Antitumor and Antimetastatic Activities of Docetaxel Are Enhanced by Genistein through Regulation of Osteoprotegerin/Receptor Activator of Nuclear Factor-κB (RANK)/RANK Ligand/MMP-9 Signaling in Prostate Cancer

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Abstract

Bone metastasis is very common in advanced prostate cancer. Docetaxel has been shown to improve survival in patients with metastatic prostate cancer. However, treatment with docetaxel is associated with a certain degree of toxicity. Genistein, derived from soybeans, has been found to inhibit cancer cell growth without toxicity. We have recently reported that genistein could potentiate the antitumor activity of chemotherapeutic agents both in vitro and in vivo. However, the molecular mechanism of this novel effect of genistein has not been fully elucidated. In this study, we found that genistein significantly potentiated the antitumor, anti-invasive, and antimetastatic activities of docetaxel both in culture and in severe combined immunodeficient (SCID)-human model of experimental prostate cancer bone metastasis. We further conducted microarray analysis, real-time reverse transcription-PCR, Western blot analysis, small interfering RNA and cDNA transfection, matrix metalloproteinase-9 (MMP-9) activity assay, and invasion assay. We found that the expression of osteoprotegerin (OPG) was induced by genistein and inhibited by docetaxel, whereas genistein significantly down-regulated the expression and secretion of receptor activator of nuclear factor-κB (RANK) ligand (RANKL) and inhibited osteoclast formation. Moreover, genistein down-regulated the expression and activity of MMP-9, which was induced by docetaxel treatment, and inhibited invasion of PC-3 cells. These results suggest that the observed potentiation of antitumor activity of docetaxel by genistein in the SCID-human model of experimental bone metastasis could be mediated by regulation of OPG/RANK/RANKL/MMP-9 signaling, resulting in the inhibition of osteoclastic bone resorption and prostate cancer bone metastasis. From these results, we conclude that genistein could be a promising nontoxic agent to improve the treatment outcome of metastatic prostate cancer with docetaxel. (Cancer Res 2006; 66(9): 4816-25)

Introduction

The prognosis of prostate cancer is mainly determined by the presence or absence of metastasis. It has been reported that 35% of patients with prostate cancer develop hematogenous metastases, in which bone metastasis of prostate cancer is the most frequent (~90%; refs. 1, 2). Bone metastasis causes morbidity, including bone pain, immobility, hematoepoietic compromises, and spinal cord compression. It is known that cancer cells spread to bone and use the local cytokine machinery to stimulate osteoclasts, resulting in bone resorption and cancer cell growth (3). The most important cytokine machinery, which is involved in bone metastasis of cancer cells, is osteoprotegerin (OPG)/receptor activator of nuclear factor-κB (RANK)/RANK ligand (RANKL)/matrix metalloproteinase-9 (MMP-9) system. The molecular triad OPG/RANK/RANKL plays important roles in bone remodeling. RANKL is expressed by osteoblasts and is necessary and sufficient for osteoclastogenesis (4). RANKL binds to its receptor RANK, present at the surface of osteoclast precursors and mature osteoclasts, inducing osteoclast formation and activation (4, 5). It has been reported that RAW264.7 cells, one of the osteoclast precursor macrophages, can differentiate to osteoclasts when cultured in the presence of 25 to 200 ng/mL RANKL (5). The main features of osteoclasts include the abilities to absorb bone, express tartrate-resistant acid phosphatase (TRAP), and express proteases, including MMP-9 (5, 6). RANKL activity can be blocked by the soluble decoy receptor OPG, resulting in prevention of bone resorption (7). The expression of OPG/RANK/RANKL has also been found in some cancer cells and active T cells, suggesting their effects on cancer bone metastasis (3, 8, 9). RANKL also enhances expression and activity of MMP-9 (6), which is a well-known protease for cancer cell invasion and metastasis. Thus, OPG/RANK/RANKL/MMP-9 system is believed to be a therapeutic target for the treatment of cancer bone metastasis (9).

Recent randomized clinical trials have shown that docetaxel-based combination chemotherapy improves survival in patients with androgen-independent or metastatic prostate cancer (10, 11). However, the combination treatment is associated with a certain degree of dose-related toxicity. Therefore, there is a need for the development of mechanism-based therapeutic strategies to improve efficacy and reduce side effects of docetaxel-based treatment. Genistein, a prominent isoflavone found in soybeans, has been proposed to be partly responsible for the low rate of prostate cancer in Asian men (12, 13). It has been found that genistein inhibits cancer cell growth in vitro and in vivo without toxicity (13). Studies from our laboratory have found that genistein can modulate the expression of genes related to apoptotic pathway, induce apoptosis, and inhibit NF-κB and Akt activation in cancer cells (14–17). In addition, it has been reported that dietary soy significantly reduces tumor cell proliferation, increases apoptosis,
and reduces microvessel density in PC-3 xenograft tumors in severe combined immunodeficient (SCID) mice (18), suggesting its antiangiogenic and antitumor activities in prostate cancer. Genistein also inhibits the in vitro invasive potential of human prostate cancer cell lines, suggesting that genistein could inhibit the metastatic growth of prostate cancer (19). Moreover, our studies using microarray gene expression profiling have shown that genistein inhibits prostate cancer cells in vitro by regulating the expression of genes that are critically involved in cell growth, cell cycle, cell signal transduction, angiogenesis, tumor cell invasion, and metastasis (20, 21). We have also reported that dietary genistein inhibits prostate cancer experimental bone metastasis in a SCID-human model (22). More importantly, we have recently found that genistein potentiates apoptosis-inducing effects of chemotherapeutic agents, such as docetaxel, doxorubicin, gemicitabine, and cisplatin, through down-regulation of NF-κB in multiple human cancer cell lines in vitro and in vivo (23, 24).

In this study, we investigated whether genistein in vitro and in vivo could potentiate the antitumor, anti-invasive, and antimetastatic activities of docetaxel in PC-3 prostate cancer cells and in a SCID-human model of experimental prostate cancer bone metastasis. The purpose of our current investigation was (a) to determine the effects of genistein and docetaxel treatment on prostate cancer cell growth and invasion in vitro; (b) to determine the effects of dietary genistein and docetaxel treatment on prostate cancer bone tumor growth in vivo; and (c) to determine the effects of genistein, docetaxel, or combination treatment on OPG/RANKL/MMP-9 system, so as to better understand the molecular mechanism(s) by which genistein and docetaxel may exert their antitumor, anti-invasive, and antimetastatic effects on prostate cancer cells both in vitro and in vivo.

Materials and Methods

PC-3 cell culture and cell growth inhibition. PC-3 human prostate cancer cells [American Type Culture Collection (ATCC), Manassas, VA] were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS). Genistein (Toronto Research Chemicals, North York, Ontario, Canada) was dissolved in 0.1 mol/L Na2CO3, to make a 10 mmol/L stock solution. Docetaxel (Aventis Pharmaceuticals, Bridgewater, NJ) was dissolved in DMSO to make 4 mmol/L stock solution. The PC-3 cells were seeded at a density of 2 × 104 per well in 96-well culture plates. After 24 hours, the cells were treated with 0 or 50 μmol/L genistein, 1 or 2 mmol/L docetaxel, 30 μmol/L genistein plus 1 mmol/L docetaxel, 0.5 mmol/L Na2CO3 (vehicle control), or 0.05% DMSO (vehicle control) for 1 to 3 days. After treatment, cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 0.5 mg/mL, Sigma, St. Louis, MO) at 37 C for two hours and then with DMSO at room temperature for 1 hour. The spectrophotometric absorbance of the samples was determined by using ULTRA Multifunctional Microplate Reader (TECAN, Durham, NC) at 595 nm.

Animal studies. SCID-human prostate cancer model of experimental bone metastasis used for our study was described previously (25). Briefly, male homozygous CB-17 SCID/SCID mice, 4-week old, were purchased from Taconic Farms (Germantown, NY). Human male fetal bone tissue was obtained by a third-party, nonprofit organization (Advanced Bioscience Resources, Alameda, CA), and written informed consent was obtained from the donor family, consistent with regulations issued by each state involved and the federal government. After 1 week of acclimatization, the mice were implanted with a single human fetal bone fragment as described previously (25). Suspensions of PC-3 cells (1 × 104 in a volume of 20 μL RPMI 1640) were injected intrasosseously by insertion of a 27-gauge needle through the mouse skin directly into the marrow surface of the previously implanted bone. The mice were divided into four groups: control, genistein, docetaxel, and genistein plus docetaxel treatment groups. In the genistein and genistein plus docetaxel treatment groups, the mice were fed a genistein-containing diet (1 g/kg diet) beginning on the 30th day after intraosseous PC-3 cell injection, and the genistein-containing diet was stopped when docetaxel treatment was completed. The mice in docetaxel and genistein plus docetaxel treatment groups received a total of three doses of docetaxel (5 mg/kg, i.v., Aventis Pharmaceuticals) in 6 days as soon as the majority of the bone implants began to enlarge (now called a "bone tumor"), as determined by caliper measurements (35th day after cancer cell injection). The control mice received the exact same diet (AIN76A phytoestrogen-free diet, Purina Mills TestDiet, Richmond, IN) but without genistein (Toronto Research Chemicals). The volume of the bone tumor in each group was determined by twice weekly caliper measurements. The body weight of mice in each group was also measured. All mice were sacrificed on the 46th day after cancer cell injection because big tumors were formed in control mice. Bone tumors were removed and subjected to ex vivo imaging on a Lo-Rad M-IV mammography unit using a magnified specimen technique. Two bone tumors from the genistein treatment group and control group were subjected to microarray analysis. As a microenvironment control, PC-3 s.c. tumors were created in SCID mice by injecting 5 × 106 cells. The mice were fed the genistein-containing diet of the same diet but without genistein. The expression of RANKL in PC-3 s.c. tumors was tested by Western blot. For statistical analysis, a one-way ANOVA was used to compare treatment groups with respect to differences in tumor volumes and Holm’s stepdown procedure was used to control type I error when testing for differences between treatment groups.

Microarray analysis for gene expression profiles. PC-3 cells were treated with 50 μmol/L genistein, 2 mmol/L docetaxel, 0.5 mmol/L Na2CO3, or 0.05% DMSO for 6, 36, and 72 hours. Total RNA from each sample was isolated by TRIzol (Invitrogen, Carlsbad, CA) and purified by RNeasy Mini kit and RNA-free DNase set (Qiagen, Valencia, CA) according to the protocol of the manufacturer. The purified RNA samples were subjected to microarray analysis using Human Genome U95 or U133A Array (Affymetrix, Santa Clara, CA) as described previously (20). The gene expression levels of samples were analyzed by using Microarray Suite, MicroDB, and Data Mining Tool software (Affymetrix). Clustering and annotation of the gene expression were analyzed by using Cluster, TreeView (26), Onto-Express (27), and GenMAPP.5

Real-time reverse transcription-PCR analysis for gene expression. The total RNA prepared for microarray was also subjected to real-time reverse transcription-PCR (RT-PCR) using the method published previously (20). The primers for MMP-9 were as follows: 5'-GGCAGCAGATGTTGACAC-3' and 5'-GCTCCAGTGGGATTTAC-3'. The primers for OPG were as follows: 5'-GCTTAACGGGAAGAAGAAG-3' and 5'-AGATCGTGTTCACTGGGGT3'. Data were analyzed according to the comparative C threshold and were normalized by glyceraldehyde-3-phosphate dehydrogenase expression in each sample. Melting curve for each PCR reaction were generated to ensure the purity of the amplification product.

Western blot analysis. PC-3 cells were treated with 30 and 50 μmol/L genistein, 1 and 2 mmol/L docetaxel, or 30 mmol/L genistein plus 1 mmol/L docetaxel for 48 and 72 hours. After treatment, the cells were lysed and protein concentration was measured using BCA protein assay (Pierce, Rockford, IL). The proteins were subjected to SDS-PAGE and electrophoretically transferred to nitrocellulose membrane. The membranes were incubated with anti-MMP-9 (1:50, Santa Cruz Biotechnology, Santa Cruz, CA), anti-OPG (1:250, R&D Systems, Minneapolis, MN), anti-RANKL (1:100, Santa Cruz Biotechnology), and anti-β-actin (1:10,000, Sigma, St. Louis, MO) primary antibodies, and subsequently incubated with secondary antibody conjugated with peroxidase. The signal was then detected using the chemiluminescent detection system (Pierce) and quantified by using Molecular Analyst System (Bio-Rad, Hercules, CA). The ratios of MMP-9, OPG, or RANKL against β-actin were calculated by standardizing the ratios of each control to the unit value.

5 www.genmapp.org.
OPG and RANKL cDNA transfection. PC-3 cells were seeded in a six-well plate (1.5 × 10^5 per well) and incubated at 37°C for 24 hours. The cells were then transiently transfected with OPG expression vector (InvivoGen, San Diego, CA), RANKL expression vector (InvivoGen), or control empty vector by ExGen 500 (Fermentas, Hanover, MD). After 5 hours, the transfected cells were treated with 50 μM genistein, 2 nmol/L docetaxel, or left untreated. After 24 hours of transfection, RANKL-transfected cells were incubated with serum-free medium supplemented with 50 μM/L genistein or 2 nmol/L docetaxel. After 48 hours of transfection, the total cellular proteins were extracted and the conditioned medium was collected. OPG or RANKL protein in cell lysate and secreted RANKL protein in conditioned medium were detected by Western blot analysis. MMP-9 activity in conditioned medium was detected by MMP-9 activity assay. OPG expression vector and pHygEGFP vector (for selection) were also stably cotransfected into PC-3 cells. The clones that highly express OPG were selected by hygromycin.

MMP-9 small interfering RNA assay. PC-3 cells were seeded in a six-well plate (1.2 × 10^5 per well) and incubated at 37°C for 24 hours. The cells were then transfected with MMP-9 small interfering RNA (siRNA; Santa Cruz Biotechnology) or control RNA duplex by LipofectAMINE 2000 (Invitrogen). After 5 hours, the siRNA-transfected cells were treated with 50 μM/L genistein or left untreated. After 24 hours of incubation, the cells were treated with 2 nmol/L docetaxel for 24 hours. Then, the total cellular proteins were extracted. MMP-9 expression was detected by Western blot analysis.

MMP-9 activity assay. PC-3 cells were seeded in a six-well plate (1.0 × 10^5 per well) and incubated at 37°C for 24 hours. After 24 hours, the complete medium was removed and the cells were washed with serum-free medium. The cells were then incubated in serum-free medium supplemented with 50 μM/L genistein and/or 2 nmol/L docetaxel for 24, 48, and 72 hours. MMP-9 activity in the conditioned medium and cell lysate was detected by using Fluorokine E Human MMP-9 Activity Assay Kit (R&D Systems) according to the protocol of the manufacturer.

Osteoclast differentiation and TRAP staining. RAW264.7 cells (ATCC) were cultured in DMEM (Invitrogen) supplemented with 10% FBS. To induce RAW264.7 cell differentiation to osteoclasts, RAW264.7 cells were seeded at 1 × 10^5/cm² density and treated with 100 ng/mL RANKL (Santa Cruz Biotechnology). These conditions have been shown to successfully stimulate RAW264.7 cell differentiation to osteoclasts (5, 28). RAW264.7 cells were also cocultured with OPG stably transfected PC-3 cells in 10:1 ratio. After 5 days of incubation, the cells were treated with 10 μM genistein and/or 0.5 nmol/L docetaxel. The lower concentrations of genistein and docetaxel were used to capture biological activity without killing prostate cancer cells. After 10 days of RANKL treatment, the formed TRAP in differentiated osteoclasts was stained using TRAP staining kit (Sigma). The purplish to dark red granules, which indicate the formation of TRAP, were photographed and the spectrophotometric absorbance of the granules was determined by using ULTRA Multifunctional Microplate Reader (TECAN).

PC-3 cell and osteoclast coculture. To emulate prostate cancer cell grown in an osteoclastic environment, PC-3 cells and RAW264.7 cells were cocultured at 1 × 10^5/cm² density in 1:1 ratio. The cells were treated with 100 ng/mL RANKL for 7 days. Then, the cells were incubated in serum-free medium supplemented with 50 μM/L genistein and/or 2 nmol/L docetaxel for 48 hours. The conditioned medium was collected and the MMP-9 activity in the medium was detected by using Fluorokine E human MMP-9 activity assay kit.
Genistein Potentiates Antitumor Activity of Docetaxel

Table 1. The average tumor size in each group

<table>
<thead>
<tr>
<th>Group</th>
<th>Average tumor size (mm³)</th>
<th>P</th>
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<tbody>
<tr>
<td>Control</td>
<td>1.930</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Genistein treatment</td>
<td>1.490</td>
<td></td>
</tr>
<tr>
<td>Docetaxel treatment</td>
<td>1.280</td>
<td>0.01</td>
</tr>
<tr>
<td>Genistein and docetaxel</td>
<td>1.042</td>
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Invasion assay. The invasive activity of PC-3 cells with different treatment was tested by using BD BioCoat Tumor Invasion Assay System (BD Biosciences, Bedford, MA) according to the protocol of the manufacturer with minor modification. Briefly, PC-3 cells (5 × 10⁵) with serum-free medium supplemented with 50 μmol/L genistein and/or 2 nmol/L docetaxel were seeded into the upper chamber of the system. Bottom wells in the system were filled with complete medium and same reagent treatment as upper chamber. After 48 hours of incubation, the cells in the upper chamber were removed, and the cells, which invaded through Matrigel matrix membrane, were stained with 4 μg/mL calcein AM in Hank's buffered saline at 37°C for 1 hour. Then, fluorescence of the invaded cells was read in ULTRA Multifunctional Microplate Reader (TECAN) at excitation/emission wavelengths of 530/590 nm. These fluorescently labeled invasive cells were also photographed under a fluorescent microscope. In another experiment, bottom wells were substituted with RANKL-stimulated RAW264.7 cells cultured in complete medium and subjected to the same reagent treatment as the upper chamber to test the invasive activity of PC-3 cells under an osteoclast growth environment.

Results

Genistein potentiated cancer cell growth inhibition caused by docetaxel in vitro. PC-3 prostate cancer cells were treated with genistein, docetaxel, or genistein in combination with a lower dose of docetaxel. The cell viability was determined by the MTT assay. The effect of genistein and docetaxol on the growth of PC-3 cells is shown in Fig. 1. We found that treatment of cells with genistein or docetaxol alone for 72 hours generally caused 50% to 65% growth inhibition in PC-3 cells. However, genistein, in combination with lower doses of docetaxol, resulted in ~75% growth inhibition in PC-3 prostate cancer cells. These results suggest that combination of genistein with lower dose of docetaxol elicited significantly greater inhibition of PC-3 cancer cell growth compared with either agent alone. Because we observed that genistein potentiated cancer cell growth inhibition caused by docetaxel in vitro, we next tested this effect of genistein in vivo.

Genistein enhanced PC-3 bone tumor growth inhibition induced by docetaxel. To test whether genistein has similar effects in vivo, we conducted an animal experiment using SCID-human model of experimental prostate cancer metastasis. We found that genistein significantly inhibited PC-3 bone tumor growth and potentiated PC-3 bone tumor growth inhibition induced by docetaxel, demonstrating an enhanced inhibitory effect of genistein and docetaxel combination treatment on the in vivo model of prostate cancer bone metastasis (Fig. 2A and B; Table 1). The body weight of mice in each group did not show significant difference. Bone tumor X-ray showed that dietary genistein significantly inhibited osteolysis and tumor growth (Fig. 2C). To explore the molecular mechanism by which genistein potentiated the antitumor and antimitastatic activity of docetaxel, we further analyzed the gene expression profiles altered by genistein or docetaxel treatment.

Regulation of OPG expression by genistein and docetaxel treatment. We have previously reported that genistein and docetaxol regulated the expression of a large number of genes, which are critical for the control of cell cycle, apoptosis, and oncogenesis (26,29). During further analysis of microarray data, we uncovered very interesting results showing that genistein and docetaxol exerted opposite effects on the expression of some genes, such as OPG and MMP-9, which might contribute to the enhancement of antitumor effect of docetaxel in the in vivo model of experimental prostate cancer bone metastasis by genistein (Table 2). Microarray data analysis showed that the expression of OPG was induced by genistein and inhibited by docetaxol (Table 2). Real-time RT-PCR and Western blot analysis were conducted to confirm the alteration in the expression of OPG. The result of RT-PCR analysis for OPG mRNA expression (Fig. 3A and B) was in direct agreement with the microarray data. Western blot analysis also showed that OPG protein was up-regulated in genistein-treated PC-3 cells, and down-regulated in docetaxel-treated PC-3

Table 2. The fold change of expression of some selected genes in genistein- or docetaxel-treated PC-3 cancer cells

<table>
<thead>
<tr>
<th>Gene name</th>
<th>PC-3 bone tumor treated with genistein</th>
<th>PC-3 cells treated with docetaxel</th>
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<tr>
<td></td>
<td>6 h</td>
<td>36 h</td>
</tr>
<tr>
<td>Down-regulated by genistein and up-regulated by docetaxel</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM_000999.1, matrix metalloproteinase 9</td>
<td>–2.5</td>
<td>1.1</td>
</tr>
<tr>
<td>NM_001265.1, cathepsin B (CTSB)</td>
<td>–1.3</td>
<td>1.2</td>
</tr>
<tr>
<td>AU149305, matrix metalloproteinase 14</td>
<td>–6.1</td>
<td>NC</td>
</tr>
<tr>
<td>Up-regulated by genistein and down-regulated by docetaxel</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AF065241.1, thioredoxin δ3 (TXN δ3)</td>
<td>2.1</td>
<td>–2</td>
</tr>
<tr>
<td>AF138501.1, OPN (TNFRSF11B)</td>
<td>2</td>
<td>–1.1</td>
</tr>
<tr>
<td>NM_002546.1, OPG (TNFRSF11B) mRNA</td>
<td>2</td>
<td>NC</td>
</tr>
<tr>
<td>AF001294.1, IPI mRNA</td>
<td>2.1</td>
<td>NC</td>
</tr>
<tr>
<td>X77598.1, laminin α3 chain</td>
<td>2</td>
<td>NC</td>
</tr>
</tbody>
</table>

NOTE: Negative values indicate decrease, whereas positive values indicate increase. Abbreviation: NC, no change.
cells (Fig. 3C). Combination treatment also up-regulated the expression of OPG by neutralizing the effect of docetaxel by genistein. These results clearly suggest that genistein and docetaxel differentially regulated mRNA transcription and protein level of OPG, which seems to have some mechanistic role in enhancing the antitumor activity of docetaxel by genistein (Fig. 3D). Because OPG/RANK/RANKL are important molecules in bone remodeling and because osteoclast plays important roles in bone metastasis, we next tested the effects of genistein on RANKL and osteoclast differentiation.

**Genistein down-regulated RANKL and inhibited differentiation of osteoclasts.** By Western blot analysis, we observed weak expression of RANKL in PC-3 cell culture and PC-3 s.c. tumor, whereas much stronger expression of RANKL was observed in SCID-human PC-3 bone tumor (Fig. 4A). Moreover, we found that genistein significantly inhibited the expression of RANKL in SCID-human PC-3 bone tumor. We also found that genistein inhibited secretion of RANKL protein into medium in RANKL-transfected PC-3 cells (Fig. 4B). In the osteoclast differentiation experiment, we observed that RANKL treatment for 7 to 10 days induced RAW264.7 cell differentiation to osteoclasts, which were characterized by the formation of multinucleated cells and the expression of TRAP (Fig. 4C-E). However, RANKL-induced RAW264.7 cell differentiation to osteoclast was inhibited by genistein treatment (Fig. 4C-E). Coculture of RAW264.7 cells and stably transfected PC-3 cells with OPG also showed similar inhibition of osteoclast differentiation (data not shown), suggesting the competitive effect of OPG with RANKL. Docetaxel treatment did not show such inhibitory effect. Because proteases play important roles in bone remodeling and cancer metastasis, we next tested the effects of genistein and docetaxel on the expression and activity of MMP-9.

**Regulation in the expression and activity of MMP-9 by genistein and docetaxel treatment.** Microarray analysis showed that genistein inhibited the expression of MMP-9, whereas docetaxel treatment induced MMP-9 in cultured PC-3 cells and PC-3 bone tumors (Table 2). This observation on the induction of MMP-9 by docetaxel was truly surprising and unexpected. Hence, real-time RT-PCR and Western blot analysis were conducted to confirm the alteration in the expression of MMP-9 both at the mRNA and protein levels. The results of RT-PCR, Western blot analysis, and siRNA assay for MMP-9 expression (Fig. 5A-D) were in direct agreement with the microarray data. Combination treatment with genistein and docetaxel also showed down-regulation of MMP-9. Furthermore, we found that genistein significantly inhibited the activity of MMP-9, whereas docetaxel induced the activity of MMP-9 in conditioned medium and cell lysate in PC-3 cell culture and coculture of RANKL-stimulated RAW264.7 and PC-3 cells (Fig. 5E and F). In addition, we also observed that genistein significantly inhibited MMP-9 activity stimulated by RANKL.
transfection (Fig. 4B). Together, these results clearly suggest that genistein and docetaxel differentially regulate MMP-9, and thus genistein could enhance the antitumor and antimetastatic activity of docetaxel by negating docetaxel-induced MMP-9 activity. To further support the role of MMP in prostate cancer metastasis, we conducted cell invasion assay.

Genistein potentiated docetaxel-induced inhibition of cancer cell invasion. It has been well known that MMP-9 is an important molecule involved in cancer cell invasion and metastasis. Because genistein inhibited the expression and activity of MMP-9 and abrogated the up-regulation of MMP-9 stimulated by docetaxel, we tested the effects of genistein and docetaxel on cancer cell invasion. We found that both genistein and docetaxel inhibited invasion of PC-3 cells through Matrigel matrix membrane in PC-3 growth or differentiated osteoclast growth environment (Fig. 6A and B). Combination treatment with genistein and docetaxel significantly inhibited invasion of PC-3 cells compared with monotreatment.

Discussion
Docetaxel, a member of the taxane family, has shown clinical activity in a wide spectrum of solid tumors, including breast, lung, ovarian, prostate cancers, etc. (30, 31). Docetaxel binds to tubulin and deranges the equilibrium between microtubule assembly and disassembly during mitosis and, in turn, impairs mitosis and cell proliferation in tumors. We have previously reported that docetaxel down-regulates some genes for mitotic spindle formation, transcription factors, and oncogenesis, suggesting pleiotropic effects of docetaxel on prostate cancer cells (29). In this study, we found that docetaxel alone inhibited PC-3 prostate cancer cells grown in SCID-human model of experimental bone metastasis, suggesting its inhibitory effect on prostate cancer bone metastasis in vivo. Furthermore, we used the antimetastatic activity and nontoxic property of genistein in combination with docetaxel treatment to enhance the antitumor and antimetastatic activities of docetaxel. Here, we showed that genistein, in combination with lower doses of docetaxel, resulted in more growth inhibition in PC-3 prostate
cancer cells compared with higher doses of docetaxel treatment alone, suggesting that lower toxicity of docetaxel could be achieved in combination treatment with better treatment outcome. To recapitulate in vitro data in the in vivo setting, we conducted animal experiments. We found that genistein significantly potentiated the antimetastasis activity of docetaxel in SCID-human model of prostate cancer bone metastasis. These in vitro and in vivo data clearly suggest that genistein combined with docetaxel could be more beneficial in patients with prostate cancer bone metastasis compared with either agent alone.

We have previously found that both genistein and docetaxel monotherapy down-regulated some genes critical to the promotion of cell cycle progress and cell proliferation, and up-regulated some genes related to the induction of apoptosis and cell death. Figure 5. MMP-9 expression was up-regulated by docetaxel and down-regulated by genistein. A, real-time RT-PCR analysis of MMP-9 mRNA expression in genistein- or docetaxel-treated PC-3 cells. B, real-time RT-PCR melting curve showing the PCR product of MMP-9 is pure (only one peak). C, Western blot and quantitative analysis of MMP-9 protein expression relative to β-actin in genistein and/or docetaxel treated PC-3 cells (C, control; G, 50 μmol/L genistein treatment; D, 2 nmol/L docetaxel treatment; G+D, 30 μmol/L genistein and 1 nmol/L docetaxel combination treatment). D, Western blot analysis of MMP-9 protein expression in genistein- or docetaxel-treated PC-3 cells with or without MMP-9 siRNA transfection. E, MMP-9 activity assay showed that MMP-9 was up-regulated by docetaxel and down-regulated by genistein in PC-3 cell lysate and conditioned medium. F, MMP-9 activity assay showed that MMP-9 was up-regulated by docetaxel and down-regulated by genistein in conditioned medium of PC-3 cell and RANKL-induced RAW264.7 cell coculture.
These results suggested that the induction of apoptosis and the inhibition of cell proliferation, cell mitosis, and cell survival could be enhanced in genistein and docetaxel combination treatment through the expression regulation of critical genes. To investigate the molecular effects of genistein and docetaxel combination treatment on human prostate cancer bone metastasis, we further analyzed the alternation of gene expression profiles in the experimental PC-3 prostate cancer bone tumors exposed to dietary genistein and the PC-3 prostate cancer cells treated with docetaxel or genistein. Surprisingly, we found that although docetaxel is an anticancer drug, it caused up-regulation of MMP-9 and down-regulation of OPG, which could promote cancer cell growth in the bone environment. We found that genistein and docetaxel exerted different effects on the expression of bone remodeling– and cancer bone metastasis–related genes, OPG/RANK/RANKL/MMP-9, which might contribute to the enhancement of tumor growth inhibition in the in vivo model of experimental PC-3 prostate cancer bone metastasis.

First of all, genistein could enhance antimetastasis activity of docetaxel by regulation of osteoclast differentiation and formation. It has been known that metastatic cancer cells release RANKL and OPG, which act on osteoclast precursor cells to regulate the production of functioning osteoclasts in the bone microenvironment (32, 33). RANKL stimulates the formation and differentiation of osteoclasts via binding to its receptor, RANK, expressed in osteoclast precursors and mature osteoclasts (32, 33). OPG is a decoy receptor that prevents binding of RANKL to RANK by competitive binding to RANKL, leading to the inhibition of osteoclast formation and osteoclast activity (4, 33) in the presence of cancer cells. Yonou et al. (34) reported that OPG decreased human prostate cancer burden in human adult bone implanted into SCID mice. In our study, we found that genistein up-regulated the expression of OPG in PC-3 culture and PC-3 prostate cancer bone tumors, which in fact provided experimental evidence supporting the data published by Yonou et al. Genistein also inhibited RANKL expression and secretion from PC-3 cancer cells, suggesting an inhibitory effect of genistein on osteoclast formation. In contrast, we found that docetaxel inhibited the expression of OPG and that the inhibition of OPG could promote the formation and the activity of osteoclast. More importantly, we found that genistein inhibited differentiation and formation of osteoclast, suggesting the inhibitory effect of genistein on osteolysis. Indeed, from ex vivo bone tumor X-ray, we observed much less osteolysis in genistein-treated bone tumor than in control bone tumor. Osteoclastic activity is believed to be a
critical target for therapy against bone metastasis. Recent reports also showed that genistein supplemented decreased the ratio of RANKL/OPG in healthy human serum and negatively regulated RANKL-induced osteoclast differentiation, supporting our findings (35, 36). Thus, regulation of the ratio of RANKL/OPG could be a mechanism by which genistein enhanced the antimetastasis activity of docetaxel in PC-3 prostate cancer bone metastasis (Fig. 3D).

Another important molecule involved in cancer invasion and metastasis is MMP-9. Studies from our laboratory and others have shown that MMP plays prominent roles in metastasis (37–39). MMP-9 has been implicated in metastasis because of its role in the degradation of basement membrane collagen. In addition, MMP activity is known to play a role in both normal and cancer-induced bone remodeling. It has been reported that RANKL enhances MMP-9 expression and pro-MMP-9 activity in osteoclasts (6). Thus, up-regulation of MMP-9 by docetaxel treatment might favor metastatic cancer cell growth and bone remodeling rather than tumor killing. In this study, we found that genistein inhibited the expression, secretion, and activation of MMP-9, suggesting that genistein could prevent bone matrix degradation and reduce prostate cancer cell proliferation in human bone implanted in SCID mice. The down-regulation of MMP-9 by genistein could be mediated by the inhibition of RANKL and the down-regulation of NF-κB (17), whose binding site has been found in the promoter of MMP-9. We have previously found that dietary genistein also inhibited the expression of other MMPs, such as MMP-2, MMP-11, MMP-13, and MMP-14 (22). These results suggest that genistein could potentiate the antitumor and antimetastasis activities of docetaxel in SCID-human model of prostate cancer bone metastasis partly through the down-regulation of MMP expression (Fig. 3D), thereby negating the MMP-9 activity induced by docetaxel. Because we observed that genistein down-regulated MMP-9, we tested the effects of genistein and docetaxel on the invasion of PC-3 prostate cancer cells. We found that genistein inhibited the invasion of PC-3 cells and significantly potentiated the inhibitory effect of docetaxel on PC-3 cell invasion. These results corresponded with MMP-9 data, showing that genistein could inhibit cancer cell invasion and potentiate the anti-invasive activity of docetaxel partly through down-regulation of MMP-9 and negating docetaxel induced MMP-9 activity.

In summary, our data suggests that genistein could potentiate the anticancer, anti-invasive, and antimetastatic activities of docetaxel in SCID-human model of experimental prostate cancer bone metastasis by regulating OPG/RANK/RANKL/MMP-9 system, which is critical for cancer metastasis and osteoclastic bone resorption. From these results, we conclude that genistein could be a promising nontoxic agent to improve the treatment outcome of docetaxel in metastatic prostate cancer. However, further in-depth studies, including clinical trials, are needed to fully appreciate the value of genistein for combination treatment of metastatic prostate cancer with docetaxel.

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Retraction: Antitumor and Antimetastatic Activities of Docetaxel Are Enhanced by Genistein through Regulation of Osteoprotegerin/Receptor Activator of Nuclear Factor-κB (RANK)/RANK Ligand/MMP-9 Signaling in Prostate Cancer

This article (1) has been retracted at the request of the editors. Following an institutional review by Wayne State University (Detroit, MI), the primary affiliation for several of the authors, it was determined that the article (1) included falsification and/or fabrication, including inappropriate cutting/pasting and image manipulation of the left eight bands of the OPG Western blot bands in Fig. 3C. As a result of these findings, the institution recommended retraction and, upon internal review, the editors agree with this recommendation.

A copy of this Retraction Notice was sent to the last known email addresses for all six authors. Four authors (O. Kucuk, M. Hussain, J. Abrams, and M.L. Cher) agreed to the retraction; the two remaining authors (Y. Li and F.H. Sarkar) did not respond.

Reference

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Antitumor and Antimetastatic Activities of Docetaxel Are Enhanced by Genistein through Regulation of Osteoprotegerin/Receptor Activator of Nuclear Factor-κB (RANK)/RANK Ligand/MMP-9 Signaling in Prostate Cancer

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