Inhibitory effect of ginsenoside-Rb2 on invasiveness of uterine endometrial cancer cells to the basement membrane

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Summary

Ginsenoside-Rb2 derived from ginseng inhibited invasiveness to the basement membrane of endometrial cancer cell lines Ishikawa, HHUA and HEC-1-A cells. These cells dominantly expressed matrix metalloproteinase (MMP)-2 (gelatinase A) among MMPs by zymography. Ginsenoside-Rb2 suppressed the expression and activity of MMP-2, but did not alter the expression of tissue inhibitors of metalloproteinase (TIMP)-1 and TIMP-2 in the cells. Therefore, ginsenoside-Rb2 might inhibit invasiveness to the basement membrane via MMP-2 suppression in some endometrial cancers, and can be used as a medicine for inhibition of secondary spreading of uterine endometrial cancers.

Key words: Ginsenoside-Rb2; Ginseng; Matrix metalloproteinase; Invasion; Endometrial cancer.

Introduction

In cases of malignant tumors, the presence or absence of metastatic lesions is critical to patient prognosis. Therefore, inhibiting the occurrence of metastatic lesions in metastasis-free patients and suppressing the advancement of metastatic lesions in metastasis-positive patients are effective treatments against tumor progression. Inhibiting invasiveness of cancer cells through the basement membrane, which is one of the metastatic processes, is recognized as an effective strategy to suppress tumor advancement. Since curative surgery can be accomplished in most uterine endometrial cancers, long-term prophylactic administration of anti-metastatic agents is a practical strategy to better patient prognosis. Thus, an agent with minimal side-effects should be selected. Ginseng, used as a tonic remedy with minimal side-effects in China, Korea and Japan for more than 4,000 years, is increasingly attracting attention for cancer treatment [1]. Ginseng possesses, among numerous other beneficial effects, antitumoral actions as follows: Ginseng showed a growth-inhibitory activity against several tumor cell lines [2]. Prolonged administration of ginseng extract inhibited the incidence and the proliferation of tumors induced by 9,10-dimethyl-1,2-benzanthracene, urethane and aflatoxin B1 [3]. Ginsenosides derived from ginseng have an inhibitory effect on tumor angiogenesis and metastasis in mice [4, 5]. The purpose of the present study was to investigate the inhibitory effect of ginsenosides on invasiveness of uterine endometrial cancer cells in addition to the molecular mechanisms of ginsenosides.

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Materials and Methods

Chemicals

Ginsenosides Rb1, Rb2, Rc, Rd, Re and Rg1 were purchased from Sigma (St Louis, MO, USA).

Cell culture

Human uterine endometrial cancer cell lines were as follows: Ishikawa [6], HHUA [7] and HEC-1-A [American Type Culture Collection (ATCC): HTB112]. The conventional culture media were 90% Eagle's MEM with 10% fetal bovine serum (FBS) for Ishikawa cells; 90% RPMI 1640 with 10% FBS for HHUA cells; and 90% McCoy's 5a with 10% FBS for HEC-1-A cells.

Assay for invasiveness to basement membrane

For invasion assay [8], the upper faces of filters in a chemotaxicell (Kurabo Biomedical, Osaka, Japan) were coated with Engelbreth-Holm-Swarm (EHS) extract [30 μg/filter, Iwaki Glass, Chiba, Japan; main ingredients: laminin, heparan sulfate, proteoglycan and entactin] [9], and collagen type IV (Iwaki Glass, 5 μg/filter) to promote cell adhesion. Each cell suspension ($5x10^4$/chamber) was put into an upper chamber, and incubated in the conventional media without FBS, and with the ginsenosides to be tested. The number of cells which passed through the gel layer was counted in 5 high power fields using a light microscope.

Assay of gelatin-degrading MMPs by zymography

The cancer cells (10^6 cells/well), treated with ginsenoside-Rb2 or untreated, were homogenized in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis sample buffer containing glycerol (10% v/v), SDS (1% w/v) and bromophenol blue by the method of Davies [10]. The homogenates were electrophoresed on 10% SDS-polyacrylamide gel containing 0.1% gelatin. After electrophoresis, the gel was washed with washing buffer (10 mM Tris-HC1, pH 8.0, 2.5% Triton X-100) for 30 min at 37 °C twice, and incubated in the reaction buffer (50 mM Tris-HC1, pH 8.0, 0.5 mM $CaC1_2$, 10 μM $ZnCl_2$) for 16 hours at 37°C. The gel was then stained with 0.2% Coomassie bril-

liant blue in 50% methanol and 10% acetic acid for 1 hour and washed in 20% methanol and 10% acetic acid. The gelatinase activities were detected as unstained bands.

Western blot analysis for MMP-2

The cancer cells were homogenized in WB-HB buffer (10 mM Tris-HC1, pH 7.4, 150 mM NaC1, 0.5% triton X-100 and 0.2 mM phenylmethyl sulfonyl fluoride) with a Polytron homogenizer (Kinematics, Luzern, Switzerland). The protein concentration of samples was measured by the method of Bradford [11]. Each sample (25 µl) containing 50 µg of protein was added to 25 µl of a sample buffer [12.5 mM Tris-HCl, pH 6.8, 2% glycerol, 0.4% sodium dodecyl sulfate (SDS) and 1.25% 2-mercaptoethanol] and analyzed by 7.5% SDS-PAGE under non-reducing conditions. The gel was transferred to a nitrocellulose membrane (Hybond ECL Western; Amersham, Arlington Heights, IL, USA). The membrane was blocked with 5% milk (from dehydrate) in a blocking buffer (20 mM Tris-HCl, pH 7.6, 137 mM NaCl and 0.1% tween 20), incubated with mouse anti-human MMP-2 antibody [1:1000] (Fuji Chemical, Takaoka, Japan) as the first antibody, washed, and then incubated with peroxidase-linked whole antibody, anti-mouse immunoglobulin from sheep [1:2000] (Amersham, Arlington Heights, IL, USA) as the second antibody. Specific bands were detected with ECL chemiluminescence reagent (Amersham), and X-ray film was exposed on the membrane at room temperature for 10 min.

Enzyme immunoassay for determination of MMP-2, TIMP-1 and TIMP-2 levels

All steps were carried out at 4°C. The cells ($2x10^6$ cells) were homogenized in HG buffer (5 mM Tris-HCl, pH 7.4, 5 mM NaCl, 1 mM $CaCl_2$, 2 mM ethyleneglycol-bis-[ß-aminoethyl ether]-N, N, N', N'-tetraacetic acid, 1 mM MgCl2, 2 mM dithiothreitol, 25 µg/ml aprotinin, and 25 µg/ml leupeptin) with a Polytron homogenizer (Kinematics, Luzern, Switzerland). This suspension was centrifuged in a microfuge at 12,000 rpm for 3 min to remove the nuclear pellet. The protein concentration of samples was measured by the method of Bradford [11] to standardize MMP-2, TIMP-1 and TIMP-2 antigen levels. MMP-2, TIMP-1 and TIMP-2 antigen levels in the samples were determined by a sandwich enzyme immunoassay using a MMP-2, human, ELISA system (Biotrak, Amersham Life Science, Buckinghamshire, England), a hTIMP-1 kit (Fuji Chemical, Takaoka, Japan) and a hTIMP-2 kit (Fuji Chemical). The levels of MMP-2, TIMP-1 and TIMP-2 were standardized with the corresponding cellular protein concentrations.

Statistics

Statistical analysis was performed with one-way ANOVA. Differences were considered significant when p was less than 0.05.

Results

Ginsenoside-Rb2 (≧ 500 ng/ml) significantly ($p < 0.05$) suppressed the migration of Ishikawa, HHUA and HEC-1-A cells in 12 hours (Figure 1). On the other hand, ginsenosides-Rb1, Rc, Rd, Re and Rg1 failed to suppress the migration of all cells tested in 12 hours (data not shown).

Gelatinase activity in Ishikawa, HHUA and HEC-1-A cells, treated with ginsenoside-Rb2 or untreated, was detected as unstained bands (72-kDa, 57-kDa and 41-kDa) produced by the degradation of gelatin in zymography (Figure 2). Three forms were detected as MMP-2 antigen in Western blotting (data not shown). Therefore, three forms of gelatinase in the endometrial cancer cells were recognized as MMP-2 zymogen (72-kDa), and active forms of MMP-2 zymogen (57-kDa and 41-kDa). Gelatinase activity in Ishikawa, HHUA and HEC-1-A cells was suppressed by ginsenoside-Rb2 (Figure 2). The levels of MMP-2 in the uterine endometrial cancer cells treated with ginsenoside-Rb2 were measured by enzyme immunoassay (Figure 3). Ginsenoside-Rb2 (≧ 500 ng/ml) significantly ($p < 0.05$) suppressed the levels of MMP-2 in Ishikawa, HHUA and HEC-1-A cells in 12 hours (Figure 3). The pattern of alteration of cell migration was similar to the alteration of MMP-2 levels by ginsenoside-Rb2 in Ishikawa, HHUA and HEC-1-A cells (Figures 1 and 3). Furthermore, there was no significant alteration in the levels of TIMP-1 and TIMP-2 in Ishikawa, HHUA and HEC-1-A cells treated with ginsenoside-Rb2 (data not shown).

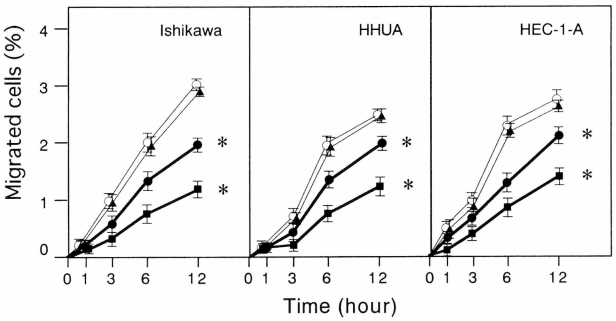

Figure 1. — Effect of ginsenoside-Rb2 on migration of lined endometrial cancer cells.
The upper face of filters was coated with 30 µg EHS extract and 5 µg collagen type IV. Endometrial cancer cell lines Ishikawa, HHUA and HEC-1-A cells were incubated in the conventional media without FBS, and with ginsenoside-Rb2. The number of cells passing through the gel layer on the filter was counted in 5 high power fields.
Data are the mean ± SD of 6 determinations.
○, control (without treatment); ▲, ginsenoside-Rb2, 50 ng/ml; ●, ginsenoside-Rb2, 500 ng/ml; ■, ginsenoside-Rb2, µg/ml; *, $p < 0.05$ versus control.

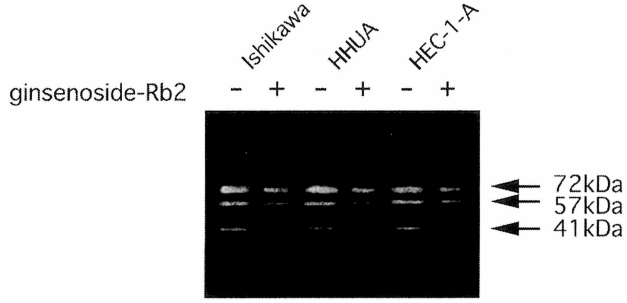

Figure 2. — Effect of ginsenoside-Rb2 on activity of gelatinase in lined endometrial cancer cells.
Gelatinase activity was detected as unstained bands produced by the degradation of gelatin in zymography. Three dominant gelatinases were detected in the uterine endometrial cancer cell lines, 72-kDa (MMP-2 zymogen), and 57-kDa and 41-kDa, which are active forms of 72-kDa gelatinase. Ginsenoside-Rb2 concentration was 500 ng/ml.

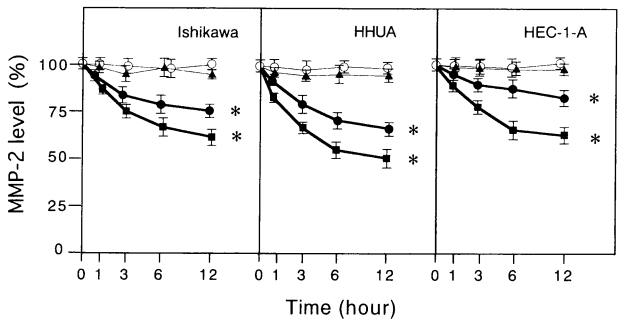

Figure 3. — Effect of ginsenoside-Rb2 on the expression of MMP-2 in lined endometrial cancer cells.
Endometrial cancer cell lines Ishikawa, HHUA and HEC-1-A cells were incubated in the conventional media without FBS, and with ginsenoside-Rb2. The levels of MMP-2 were measured by the ELISA for MMP-2 (BIOTRAK, Amersham Life Science). Data are the mean ± SD of 6 determinations.
○, control (without treatment); ▲, ginsenoside-Rb2, 50 ng/ml; ●, ginsenoside-Rb2, 500 ng/ml; ■, ginsenoside-Rb2, μg/ml; *, $p < 0.05$ versus control.

Discussion

MMP-2 zymogen and its active forms were detected in uterine endometrial cancer cell lines Ishikawa, HHUA and HEC-1-A cells as enzyme activity to degrade gelatin by zymography. The presence of MMP-2 zymogen has been demonstrated in uterine endometrial cancer tissues and cell lines by other authors [12-14]. MMP-2 possesses a potential to degrade collagen IV, which contributes to invasiveness of cancer cells [10, 15, 16]. Active forms of MMP-2 apart from other MMPs have been detected in cancer tissues [10, 17]. Furthermore, the level of MMP-2 active forms correlates with grades of invasiveness in gastric and breast cancers [18]. Therefore, MMP-2 in uterine endometrial cancer cell lines might dominantly work on invasiveness to the basement membrane.

In the present study, ginsenoside-Rb2 suppressed invasiveness along with MMP-2 expression and activity, but did not alter the expression of TIMP-1 and TIMP-2 in uterine endometrial cancer cell lines. Ginsenoside-Rb2 can be used to inhibit occurrence of metastatic lesions in metastasis-free patients and to suppress the advancement of metastatic lesions in metastasis-positive patients in some uterine endometrial cancers. Especially noteworthy is that since ginsenoside-Rb2 derived from ginseng has minimal side-effects, it is appropriate for long-term prophylactic administration as an anti-metastatic agent.

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