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Genistein Suppresses Tumor Necrosis Factor-α-Induced Proliferation via the Apoptotic Signaling Pathway in Human Aortic Smooth Muscle Cells

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The proliferation of vascular smooth muscle cells (VSMCs) plays a key role in the development of atherosclerosis. Abnormal VSMC proliferation induces vascular dysfunction and several other pathological processes. The present study investigated the apoptotic effects of genistein on tumor necrosis factor-α (TNF-α)-induced proliferation in human aortic smooth muscle cells (HASMCs). The apoptotic effects of genistein were assessed to determine the mechanism(s) of its antiproliferative activity, including MTT, LDH assay, morphological change of cell, DNA fragmentation, and expression levels of pro- or anti-apoptotic molecules by RT-PCR and Western blots. The results show that genistein significantly reduced cell proliferation in TNF-α-induced HASMCs. Genistein also reduced intracellular nuclei staining with DAPI in a dose-dependent manner. In addition, genistein increased nucleosomal DNA fragmentation, increased the expression levels of Bax and c-Myc, and decreased the expression levels of Bcl-2 and Bcl-xL in TNF-α-induced HASMCs. Taken together, these findings indicate that genistein regulates the activation of apoptosis-related molecules in TNF-α-induced HASMCs, leading to the suppression of proliferation and induction of apoptosis.

KEYWORDS: Genistein; tumor necrosis factor-α; human aortic smooth cells; apoptosis

INTRODUCTION

Atherosclerosis involves proliferative smooth muscle lesions, which may result from various forms of endothelial injury or dysfunction associated with different risk factors (*1*). These factors can alter both phenotype and proliferation of vascular smooth muscle cells (VSMCs) (*2*), which can result in the formation of advanced atherosclerotic lesions (*3*). The functions and activities of VSMCs in the artery are dependent on the extracellular environment and on other factors such as platelets, macrophages, and various substances released by these cells (*4*). Therefore, abnormal VSMC proliferation may be an important event during the development of atherosclerosis (*5*−*7*). Generally, vascular lesions form during several pathological processes that involve accumulation of inflammatory cells and release of cytokines (*8*). The cytokine tumor necrosis factor-α (TNF-α) is secreted by VSMCs in the neointima after balloon catheter injury (*9*). TNF-α also serves as a marker for changes in VSMC phenotype after balloon injury and may contribute to local cellular activation and proliferation of VSMCs (*10*). Recent studies have revealed that emodin and rhein inhibit the proliferation of TNF-α-induced human aortic smooth muscle cells (HASMCs) via apoptotic pathways (*11*).

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Apoptosis is defined as cell death by suicide, also known as programmed cell death, and is an energy-dependent process of cellular self-destruction. It is characterized by a number of well-defined characteristics including changes in cell morphology, condensation of nuclear contents, nuclear fragmentation, packing of the nuclear fragments, and activation of a family of apoptosis related proteins known as Bcl-2 (*12*). The Bcl-2 family consists of pro-apoptotic and anti-apoptotic proteins such as Bax, Bak, Bcl-2, and Bcl-xL. It has been well established that the Bcl-2 family and related cytoplasmic proteins affect the integrity of mitochondria and are critical regulators of apoptosis (*13*). Pro-apoptotic Bax directly induces the release of cytochrome c from the mitochondria (*14*). In contrast, members of the anti-apoptotic Bcl-2 protein family, Bcl-2 and Bcl-xL, are important proteins that contribute to the inhibition of apoptosis (*15*). During the activation of multiple cell death, Bcl-xL suppresses cytochrome *c* release, but not mitochondrial depolarization (*16*). The proto-oncogene c-Myc is widely known as a critical regulator of cell proliferation in normal and neoplastic cells. Additionally, c-Myc induces apoptosis through separate cell death mechanisms (*17*).

Genistein, one of the principal soybean isoflavones, has been reported to favorably affect cancer chemotherapy and to have anticancer, antioxidant, and anti-inflammatory activities (*18*). Furthermore, it has been reported that genistein inhibits cell growth and DNA synthesis in aortic smooth muscle cells from hypertensive animal model and induces apoptosis in chemoresistant

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cancer cells through the modulation of cell signaling pathways (*19*). There are several reports on the pretreatment of genistein-inhibited epidermal growth factor (EGF)-induced activation of Rac, PAK, and JNK in VSMCs (*20*). Moreover, genistein completely inhibited oxidized low-density lipoprotein (ox-LDL)-stimulated tissue factor synthesis in human umbilical vein endothelial cells (HUVECs) (*21*). However, the role of genistein in the inhibition of TNF-α-stimulated HASMC proliferation remains to be fully determined. This paper describes genistein-suppressed TNF-α-stimulated proliferation via the apoptotic pathway in HASMCs.

MATERIALS AND METHODS

Materials. Genistein from *Glycine max* (soybean) was obtained from Sigma Chemicals (98.5% (v/v), St. Louis, MO). Medium for smooth muscle cell (SMCM) and supplemented SMC growth factors were obtained from ScienCell (Carlsbad, CA). Recombinant human tumor necrosis factor-α (TNF-α) was purchased from R&D Systems (Minneapolis, MN). Non-radioactive lactate dehydrogenase (LDH) assay kit, teminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick-end labeling (TUNEL) system, and reverse transcription system were purchased from Promega (Madison, WI). TRI reagent was obtained from MRC (Cincinnati, OH). Polyclonal antibodies against Bax, Bcl-2, Bcl-xL, and c-Myc were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal β-actin antibody was purchased from Cell Signaling Technologies (Beverly, MA). Dimethyl sulfoxide (DMSO), 4,6-diamidino-2-phenylindole (DAPI), and 2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma.

Cell Cultures. HASMCs were cultured in SMCM containing growth factors and 5% CO_2 humidified atmosphere at 37 °C. For all experiments, early passage (passage 10) HASMCs were grown to 80−90% confluence and made quiescent by starvation for at least 24 h.

Proliferation Assay. Cell proliferation was determined by MTT assay. Briefly, HASMCs were seeded on a 96-well plate at 7×10^3 cells/well and allowed to attach for 24 h. After incubation, they were treated with various concentrations of genistein (5, 10, 20, and 30 μM) for 24 h without or with TNF-α (100 ng/mL). The medium was carefully discarded and replaced with 90 μL of new medium, and 10 μL of 0.5 mg/mL MTT solution was added to each well. After 4 h of incubation, the medium was then removed and the precipitate was solubilized by DMSO/ethanol (1:1, v/v). The amount of formazan formed was determined by measuring the absorbance at 570 nm using an ELISA plate reader (Molecular Devices, Sunnyvale, CA).

LDH Assay. Cytotoxicity was assessed by LDH assay in the supernatant medium using a non-radioactive cytotoxicity kit according to the protocol of the manufacturer. HASMCs were seeded on a round-bottom 96-well culture plate in triplicate sets of wells. After incubation, cells were treated with various concentrations of genistein (5, 10, 20, and 30 μM) for 24 h without or with TNF-α (100 ng/mL). HASMCs were lysed followed by two freeze/thaw cycles (freezing at −70 °C for 45 min and thawing at 37 °C for 15 min). The plate was centrifuged at 250*g* for 4 min, and 50 μL of supernatant was transferred from each well to a fresh 96-well flat-bottom plate. Then 50 μL of the reconstituted substrate mix was added to each well of the plate. The plate was covered with foil to protect it from light, and then the plate was incubated at room temperature for 30 min. The reaction was stopped by the addition of 50 μL of stop solution, and LDH was determined by measuring the absorbance at 490 nm. The fold activity of LDH was calculated from the following equation: [(experimental LDH release)/(vehicle LDH release)].

Microscopic Images. HASMCs were seeded on a 6-well culture plate at 1×10^5 cells/well and allowed to attach for 24 h. After incubation, they were treated with various concentrations of genistein (10, 20, and 30 μM) for 24 h without or with TNF-α (100 ng/mL). The morphological change of cells was visualized in microscopic images using a model CKX41 (Olympus, Japan).

Morphological Assessment of Apoptotic Cells. To assay nuclear morphology, HASMCs were grown on Lab-Tek II chamber slides (Nalge Nunc, IL), and cells were washed twice with PBS. The cells were fixed in 4% paraformaldehyde for 30 min, and then the slides were permeabilized with 0.1% Triton X-100 in PBS (PBST) for 10 min at room temperature. After permeabilization, cells were stained with DAPI, and the TUNEL assay was performed with apoptosis detection system according to the protocol of the manufacturer. A fluorescence microscope (Olympus, BX50) fitted with appropriate filters and a CCD digital camera (Olympus, DP71) linked to image processing software was used to capture the fluorescent images.

DNA Fragmentation Assay. HASMCs were treated without or with TNF-α (100 ng/mL) or various concentrations of genistein (10, 20, and 30 μM) for 24 h and then suspended in lysis buffer (50 mM Tris, pH 7.5, 10 mM EDTA, and 0.3% Triton X-100) for 30 min on ice. Cell lysates were treated with RNase (100 μg/mL) for 30 min at 55 °C and then treated with proteinase K (400 μg/mL) for 1 h at 55 °C. The supernatant was extracted with phenol/chloroform (1:1, v/v). The fragmented DNA was precipitated using by centrifugations and electrophoresed on 1.5% agarose gels. The gel containing fragmented DNA was photographed under UV light.

Reverse Transcription−Polymerase Chain Reaction (RT-PCR). Expression of apoptosis genes such as Bax, Bcl-2, Bcl-xL, and c-Myc was studied using RT-PCR. HASMCs were treated with various concentrations of genistein (10, 20, and 30 μM) without or with TNF-α (100 ng/mL) for 6 h. After incubation, total RNA was extracted from HASMCs with TRI Reagent according to manufacturer's instructions. For RT-PCR, a cDNA was synthesized from 1 μg of total RNA using Improm-II reverse transcriptase system (Promega) according to the manufacturer's protocol. The cDNA was amplified by PCR with the following primers: Bax (366 bp, accession no. L22473), 5′-AAGCTGAGCGAGTGTCTCAAGCGC-3′ (sense) and 5′-TCCCGCCACAAAGATGGTCACG-3′ (antisense); Bcl-2 (367 bp, accession no. M13994), 5′-AGATGTCCAGCCAGCTGCACCTGAC-3′ (sense) and 5′-AGATAGGCACCCAGGGTGATGCAAGCT-3′ (antisense); Bcl-xL (545 bp, accession no. Z23115), 5′-ATGGCAGCAGTAAAGCAAGCGC-3′ (sense) and 5′-TTCTCCTGGTGGCAATGGCG-3′ (antisense); c-Myc (505 bp, accession no. V00568), 5′-CCAGGACTGTATGTGGAGCG-3′ (sense) and 5′-CTTGAGGACCAGT GGGCTGT-3′ (antisense); GAPDH (482 bp, accession no. M33197), 5′-GAG TCAACGGATTTGGTCGT-3′ (sense) and 5′-GTTGTCATGGATGACCTTGG-3′ (antisense). PCR products were analyzed by agarose gel electrophoresis and visualized by treatment with ethidium bromide. Expression of Bax, Bcl-2, Bcl-xL, or c-Myc mRNA was normalized relative to GAPDH mRNA.

Western Blot Analysis. HASMCs were treated with various concentrations of genistein (10, 20, and 30 μM) without or with TNF-α (100 ng/mL) for 24 h. After incubation, the cells were lysed in protein lysis buffer consisting of 50 mM Tris pH 7.4, 159 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.1% SDS, and 2 mM PMSF. Protein concentrations were determined using the Bradford method. Twenty microliters of protein was boiled for 5 min, and then each sample was separated by 12% SDS−polyacrylamide gel electrophoresis and electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes (Pall, FL). The membrane was blocked with 5% nonfat dry milk in PBST buffer and incubated overnight at 4 °C with specific primary antibodies including anti-Bax, anti-Bcl-2, anti-Bcl-xL, c-Myc, and anti-β-actin. Subsequently, the membrane was washed with PBS and incubated with horseradish peroxidase-conjugated secondary antibodies. Determinations of Western blot analysis were performed using enhanced chemiluminescence (ECL) kits (Amersham Pharmacia Biotech, U.K.).

Statistical Analysis. Results are expressed as mean ± SD. The differences between groups were evaluated by a Student's *t* test using PRISM statistical analysis software (GraphPad Software, Inc., San Diego, CA), and all data are representative of at least three independent experiments.

RESULTS AND DISCUSSION

In the present study, the inhibitory effect of genistein on TNF-α-induced HASMC proliferation was investigated by an MTT assay. TNF-α markedly increased HASMC proliferation to 144.5%, and this abnormal proliferation was significantly inhibited by genistein in a dose-dependent manner (**Figure 1A**). Next, the cytotoxicity of genistein in HASMCs using by LDH assay was investigated. The concentration of 30 μM genistein with TNF-α resulted in a 4.3-fold increase in activity (**Figure 1B**). Genistein alone had no effect on HASMC proliferation and cytotoxicity. Microscopic observations revealed morphological changes on

TNF-α-induced HASMC proliferation (**Figure 2A**). However, genistein alone (30 μM) did not cause the change in cell morphology. Next, DAPI staining was conducted to investigate the effect of genistein on TNF-α-induced cell apoptosis. HASMCs exhibited typical morphological features of apoptotic cells in a dose-dependent manner, owing to the genistein suppression (**Figure 2B**). A TUNEL assay was used to further characterize the morphological properties observed. The assay results indicate that genistein (10, 20, and 30 μM) induced weak, condensed, and/or fragmented nuclei morphologies in TNF-α-stimulated HASMCs (**Figure 2C**). To determine whether the antiproliferative effect of genistein in TNF-α-induced HASMCs acts through cell apoptosis induction or not, we conducted a DNA fragmentation assay. Genistein increased DNA fragmentation in HASMCs treated with TNF-α (100 ng/mL) in a dose-dependent manner (**Figure 3**). It is important to note that although genistein (10, 20, and 30 μM) strongly induced DNA fragmentation by endonuclease activation during the apoptosis process, genistein alone (30 μM) did not induce DNA fragmentation. The expression of apoptotic genes such as the pro-apoptotic genes Bax and c-Myc and the anti-apoptotic genes Bcl-2 and Bcl-xL, is an active process in cell suicide that occurs in response to apoptosis regulatory factors. Thus, the present study examined the effects of genistein on apoptotic gene expression in TNF-α-stimulated HASMCs by semiquantitative RT-PCR. As shown in **Figure 4**, in TNF-α-stimulated HASMCs, genistein

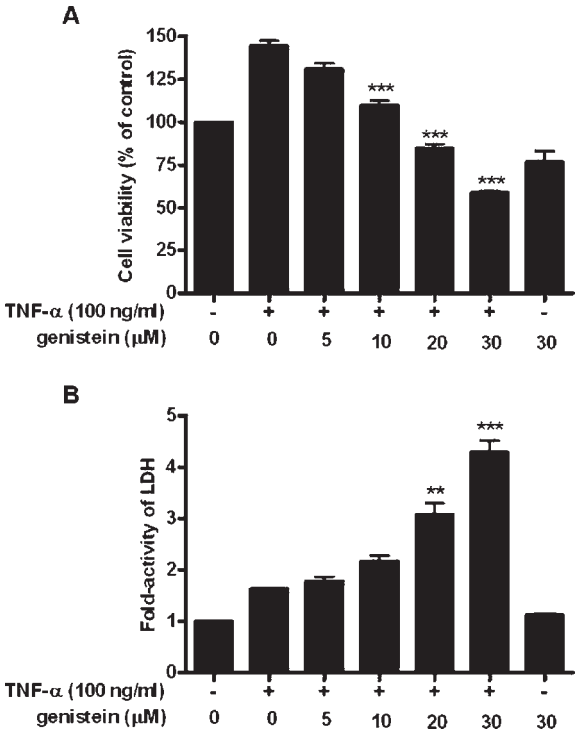

Figure 1. Effects of genistein on the growth of TNF-α-induced HASMCs. (**A**) HASMCs were incubated with 5, 10, 20, and 30 μM genistein for 30 min prior to treatment with 100 ng/mL TNF-α for 24 h, and their proliferation was assessed by MTT assay. (**B**) HASMCs were treated to the indicated concentration of genistein for 30 min prior to treatment with 100 ng/mL TNF-α for 24 h. LDH activity in the culture medium was measured as a marker for loss of HASMC membrane integrity. The control group was not treated with TNF-α or genistein. Data shown are representative of at least three independent experiments. Data represent the means ± SD; **, $p < 0.01$, and ***, $p < 0.001$, versus TNF- α alone group.

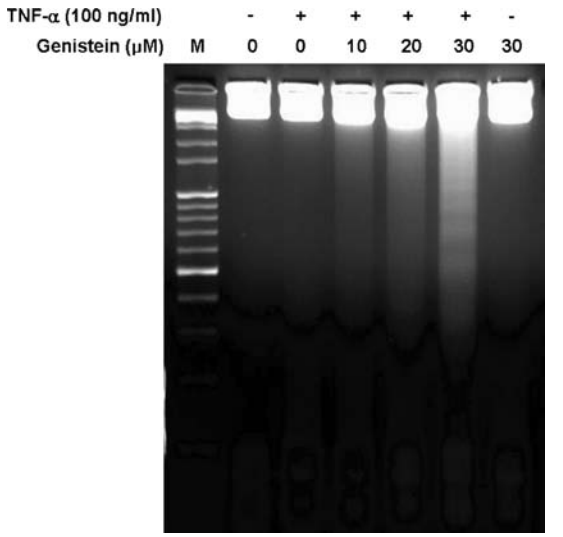

Figure 3. Effects of genistein on DNA fragmentation. HASMCs were incubated with 10, 20, and 30 μM genistein for 30 min prior to treatment with TNF-α (100 ng/mL) for 24 h, and genomic DNA was isolated from the cells. DNA fragmentation was evaluated by electrophoresis on 1.5% agrose gel containing ethidium bromide, which was photographed under UV light. Lane 1 (M) represents DNA molecular mass markers with sizes indicated (Promega DNA 100 bp ladder). DNA fragmentation was determined as described under Materials and Methods.

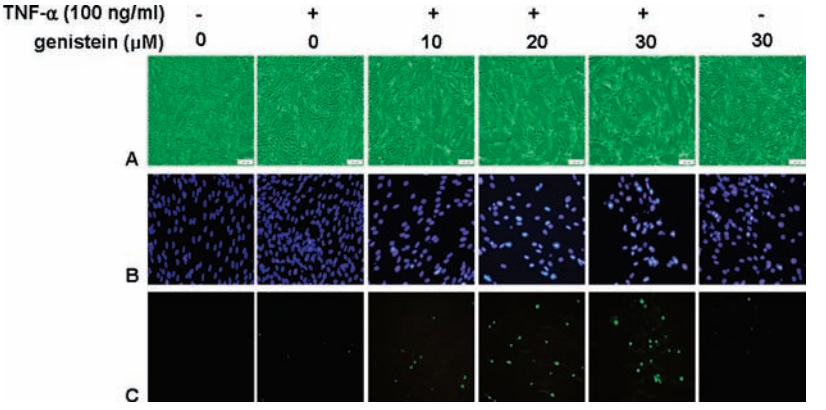

Figure 2. Microscopic observations and effects of genistein on apoptosis. HASMCs were treated with 10, 20, and 30 μM genistein for 30 min prior to treatment with 100 ng/mL TNF-α or without of TNF-α for 24 h. (**A**) The morphology of cells was detected by a microscopic observation. (**B**, **C**) HASMCs were harvested and washed with ice-cold PBS. HASMCs were fixed in 4% paraformaldehyde and permeabilized in 0.1% Triton X-100 for 30 min, respectively. Cells were treated with 1 μg/mL DAPI or TUNEL reaction mixture, and stained HASMCs were examined using fluorescence microscopy.

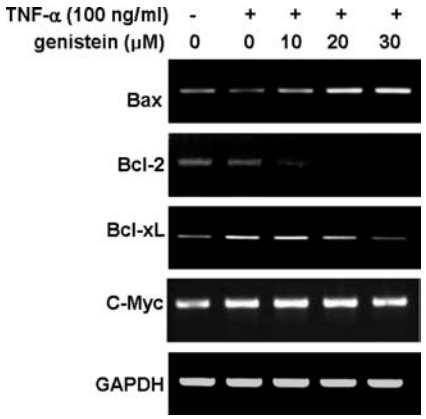

Figure 4. Effects of genistein on apoptotic genes expression. HASMCs were treated with 10, 20, and 30 μM genistein for 30 min prior to treatment with TNF-α (100 ng/mL) for 6 h. After treatment, cells were harvested, and total RNA was isolated by the guanidine isothiocyanate method using TRI-Reagent (MRC). RT-PCR was performed using ImProm-II (Promega) according to the manufacturer's instructions, as described under Materials and Methods. PCR products were resolved on a 1.0% agarose gel and visualized using ethidium bromide, which was photographed under UV light.

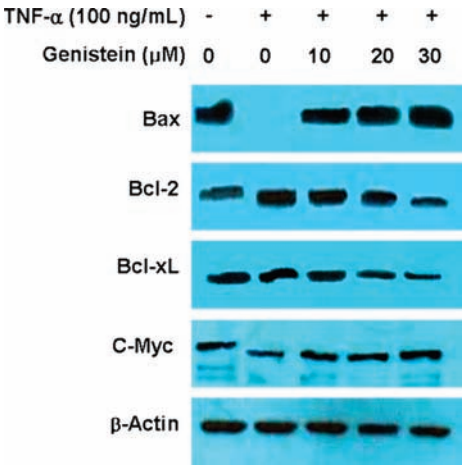

Figure 5. Effects of genistein on apoptotic protein expression levels. HASMCs were treated with 10, 20, and 30 μM genistein for 30 min prior to treatment with TNF-α (100 ng/mL) for 24 h. After treatment, whole cell protein extracts were obtained, and equal amounts of total protein were resolved by 12% SDS−polyacrylamide gels. Western blot analysis was performed using specific antibodies against Bax, Bcl-2, Bcl-xL, c-Myc, and β-actin proteins, as described under Materials and Methods.

(10, 20, and 30 μM) increased the mRNA expression levels of Bax and c-Myc, whereas it decreased the mRNA expression levels of Bcl-2 and Bcl-xL in a dose-dependent manner. The expression levels of Bax, c-Myc, Bcl-2, and Bcl-xL apoptosis-related proteins were analyzed by Western blotting assay. As shown in **Figure 5**, genistein (10, 20, and 30 μM) increased Bax protein levels in TNF-α-stimulated HASMCs in a dose-dependent manner. In contrast, genistein significantly decreased Bcl-2 and Bcl-xL protein levels in TNF-α-stimulated HASMCs in a dose-dependent manner. Genistein also increased the c-Myc protein levels in TNF-α-stimulated HASMCs.

The proliferation of VSMCs is an important contributing factor in the development of atherosclerosis and restenosis after vascular injury (*22*). Following vascular injury, the resulting cellular pathophysiologic response induces VSMC proliferation and is thought to play a critical role in pathogenesis of vascular diseases, including atherosclerosis (*5*, *23*). Therefore, abnormal VSMC proliferation accelerates atherosclerosis in the early stages of a spontaneous event. Additionally, atherosclerosis is known to be associated with a vascular redox state of the arterial wall and with the inflammatory response (*24*). It is well-known that inflammatory cytokines such as TNF-α play an important role in the induction of inflammation in VSMCs and their migration and proliferation in VSMCs (*9*, *10*). Aggarwal (*25*) suggested that TNF-α induces cellular proliferation, survival, and differentiation in VSMCs. Furthermore, previous studies have shown that quercetin, resveratrol, and wogonin suppress TNF-α-induced HASMC proliferation by cell-cycle regulation and inhibition of MMPs activity. Heo et al. (*11*) also suggested that emodin and rhein inhibit TNF-α-induced HASMC proliferation via mitochondrial-dependent apoptosis. However, the effect of genistein on TNF-α-stimulated HASMC proliferation through an apoptotic pathway is not well understood. In the present study, genistein significantly inhibited TNF-α-induced cell proliferation in HASMCs (**Figure 1A**). Wang et al. (*26*) reported that genistein inhibits cell growth and induces apoptotic processes in pancreatic cancer cells. The present results are consistent with those of other studies that have been carried out with either similar or different phytochemicals (*11*, *12*). Treatment with genistein alone (30 μM) had no effect on cytotoxiciy, but it increased the release of LDH in TNF-α-treated cells (**Figure 1B**). Choi et al. (*27*) found that genistein treatment decreased cell viability when compared with TNF-α-treated cells, suggesting that the antiproliferative effect of genistein might be due to the ability of genistein to induce programmed cell death. To determine whether the inhibition of HASMC growth by genistein is related to apoptosis, nuclei staining and DNA fragmentation were measured as markers of cell apoptosis. The results indicate that genistein induced nuclear apoptotic and nucleosomal DNA fragmentation in HASMCs (**Figures 2** and **3**) in a dose-dependent manner. The fragmentation of genomic DNA is considered to be an important feature of cell death, and this cleavage produces a ladder of DNA fragments that are the size of integer multimers of approximately 180−200 bp (*28*). The expression of apoptosis-related genes, for example, Bcl-2 family members such as Bax, Bcl-2, and Bcl-xL, is an important factor that is influenced by pro- and anti-apoptotic regulators in many cells (*15*, *16*). Moreover, the pro-to-oncogene c-Myc has been known to regulate apoptotic cell death (*17*). As shown in **Figures 4** and **5**, genistein induced the pro-apoptotic gene Bax and the proto-oncogene c-Myc in TNF-α-stimulated HASMCs according to both RT-PCR and Western blot analysis. In contrast, genistein inhibited anti-apoptotic molecules such as Bcl-2 and Bcl-xL at the gene or protein levels.

In conclusion, genistein significantly suppresses TNF-α-induced cell proliferation in HASMCs. Furthermore, genistein induces cell apoptosis through the mitochondrial-dependent apoptosis pathway in TNF-α-induced HASMC proliferation. These findings suggest that genistein may exert therapeutic effects through inhibition of TNF-α-induced abnormal growth of HASMCs.

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