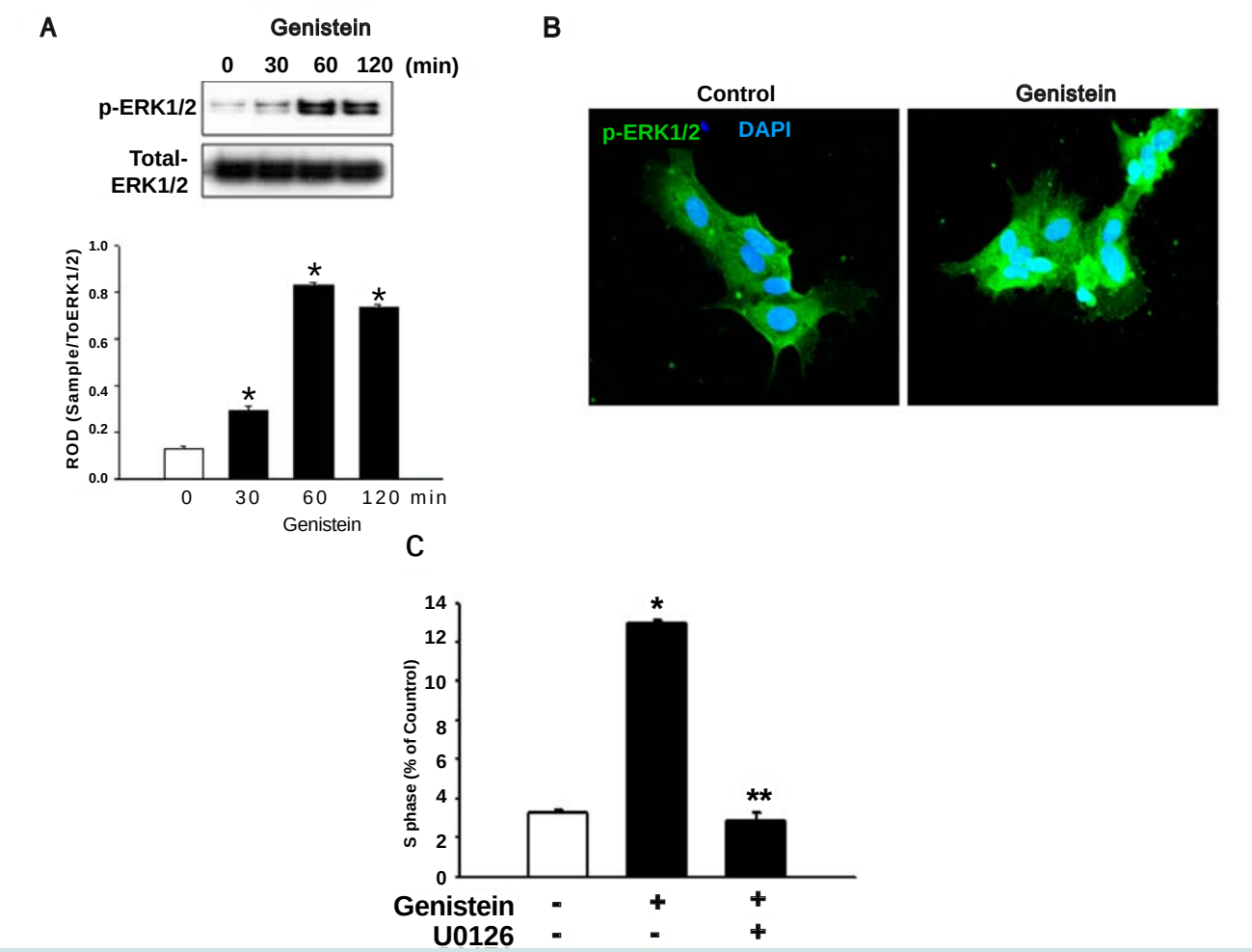

Figure 2: Involvement of extracellular signal-regulated kinase (ERK)1/2 on genistein-induced human mesenchymal stem cell (hMSC) proliferation. A, B: hMSCs were treated with genistein for different times (0-120 min). Phosphorylation of ERK1/2 was detected using Western blotting and immunostaining. **C:** The hMSCs were pretreated with U0126 (an ERK1/2 inhibitor, 10^{-6} M) for 30 min before 12 h of genistein treatment and then washed with phosphate-buffered saline (PBS), fixed, stained, and analyzed using flow cytometry. Gates were manually configured to determine the percentage of cells in the S phase based on DNA content. *P < 0.05 vs. control, **P < 0.05 vs. genistein. The lower parts of (A) show protein levels as the means ± standard error of 3 experiments for each condition as determined by densitometry relative to β-actin. *P < 0.05 vs. 0 time point.

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

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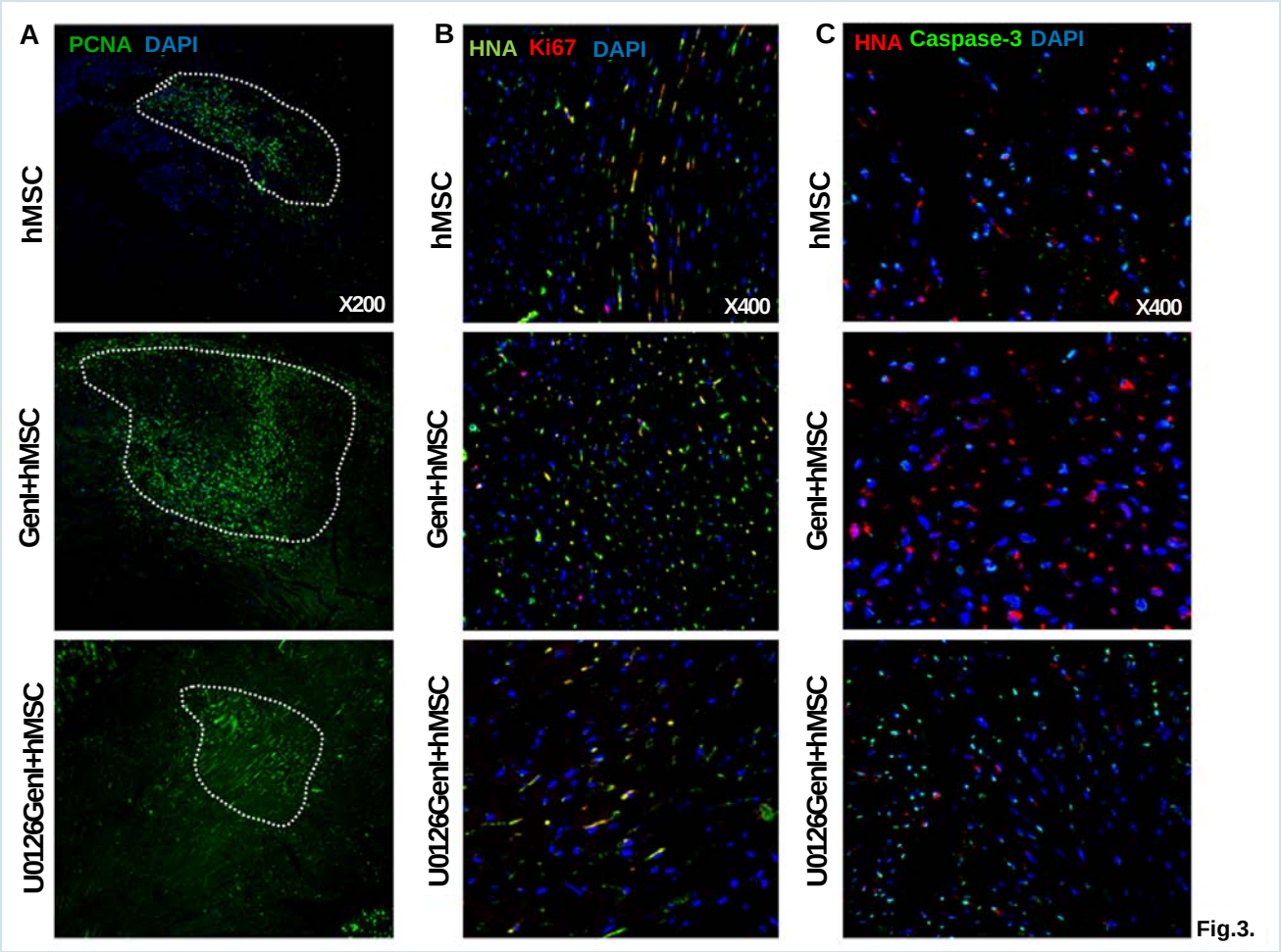

Figure 3: Extracellular signal-regulated kinase (ERK)1/2-mediated genistein-induced human mesenchymal stem cell (hMSC) proliferation and survival in the borderzone of a left ventricular infarct 3 days after myocardial ischemia. hMSCs were pretreated with U0126 for 30 min prior to a 12 h genistein (10^{-10} M) treatment, and the cells were transplanted into the ischemic region. **A:** Proliferating cell nuclear antigen (PCNA) staining for detection of proliferation of MSCs. **B:** Co-immunofluorescence staining for detection of proliferation (Ki67; Proliferation Marker, red) and hMSCs (human nuclear antigen (HNA)-positive cells, green), and 4′,6-diamidino-2-phenylindole (DAPI; blue) for nuclear staining. **C:** Co-immunofluorescence staining for detecting apoptosis (caspase-3, apoptosis marker, green) and of human endothelial progenitor cells (HNA-positive cells, red), and DAPI (blue) for nuclear staining (n = 8).

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

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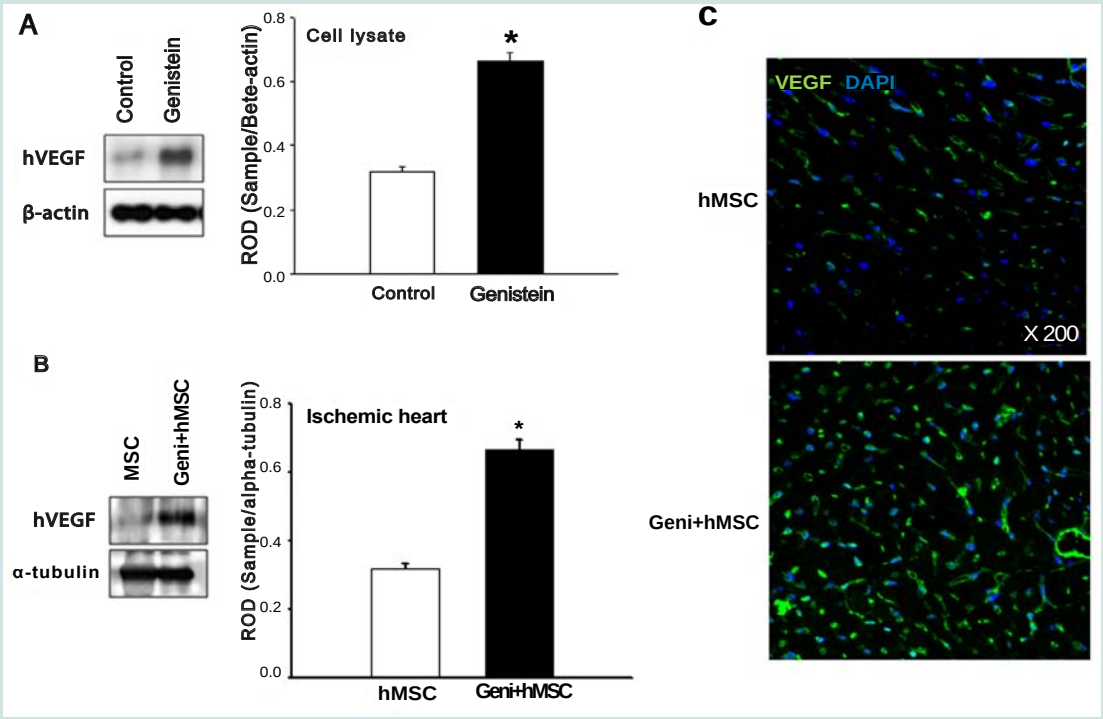

Figure 4: Enhanced secretions of angiogenic growth factors from human mesenchymal stem cells (hMSCs) stimulated with genistein in the ischemic borderzone. A: The hMSCs were cultured in serum-free medium for 24 h, and then treated with genistein for 12 h. Human-specific vascular endothelial growth factor (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).

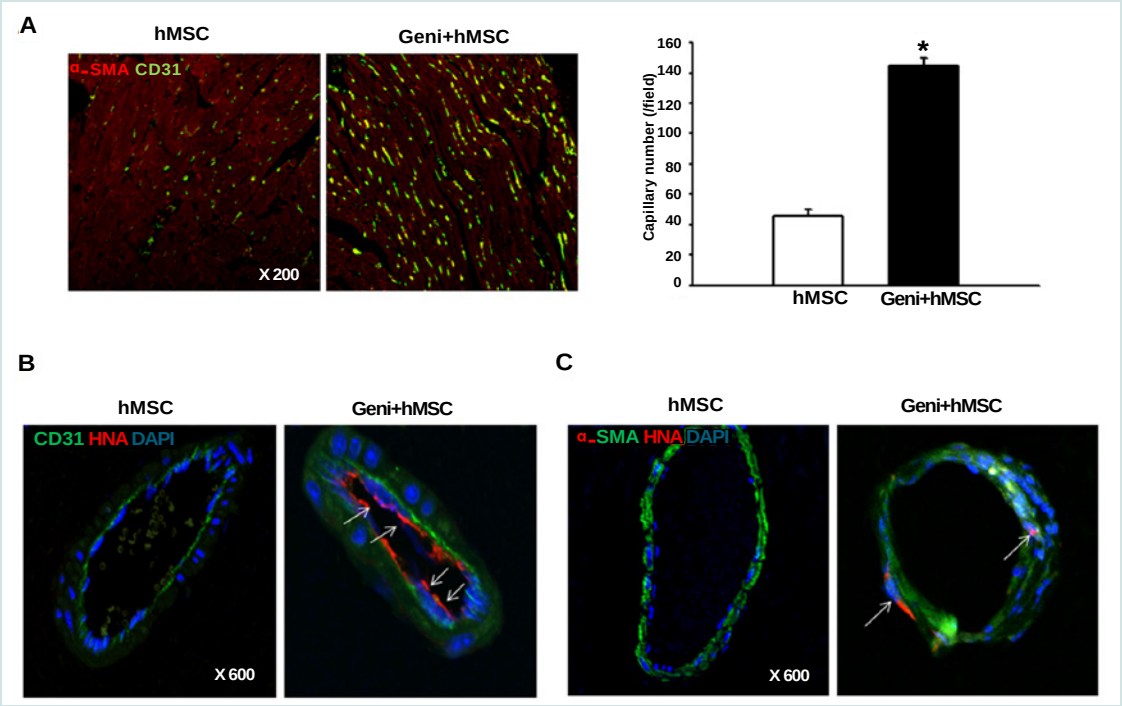

Figure 5: Enhancement of neovascularization in the ischemic borderzone by transplantation of human mesenchymal stem cells (hMSCs) stimulated with genistein. A: Immunofluorescence staining of alpha-smooth muscle actin (α-SMA; red) and CD 31 (green) in ischemic heart tissue 28 days after grafting of genistein 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).

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+ hMSCs) group had a significantly upregulated level of hVEGF, indicative of an ongoing angiogenic process as late as 4 weeks after hMSC transplantation. In the present study, mice receiving genistein stimulate-hMSCs (geni + hMSCs) showed enhanced neovascularization and colocalization of hMSCs to CD31+ and alpha-smooth muscle actin vascular structures. These hMSCs were either seen near the existing vessels or incorporated into the vascular structures, suggesting the possibility of both engraftment into vascular structures and paracrine mechanisms contributing to neovascularization. To the best of our knowledge, this is the first study to show the effect of genistein on hMSCs.

In conclusion, because of the enhanced survival and anti-apoptotic capabilities of geni + hMSCs, this combination holds great promise for improving myocardial repair after ischemic injury. We believe that this strategy could be a novel cell-based therapy for treating ischemic myocardial injury.

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