Genistein Inhibits the Expression of Vascular Endothelial Growth Factor in MDA–MB–453 Breast Cancer Cells

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Abstract **Objective** To investigate the effects of genistein on the expression of vascular endothelial growth factor (VEGF) in breast cancer cells, and further to explore the molecular mechanism of genistein´s anti-angiogenesis in HER-2/neu-overexpressing breast cancer. **Methods** MDA-MB-453 cells with HER-2/neu overexpression were incubated with/out 50μmol/l genistein for 24, 48, and 72h. Then, the expression of VEGF mRNA and protein were detected with Western blot, immunocytochemical, and reverse transcription-polymerase chain reaction (RT-PCR) techniques, respectively. **Results** The level of VEGF protein expression in genistein-loaded MDA-MB-453 cells was significant decreased compared with normal level, which had time-dependence. Furthermore, the level of VEGF mRNA expression was consistent with the alteration of level of protein expression. **Conclusion** Genistein may down-regulates the expression of VEGF at transcription and translation levels in breast cancer cells, which might be a part of molecular mechanism of genistein anti-angiogenesis in HER-2/neu-overexpressing breast cancer.

Key words Genistein; Vascular endothelial growth factor; MDA-MB-453 breast cancer cell

Genistein, as an important composition of isoflavones mainly distributed in soya bean, inhibits the angiogenesis of proliferating tumor, which evokes tremendous interest for many researchers exploreing its anti-tumor [1]. The angiogenesis of tumor is the foundation for further growth and metastasis, in which vascular endothelial growth factor (VEGF) plays a key role. Previous studies have demonstrated that breast cancers with HER-2/neu-overexpressing possess the characteristics of rapid growth, powerful ability of angiogenesis, easy metastasis, and bad prognosis[2]. Previous results from our laboratory implied that genistein effectively inhibits the angiogenesis of xenograft tumor with HER-2/neu-overexpressin breast cancer cells in BALB/c nude mouse[3]. However, the molecular mechanism of genistein´s anti-angiogenesis has not yet been fully substantiated by experimental evidence. Here, we conducted this study to investigate whether genistein regulates the VEGF expression in MDA-MB-453 cells with HER-2/neu-overexpressing, and to further explore its possible molecular mechanisms involving anti-angiogenesis.

MATERIALS AND METHODS

Cell culture and treatments

The human originated MDA-MB-453 breast cancer cells, with estrogen receptor negative-expression and HER-2/neu overexpression, were purchased from Shanghai Cell biology Institute of Chinese Academy of Sciences and cultured in Dulbecco´s modified Eagle´s medium (Invitrogen), supplemented with 10% fetal bovine serum (Hyclone), and 100U/ml penicillin and l00mg/ml streptomycin (Invitrogen) in a humidified atmosphere of 5% CO_2 at 37℃. MDA-MB-453 cells

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were grown in 25cm^2 culture flask and incubated without(control) or with 50μmol/l genistein(Sigma) pre-dissolved into dimethyl sulfoxide (DMSO) for 24, 48, and 72h, as our previous method [3]. And the equal volume solvent DMSO was added in control group.

The expression of VEGF protein with Western blot

To analyze VEGF protein expression, total protein extracts of MDA-MB-453 cells incubated with/out or with 50μmol/l genistein for each experimental condition were made by homogenization in 5 volumes of ice-cold Tris-buffered saline (0.15mol/l of sodium chloride and 20mmol/l of Tris-HCl, pH7) containing 1% Triton X-100, 1mmol/l phenylmethylsulfonylfluoride and 1mg/l aprotinin, and were measured with Bradfod method. Extracts containing 40μg of total protein were loaded onto 12% SDS-PAGE using a protein assay (Bio-Rad Laboratories, Hercules, CA), and the separated proteins were electrophoretically transferred to a nitrocellulose filter membrane. The membrane was blocked in Tris-buffered saline with 0.1% Tween-20/5% nonfat milk and probed with 1:400 diluted antibody (anti-VEGF or GAPDH, DAKO Co.) overnight, followed by a horseradish peroxidase linked secondary antibody (1:1,000 dilution). Specific protein bands were revealed by 3,3′-diaminobenzidine (DAB, Zhongshan Co., China) and further quantified by scanning the gray area with imaging analytical system (Bio-Rad Laboratories, Hercules, CA).

The expression of VEGF protein with immunocytochemistry

To further determine the alteration of VEGF protein expression by genistein, the immunocytochemistry method was used. MDA-MB-453 cells were seeded on cover slips pre-located in 6-well microtiter plates and allowed to attach for 24h. The 50μmol/l genistein was added to the wells for incubation additional 24-72h. After removal of the medium, the cells were fixed with 4% paraformaldehyde 15min at room temperature. The slips were washed in PBS, incubated with 3% hydrogen peroxide solution in PBS for 5-10 min and blocked with goat normal serum. Immediately prior to staining, primary antibody (mouse anti-human VEGF antibody, 1:100 dilution) was added and then incubated 2h at 37℃. Secondary antibody (anti-mouse IgM-biotin, 1:100 dilution) and streptavidin-HRP (1:200 dilution) were sequentially incubated for 30min at room temperature. Color was developed using 3,3′-diaminobenzidine (DAB) and slides were dehydrated in ethanol, cleared in xylene, mounted with resin and photographed.

Expression of VEGF mRNA with RT-PCR

VEGF mRNA expression were analyzed by semi-quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR). Total RNA of MDA-MB-453 cells treated with/out 50μmol/l genistein was extracted with Trizol reagent (Invitrogen, Carlsbad, CA). β-actin gene was used as an internal control. Specific primers, for VEGF and β-actin according to the literature [4], were as follows: for VEGF, sense, 5′-TTG CTG CTG TAC CTC CAC-3′ and anti-sense, 5′-AAT GCT TTC TCC GCT CTG-3′, which afforded a 418bp fragment; for β-actin, sense, 5′-AGG GGC CGG ACT CGT CAT ACT-3′ and anti-sense, 5′-GGC GGC ACC ACC ATG TAC CCT -3′, which produce a 202bp fragment. All primers were gained from Shanghai bioengineering Corporation. The amplification system (total valume 50μl) was constituted of 3μl cDNA template, 3μl sense and anti-sense primer, 2μl $MgCl_2$ buffer, 1μl dNTP, 5μl 10×PCR buffer, 1μl Taq DNA polymerase, and 32μl sterilizing H_2O. The reaction parameter was set as 30s at 94℃, 50s at 58℃ for VEGF or at 60℃ for β-actin, 1min at 72℃, total 40 cycles for VEGF or 35 cycles for β-actin, 10min at 72℃ after the last cycle. PCR products were visualized with electrophoresis in 2% agarose gels stained with ethidium bromide (EB), and the relative expression of VEGF to β-actin was further quantified with imaging analytical system (Bio-Rad Laboratories, Hercules, CA).

Data analysis

Results were expressed as means and standard deviation. The data of relative levels of VEGF protein and mRNA expression between control and 50μmol/l genistein-loaded MDA-MB-453 cells for 24, 48, and 72h was compared by single-factor analysis of variance

(ANOVA) and post hoc Students´s t−tests. Difference was considered significant if $P<0.05$. SPSS version 10.0 was used for statistical analysis.

RESULTS

Genistein decreased expression of VEGF protein in MDA−MB−453 cells measured with Western blot

The results from Western bolt were judged by the degree of brown strap on the nitrocellulose. That is, the deeper brown implied the corresponding protein with higher expression. As shown in Fig.1 (A), on the nitrocellulose, the brown strap of VEGF in the control was deeper than that of cells incubated with 50μmol/l genistein. Additionally, a gradual light strap was observed with the incubation time from 24 to 72h. The relative level of VGEF protein expression to GAPDH was shown as Fig.1 (B). These results demonstrated that genistein inhibited VEGF protein expression of MDA−MB−453 cells with a time−dependent pattern, which suggested that genistein down−regulates VEGF protein expression at translational level.

The decreased expression of VEGF protein by genistein in MDA−MB−453 cells detected with immunocytochemistry

To further determine the alteration of VEGF protein expression by genistein, immunocytochemistry method

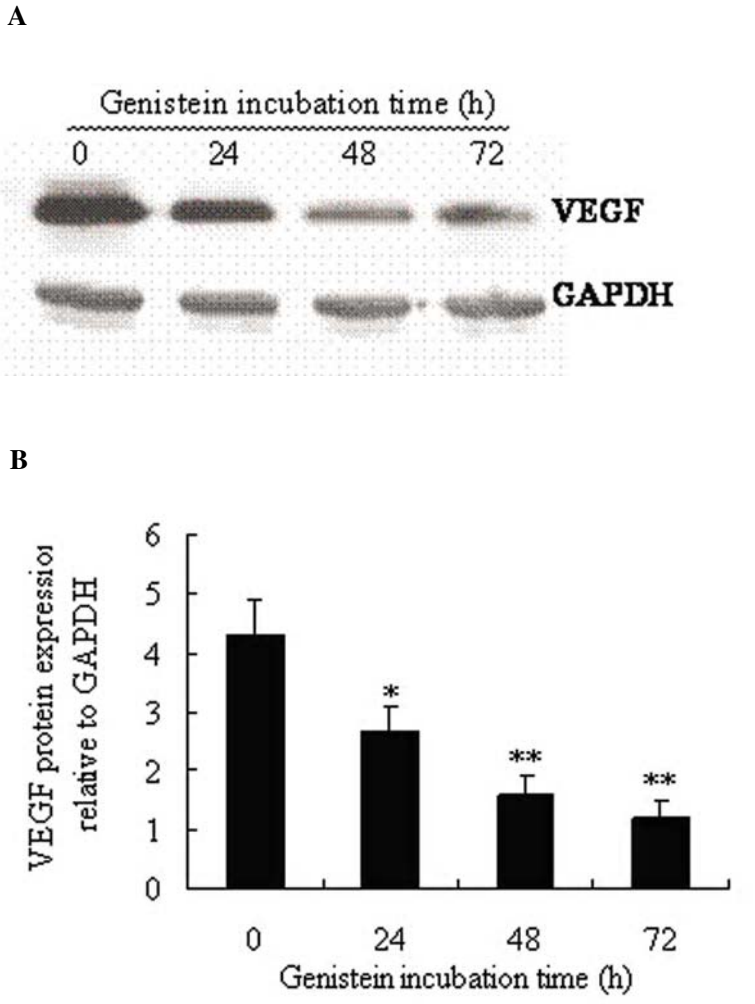

Fig. 1 Genistein inhibited expression of VEGF protein in MDA−MB−453 cells measured with Western blot

(A). the representative dying straps of anti−VEGF antibody

(B). the relative levels of VEGF protein expression, normalized for corresponding glyceralde−3−phosphate dehydrogenase(GAPDH) levels, were obtained by gray scale. Values are means for three determinations for each time point, with standard deviations represented by vertical bars. ⋆$P<0.05$, ⋆⋆$P<0.01$.

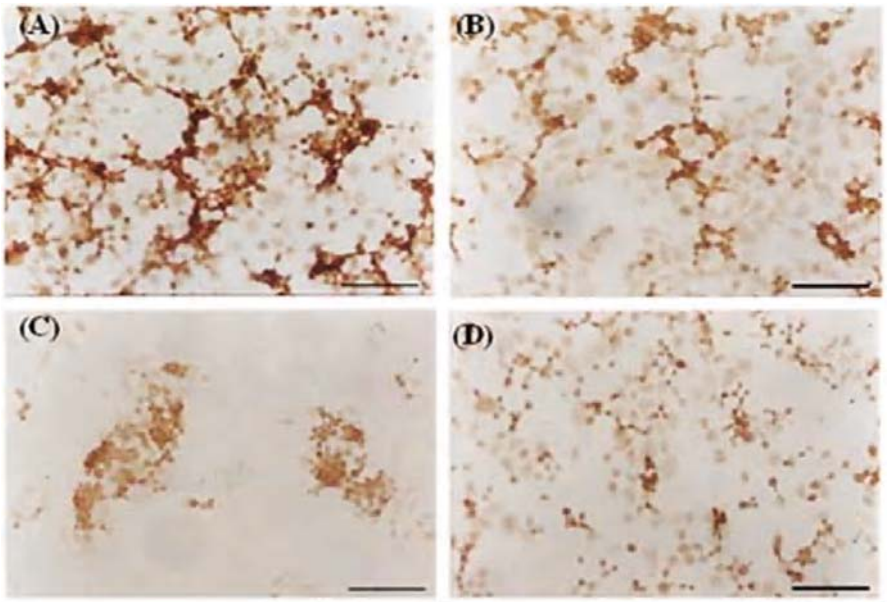

Fig. 2 The representative photographs of anti−VEGF anti−body staining in 50μmol/l genistein−loaded MDA−MB−453 cells for 0(A), 24(B), 48(C), and 72h(D) with immunocytochemistry (Bar=100μm).

was applied by us in this experiment. The cells with brown granules on the membrane or in the intracytoplasm were considered as positive immunereaction. As shown in Fig.2, the degree of grown staining in MDA−MB−453 cells with normal medium was deeper compared to 50μmol/l genistein−loaded cells for 24, 48, and 72h. A gradual decreased expression of VEGF protein was also found with genistein incubation time. These results are concordant with the data from Western blot.

Genistein down−regulated expression of VEGF mRNA determined with RT−PCR technique

To analyze weather the alteration of VEGF protein is relative to level of mRNA expression, the VEGF mRNA of MDA−MB−453 cells incubated with/out genistein was determined with RT−PCR technique (Fig.3). The brightness of 2% agarose gels electrophoresis strip in 50μmol/l genistein−loaded MDA−MB−453 cells was descendent than that of in control, and the down−regulation of VEGF mRNA by genistein was gradual light with incubation time from 24 to 72h(Fig.3 (A)). The relative level of VEGF mRNA expression to β−actin was shown as Fig.3 (B). These results suggested that genistein also down−regulates VEGF mRNA expression of MDA−MB−453 cells with time−dependent manner.

DISCUSSION

In recent years, both in vivo and in vitro, numerous researches have demonstrated that VEGF is the most important factor promoting angiogenesis. VEGF secreted by cancerous cells facilitates angiogenesis of tumor by autocrine or paracrine style[5, 6]. VEGF expression is positively correlative to the microvessel density (MVD) in xenograft tumor with both MDA−MB−231 and MCF−7 breast cancer cells [5~7]. HER−2/neu monoclonal antibody(MAbs) administration has the potential anti−angiogenesis by down−regulating VEGF expression in HER−2/neu overexpression breast cancer [1,8], and also exhibits inhibiting VEGF secretion through multi−pathways in tumoral cells with HER−2/neu everexpression [9]. Based on clinical data, the level of VEGF in malignant breast cancer tissue is higher than that in nonmalignant tissue, and there is a positive correlation between VEGF expression and MVD in malignant breast cancer. The application of recombine human VEGF monoclone antibody (rhuMab) has an efficacious therapeutics for breast cancer with HER−2/neu overexpression. A contrasting research about 107 malignant and 22 nonmalignant breast cancer patients has demonstrated that the

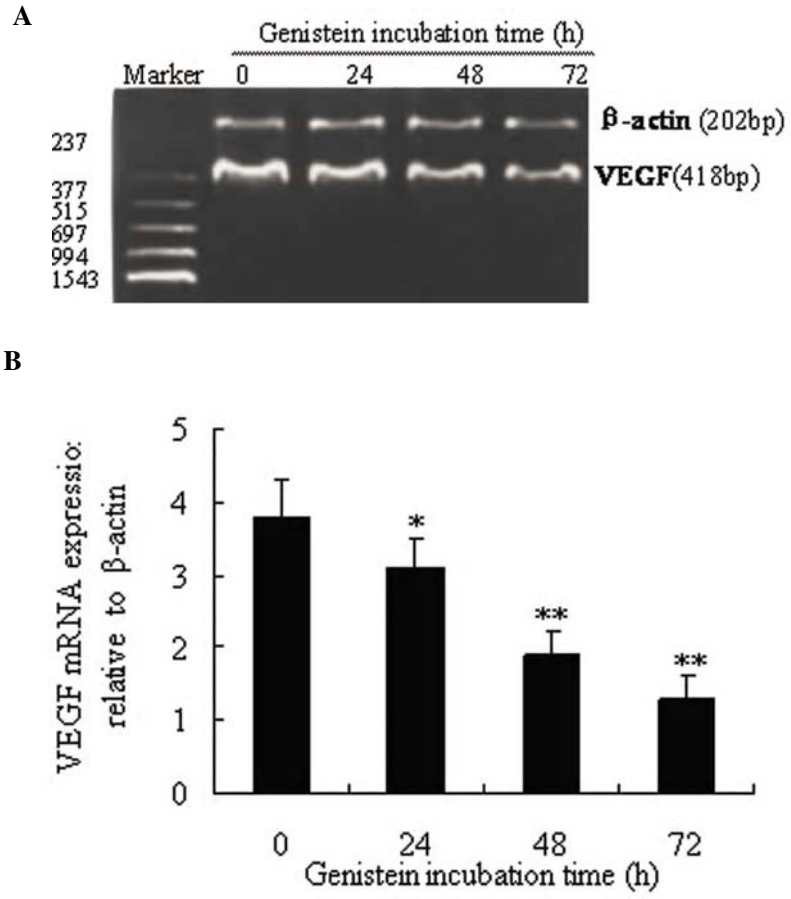

Fig. 3 Genistein down−regulated expression of VEGF mRNA in MDA−MB−453 cells with RT−PCR.
(A). the representative amplification straps of special primer for VEGF in 2% agarose gels stained with ethidium bromide (EB)
(B). the relative levels of VEGF mRNA expression, normalized for corresponding β−actin levels, were obtained by gray scale. ⋆P<0.05, ⋆⋆P<0.01.

levels of VEGF−C and VEGF−D expression in the former are higher than that in the latter, and a positive correlation is found between the levels of VEGF−A, VEGF−C, VEGF−D and the level of HER−2/neu [10,11]. These findings confirm that the up−regulation of VEGF expression, a promoting angiogenesis factor, is relevant with HER−2/neu expression.

The inhibited expression of HER−2/neu−mediated increased VEGF by blocking any one of the ring−joints may prevent tumoral angiogenesis. Genistein, as a potent inhibitor of protein tyrosine kinase (PTK), is a non−nutrition composition mainly distributed in soya bean. Because of its potential anti−tumoral effect, it has been attracting worldwide researchers for many years. Genistein exerts two properties including both competitive inhibition with ATP and noncompetitive inhibition with protein substrates, and provides the property of inhibiting tumor growth[12]. The protein kinases involving in growth factor−mediated signal pathway, such as Grb2mSOS compounds, Ras, MAPK, PI3K and PKB/AKT, may be the target for genistein exercising its biological effects. Genistein can effectively regulate the expression of VEGF mRNA and protein through inhibiting the PTK activity of HER−2/neu receptor and further interfereing the downstream signal cascade. Recently, genistein also exhibits the expression inhibition of VEGF mRNA and protein in prostatic carcinoma PC3 cells with cDNA microarray, RT−PCR and Western blot techniques [13].

MDA−MB−453 breast cancer cells, which were originated from mammary gland epithelium with negative estrogen receptor and HER−2/neu overexpression, were used in vitro as material in the present experiments. Our results demonstrated that 50μmol/l genistein down−regulates the expression of VEGF at transcription and translation levels. Additionally, a significant

decreased level of tyrosine phosphorylation of HER−2/neu receptor and PTK activity in genistein−loaded MDA−MB−453 cells was conducted in previous research at our laboratory[3,14]. Herein, we presume the hypothesis that genistein inhibits angiogenesis in breast cancer through decreasing PTK activity of HER−2/neu, further blocking HER−2/neu receptor signal transduction pathway, at last down−regulating VEGF expression at transcription and translation levels. However, the underlying mechanism whereby genistein protects against breast tumor remains to be determined.

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