Genistein Inhibits p38 Map Kinase Activation, Matrix Metalloproteinase Type 2, and Cell Invasion in Human Prostate Epithelial Cells

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Abstract

Epidemiologic studies associate consumption of genistein, in the form of dietary soy, with lower rates of metastatic prostate cancer. We have previously shown that genistein inhibits prostate cancer cell detachment in vitro, that it is well tolerated in an older cohort of men with prostate cancer, and that it alters cell signaling in that same cohort. We have also shown that p38 mitogen-activated protein kinase (MAPK) is necessary for transforming growth factor β (TGF-β)-mediated increases in prostate cancer adhesion. Although cell invasion is closely linked to metastatic behavior, little is known about how this process is regulated in prostate cancer or what effect, if any, genistein has on associated processes. We now show that genistein inhibits matrix metalloproteinase type 2 (MMP-2) activity in six of seven prostate cell lines tested, blocks MMP-2 induction by TGF-β, and inhibits cell invasion. Efficacy was seen at low nanomolar concentrations, corresponding to blood concentrations of free genistein attained after dietary consumption. Inhibition of p38 MAPK by either SB203580 or dominant-negative construct blocked induction of MMP-2 and cell invasion by TGF-β. Genistein exerted similar effects and was found to block activation of p38 MAPK by TGF-β. This study shows that p38 MAPK is necessary for TGF-β–mediated induction of MMP-2 and cell invasion in prostate cancer and that genistein blocks activation of p38 MAPK, thereby inhibiting processes closely linked to metastasis, and does so at concentrations associated with dietary consumption. Any potential causal link to epidemiologic findings will require further investigation. (Cancer Res 2005; 65(8): 3470-8)

Introduction

Consumption of dietary genistein (4',5,7-trihydroxyisoflavone) in the form of soy has been associated with lower rates of metastatic prostate cancer as well as mortality from prostate cancer (1, 2). Genistein exhibits activity in several preclinical model systems that relate to cancer prevention and is considered a cancer chemopreventive agent (for reviews, see refs. 3, 4). Genistein is being tested in clinical trials by us (5) and by others (6–8). Although a variety of disparate mechanisms have been ascribed to genistein (3, 4), mechanistic studies typically use concentrations that greatly exceed those seen in humans who subsist on soy, no single mechanism has been closely linked with chemoprevention, and no clinical efficacy has been shown. Because chemopreventive agents must be administered over long periods without toxicity, yet must be given at doses associated with effective concentrations, mechanistic studies need to take these parameters into consideration (9). Studies of genistein that take into consideration mechanisms that operate at physiologically relevant concentrations are needed and can shed light on a widely consumed agent that may have cancer chemopreventive activity.

The pharmacokinetic parameters of oral genistein have been extensively evaluated in animals (10), in human dietary studies (11), and in prospective human studies, by us (5) and others (6–8). As a result of first-pass metabolism in the liver, only 1% to 10% of total circulating genistein is free (i.e., nonconjugated). Even after dosing with amounts of genistein that represent at least an 8-fold excess over that associated with daily dietary consumption (i.e., 8 mg/kg/d), peak concentrations of free genistein in the blood are only in the low to mid nanomolar range (5–8). Average steady-state blood levels in Japanese men, who subsist on a soy-based diet, were 0.28 μmol/L for total genistein, whereas concentrations of free genistein were much lower and ranged between 3 and 15 nmol/L (11). In the same study, concentrations in red meat–consuming Westerners were shown to be 2 logs below these levels. Taken together, these studies show that differential consumption of genistein is associated with differential concentrations in the blood. This is a necessary prerequisite if in fact genistein were exerting cancer chemopreventive effects as a function of differential dietary consumption. These data also suggest that relevant concentrations of free genistein are in the low nanomolar range.

Epidemiologic studies suggest that age-adjusted rates of clinical (i.e., metastatic) prostate cancer are ~10-fold lower among soy-consuming Southeast Asians, as compared with non–soy consumers in the United States (1, 2). However, after migration to the United States, immigrant risk approaches that seen in the West, suggesting that differences are not entirely genetic. Interestingly, the prevalence of primary or organ-confined prostate cancer may not account for this difference, because some studies suggest equal rates between Western and Eastern cultures (1), whereas more recent ones suggest that Chinese born in China seem to have rates of primary prostate cancer only 2-fold lower than those of American-born Chinese (12). Although there are clear limitations to epidemiologic studies that seek to associate individual dietary constituents with specific types of cancer, existing data are consistent with the notion that epigenetic factors, including dietary constituents, may modulate prostate cancer metastatic behavior and thus mortality.
We have done a series of mechanistic studies that support the notion that genistein may be acting to inhibit prostate cancer metastasis (13–15). Genistein was first shown to inhibit prostate cancer cell detachment, an initial and necessary step in the metastatic cascade (13). Effects were time and concentration dependent and involved physiologically relevant cellular mechanisms, necessary characteristics of a specifically acting drug. Specifically, genistein treatment caused focal adhesion kinase to form a molecular complex with β1-integrin (13), a transmembrane adhesion protein important in regulating prostate cancer cell adhesion (15, 16), with later studies demonstrating that complex formation was an early event in focal adhesion complex formation (15). Focal adhesion kinase is a protein-tyrosine kinase that regulates cell adhesion and is up-regulated during prostate cancer progression (17). Since our initial report, others have described effects by genistein in a variety of model systems that directly support an anti metastatic mechanism, including decreased intestinal metastasis in rats (18), decreased invasion and lung metastasis of melanoma cells (19, 20), and decreased invasion with breast cancer (21) and glioblastoma cell lines (22). In a separate series of investigations, we showed that genistein would induce apoptosis in human prostate cancer cells (14), but went on to show that growth-inhibitory effects were only observed with supramicromolar concentrations (14, 17), thus calling into question the clinical relevance of growth inhibition. Although genistein induces apoptosis in human prostate cancer cells (14), but went on to use a p38AGF mutant (31). The mutant p38 was created by converting the TGY motif in p38 to AGF by site-directed mutagenesis, using a commercial kit (QuickChange, Stratagene). The resulting p38AGF mutant is identical to a previously described one (32).

Constitutively active β-galactosidase expression vector, pCMV-βgal, was from Stratagene (La Jolla, CA). p38α subcloned into the pcDNA vector (provided by Dr. Peter R. Young, SmithKline Beecham, King of Prussia, PA) was used to generate a p38AGF mutant (31). The mutant p38 was created by converting the TGY motif in p38 to AGF by site-directed mutagenesis, using a commercial kit (QuickChange, Stratagene). The resulting p38AGF mutant is identical to a previously described one (32).

**Cell culture and transfection.** The origin, characteristics, and culture conditions for PC3, PC3-M, and DU-145 established cell lines, as well as for human papilloma virus (HPV) transformed primary 1532NPTX (normal, 1532CPTX (cancer), 1542NPTX (normal), and 1542CP3TX (cancer) cell lines, have previously been described (17). All cells were maintained at 37°C in a humidified atmosphere of 5% carbon dioxide, with biweekly media changes. Cells were grown to subconfluence, trypsinized and replated on a standardized periodic basis. All cells were routinely monitored for Mycoplasma. Cell viability was determined by counting the number of trypan blue–excluding cells under an inverted microscope, using a hemocytometer.

For transfection studies, cells were plated into 24-well plates the previous day and were then transfected using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) per manufacturer’s instructions, using 600 ng of the indicated expression plasmid, along with 200 ng of pCMV-βgal. After a 24-hour recovery period, cells were replated and 24 hours later treated as indicated. The use of a constitutively active β-galactosidase vector allows identification of transfected cells in the context of a three-dimensional matrix used in invasion assays.

**Zymography.** Twenty-four hours after plating, cells were washed thrice with serum-free medium, placed into serum-free medium, treated as indicated, cultured for an additional 24 hours, and conditioned medium centrifuged at 3,000 rpm for 10 minutes to remove debris. In some studies, media were concentrated, thus allowing detection of both MMP-2 and MMP-9 (MMP-9 is present at much lower levels). Matrix metalloproteinases were concentrated by placing a Microcon YM-10 centrifugal filter (Millipore, Billerica, MA) and spinning at 14,000 × g for 30 minutes. For other studies, media were not concentrated, thus optimizing comparison of treatment-related effects on MMP-2. Conditioned media were separated by mixing with 2× sample dilution buffer [125 mmol/L Tris (pH 6.8), 1% SDS, 0.002% bromophenol blue, 10% glycerol], incubating 15 minutes at room temperature, and then separating on an 8% SDS polyacrylamide gel containing 1 mg/mL gelatin under nonreducing conditions. Gels were then washed with 2.5% Triton X-100 in water for 30 minutes, rinsed for 15 minutes with 15 mmol/L Tris-HCl (pH 7.4), washed once in water, and incubated for up to 48 hours at 37°C in 20 mmol/L glycine (pH 8.3), 10 mmol/L CaCl2, and 1 mmol/L ZnCl2. Gels were then stained with 0.5% Coomasie Brilliant Blue G solution containing 10% acetic acid and 20% methanol for 30 minutes and destained with 10% acetic acid and 20% methanol. Areas of MMP activity were detected as clear bands against the blue-stained gelatin background. Dov 13 ovarian cancer cells express high levels of both MMP-9 and MMP-2, and their conditioned media served as a positive control. Cell viability was closely monitored for all experiments and treatment conditions described in this article, and was not adversely affected.

**Cell invasion assays.** Cell invasion assays were done as previously described, with modifications (23). Briefly, 24 hours after replating, cells were treated as indicated, detached by treatment with trypsin/EDTA, washed, resuspended in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) with 0.1% bovine serum albumin, and 52 μL of cell suspension were placed into

**Materials and Methods**

**Materials.** Genistein, phosphatase inhibitor mixture I and II, and gelatin (used in invasion assays) were all purchased from Sigma (St. Louis, MO). The p38 MAPK inhibitor, SB203580, and the structurally related inactive analog, SB202474, were from CalBiochem (San Diego, CA). Genistein, SB203580, and SB202474 were stored as stocks in DMSO, and were thawed just before use. Antibodies were obtained from the following sources: pan-specific TGF-β neutralizing antibody, clone 1D11, R&D Systems (Minneapolis, MN); p38 MAPK, clone C-20, and β-tubulin, clone D-10, Santa Cruz Biotechnology (Santa Cruz, CA); phospho-p38 MAPK (recognizes Thr180 and Tyr182), clone 9211S, Cell Signaling Technology (Beverly, MA); anti-mouse immunoglobulin horseradish peroxidase (HRP) and anti-rabbit immunoglobulin HRP, were part of the ECL Western blotting system (Amersham Pharmacia Biotech, Piscataway, NJ), and were used to detect proteins in Western blots. Recombinant human TGF-β1 (R&D Systems) was resuspended and stored according to manufacturer’s instructions and was used at a final concentration of 2 ng/mL, unless otherwise stated.

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the upper chamber of a 48-well Boyden chamber unit (i.e., 1 × 10^6 cells per well). Cells were allowed to migrate for 9 to 15 hours through a Nuclepore Track-Etch Membrane (NC 983-1643; Whatman, Clifton, NJ), which contained 8-μm pores and was coated with 0.1% gelatin, toward serum-free NIH 3T3 conditioned medium present in the lower chamber. Cells were then fixed and stained according to manufacturer’s instructions, using Diff-Quick cell-staining kit (Dade Behring AG; Dudingen, Switzerland). Membranes were then mounted onto slides, using Permount (Fisher Scientific, Hampton, NH). Using predetermined field coordinates, the number of invading and noninvading cells were then counted in each of five prospectively determined high-power fields (i.e., ×100) for a given well, ×4 cells for each treatment condition (i.e., n = 4). All statistical tests of invasion were two-sided, and changes were only considered statistically significant for P values < 0.05.

In some instances, cells were transfected with expression vectors, along with a constitutively active β-galactosidase expression vector, thus allowing detection of transfected cells. For these experiments, membranes were fixed with 2% formaldehyde and 0.2% glutaraldehyde in PBS for 10 minutes at room temp, as above, rinsed with PBS, and β-galactosidase detected using a β-galactosidase staining kit from Stratagene according to manufacturer’s instructions. Cells were then stained as above with Diff-Quick, except that cells were only stained with solution 1 (i.e., xanthane dye). Use of dye solution 2 (thiazine dye) would have stained nuclei blue, thus interfering with the blue stain generated by β-galactosidase.

**Cell lysis and Western blot analysis.** Cells were lysed and Western blots done as previously described (27), with modifications. Briefly, cells were lysed at 4°C in radioimmunoprecipitation lysis buffer [20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 0.5% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerolphosphate] in the presence of protease inhibitors (1 μg/mL leupeptin, 1 μg/mL aprotinin, 1 mmol/L phenylmethylsulfonylfluoride, all from Sigma) and phosphatase inhibitors (10 mmol/L NaF, 1 mmol/L orthovanadate, phosphatase inhibitor mixture I and mixture II, both at 1:100 dilution; all from Sigma). The resultant clarified lysates, normalized for protein, were separated on a 10% SDS polyacrylamide gel under reducing conditions and transferred onto 0.45-μm nitrocellulose (Schleicher & Schuell, Keene, NH) in a wet transfer cell. Blots were blocked with 20% bovine serum albumin (fraction V, Sigma) in TBST [10 mmol/L Tris-HCl (pH 7.6), 80 mmol/L NaCl, 0.1% Tween 20] for 1 hour at room temperature and probed overnight at 4°C with anti–phospho-p38 MAPK, diluted 1:1000 in TBST at room temperature. After blocking, membranes were reexposed (after readdition of HRP substrate) to ensure removal of antibody and then reprobed for total p38 MAPK, using antibody clone C-20 diluted 1:500, as well as for β-tubulin, using antibody diluted 1:750.

**Results**

**Genistein decreases matrix metalloproteinase activity and inhibits cell invasion.** MMPs are a family of enzymes whose function primarily relates to degradation of extracellular matrix proteins (28). Degradation of extracellular matrix is necessary for cell invasion, and changes in MMP activity are associated with metastatic behavior in a variety of cell types. In human prostate, increases in MMP-2 expression are seen in cancer, as compared with normal cells (29). If genistein were acting to inhibit cell invasion, then MMP-2 would be a likely target. The ability of genistein to inhibit MMP-2 activity was therefore tested in a panel of prostate cell lines recently characterized by us (17). Together, this panel contains members that span the spectrum of prostatic carcinogenesis, including HPV transformed primary cell lines as well as established metastatic cell lines. As can be seen in Fig. 1, after a treatment period of only 24 hours, genistein decreased MMP-2 activity in six of seven cell lines tested. More importantly, efficacy was observed in normal (1532NPTX and 1542NPTX), early cancer (1532CP1TX and 1542CP3TX), and established cancer (PC3 and PC3-M) cell lines. MMP-9 activity was low overall, with genistein decreasing activity in only two of four MMP-9–expressing cells. Similar findings were seen in replicate experiments done at a separate time.

The above findings show that the effect of genistein is not limited to a single cell line and that it has broad activity in human prostate. In-depth investigations were therefore undertaken to elucidate the mechanism by which human prostate cells regulate MMP-2 and invasion and to evaluate the effect of genistein on those regulatory pathways. PC3 and PC3-M cells were chosen for mechanistic studies because they have been extensively characterized by us and others, and we in particular have characterized their...
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response to genistein and TGF-β under a variety of experimental conditions (13–15, 17, 27, 33). In addition, early-stage prostate cell lines were all HPV transformed, raising concerns about their use in transfection experiments. Finally, relative to other cell lines, both PC3 and PC3-M exhibited relatively low levels of MMP-2 and seemed to be less sensitive to the effects of genistein. As such, they would provide a more rigorous test model.

TGF-β is a physiologically important cytokine in human prostate cancer (27–33), and has been previously shown to increase MMP-2 activity in PC3 cells (38), and is produced by prostate cells, including PC3 and PC3-M cells (17). If genistein were in fact modulating MMP-2 activity in humans, then it should inhibit cell invasion. This was tested by pretreating cells with different concentrations of genistein (geni) for 1 hour before adding TGF-β, and then incubated for 24 hours (B). MMP activity was then measured in unconcentrated media. NP, no protein, loading buffer only (control lane).

Figure 2. Genistein inhibits TGF-β-dependent activation of MMP-2. After changing to serum-free media, PC3 and PC3-M cells were treated (or not) with 2 ng/mL TGF-β for 24 hours (A), or were pretreated with the indicated concentrations of genistein (geni) for 1 hour before adding TGF-β, and then incubated for 24 hours (B). MMP activity was then measured in unconcentrated media. NP, no protein, loading buffer only (control lane).

In addition, experimental conditions (data not shown).

Genistein inhibited TGF-β-mediated induction of MMP-2 was shown by pretreating cells with different concentrations of genistein, then treating with TGF-β and measuring their ability to invade through gelatin toward conditioned media (Fig. 3). Genistein decreased invasion in a time- and concentration-dependent fashion in both cell lines tested. More importantly, after treatment for 72 hours, efficacy was observed with genistein concentrations as low as 10 nmol/L in both cell lines tested (two-sided p value <0.05). Thus, genistein will decrease prostate cell invasion at concentrations attained in the blood after dietary consumption.

p38 mitogen-activated protein kinase regulates matrix metalloproteinase type 2 and cell invasion in human prostate. The above results show that TGF-β up-regulates MMP-2 activity in human prostate, thus linking it to the regulation of cell invasion. Cell adhesion and invasion are closely linked cellular processes. We have recently shown that p38 MAPK is necessary for TGF-β–mediated increases in cell adhesion in human prostate cells (27). We therefore investigated the possibility that p38 MAPK may also be important in regulating TGF-β–dependent effects on MMP-2 and cell invasion in human prostate.

p38 MAPK is a serine/threonine kinase and is a member of the MAPK family of signaling proteins (for a review, see ref. 39). SB203580 is a specific inhibitor of p38 MAPK (40, 41). We have previously shown that SB203580 will block TGF-β–mediated increases in cell adhesion (27). SB203580 was therefore used in the current study and was shown to inhibit cell invasion in both PC3 and PC3-M cells in a concentration-dependent fashion, relative to that of untreated control cells, as well as relative to that of cells treated with the inactive chemical homologue, SB203474 (Fig. 4A). Next, SB203580 was shown to block TGF-β–mediated increases in MMP-2, relative to cells treated with inactive chemical homologue (Fig. 4B).

Although SB203580 is a specific inhibitor of p38 MAPK activity, it was important to evaluate the possibility that it may be acting as a nonspecific inhibitor of serine/threonine kinases and thereby preventing activation of p38 MAPK. Of particular concern were the transmembrane TGF-β receptors (TGF-βR). TGF-β initially binds to the type II TGF-β receptor (TGF-βRII; a serine/threonine kinase), which then phosphorylates TGF-βRI (a serine/threonine kinase), which in turn phosphoraylates (i.e., activates) downstream members of the TGF-β signaling cascade, including p38 MAPK (42–44). If SB203580 were nonspecifically inhibiting kinases upstream of p38 MAPK, then it would prevent phosphorylation of p38 MAPK. p38 MAPK is activated by phosphorylation on Thr<sup>180</sup> and Tyr<sup>182</sup>, and this can be detected by Western blot using an activation-specific p38 MAPK antibody directed against both the Thr and Tyr phosphorylation sites. To evaluate whether SB203580 affected activation of p38 MAPK, cells were pretreated with SB203580 (or not), then treated with TGF-β (or not), and p38 MAPK phosphorylation was evaluated by Western blot. As can be seen in Fig. 4C, TGF-β–mediated phosphorylation of p38 MAPK was not inhibited by SB203580. This shows that SB203580 was not inhibiting regulatory elements upstream of p38 MAPK.

To confirm the role of p38 MAPK, its function in vivo was blocked by use of dominant-negative p38 MAPK, as previously described (27). The p38AGF mutant has point mutations at Thr<sup>180</sup> and Tyr<sup>182</sup>, and thus it cannot be activated. For these experiments, prostate cells were first transfected with either wild-type (WT) or dominant-negative p38 MAPK constructs or vector control, and treated with TGF-β (or not). Because TGF-β treatment has been shown to increase levels of both total p38 MAPK (p38), as well as activated p38 MAPK (pp38, i.e., phospho-p38) in human prostate treated with genistein for either 24 or 72 hours and measuring their ability to invade through gelatin toward conditioned media (Fig. 3). Genistein decreased invasion in a time- and concentration-dependent fashion in both cell lines tested. More importantly, after treatment for 72 hours, efficacy was observed with genistein concentrations as low as 10 nmol/L in both cell lines tested (two-sided p value <0.05). Thus, genistein will decrease prostate cell invasion at concentrations attained in the blood after dietary consumption.
Next, the ability of dominant-negative p38 MAPK to inhibit cell invasion was evaluated. For these experiments, cells were cotransfected with constitutively active β-gal, along with either WT or dominant-negative p38 MAPK. In this manner, invading and noninvading cells, transfected with dominant-negative or WT p38 MAPK, could be identified (Fig. 5B). TGF-β increased cell invasion in p38 MAPK WT transfected cells (Fig. 5C). However, TGF-β–mediated increases in cell invasion were completely abrogated in dominant-negative p38 MAPK transfected cells, thus demonstrating that p38 MAPK is necessary for TGF-β–mediated increases in cell invasion. It can also be seen in Fig. 5C that SB203580, the chemical inhibitor of p38 MAPK, did not significantly decrease cell invasion compared with cells treated with inactive chemical control, SB202474, in cells transfected with dominant-negative p38 MAPK. This provides further evidence that SB203580 is not acting in a nonspecific fashion. Finally, in dominant-negative p38 MAPK transfected cells, genistein did not significantly decrease cell invasion, raising the notion that genistein may be inhibiting p38 MAPK related signaling.

**Genistein blocks activation of p38 mitogen-activated protein kinase.** A series of experiments was next conducted to investigate the relationship between genistein and p38 MAPK. First, both genistein and SB203580 were shown to inhibit cell invasion as well as TGF-β–mediated increases in cell invasion (Fig. 6A). More importantly, when cells were treated with both genistein and SB203580, no further decrease in cell invasion was observed (Fig. 6B). This finding suggested that both genistein and SB203580 were acting on the same signaling pathway. Next, cells were pretreated with genistein (or not), then treated with TGF-β (or not), and activation of p38 MAPK measured by Western blot (Fig. 6C). Treatment with TGF-β alone increased phosphorylation of p38 MAPK. This effect was blocked when cells were preincubated with TGF-β blocking antibody. More importantly, pretreatment of cells with genistein inhibited TGF-β–mediated activation of p38 MAPK. Under the current assay conditions, inhibition was complete for PC3-M cells, and partial for PC3 cells.

## Discussion

MMPs are important regulators of cell invasion and are necessary for metastasis (28). MMP-2 in particular has been shown to be up-regulated during prostate cancer progression (29, 30). We show here for the first time that genistein is a broadly active inhibitor of MMP-2 activity in human prostate. Because cancer chemopreventive agents target processes associated with early phases of carcinogenesis (9), it was important that we showed efficacy in early transformed cells. Note that in addition to the seven cell lines evaluated for genistein-mediated decreases in MMP, we also attempted to evaluate LNCaP and normal prostate epithelial cells. However, these cells died upon serum withdrawal, precluding zymographic analysis. It was also important that we showed that genistein inhibited TGF-β–mediated activation of MMP-2, and that activity was observed with low nanomolar concentrations of genistein. TGF-β is a physiologically relevant activator of MMP-2 and is recognized as an important mediator of cell invasion (24–26, 38). Dietary consumption of genistein, in the form of soy, is associated with blood concentrations of free genistein, which range between 3 and 15 nmol/L (11). These concentrations directly overlap with lower limits of efficacy observed in the current study for genistein-mediated decreases in invasion.

Operation through physiologically relevant regulatory pathways is a basic property of effective drugs. Efficacy at clinically relevant concentrations is a basic property of any drug, and is of particular...
importance for cancer chemopreventive drugs (9, 45). With typical anticancer cytotoxic agents, escalation to clinical toxicity allows optimization of dose and efficacy. In contrast, chemopreventive agents are administered at nontoxic doses, and efficacy is only apparent after extended periods of administration (3). Taken together, the current study shows that genistein, given in dietary amounts, is capable of inhibiting MMP-2 and cell invasion. If operating in humans, this mechanism would explain, at least in part, epidemiologic findings that show reduced rates of prostate cancer metastasis and death in soy consumers (1, 2, 12, 46).

It is important to note, however, that epidemiologic studies provide associations between clinical outcome and behavior, in this case diet, which span a lifetime. There are significant limitations to extrapolating lifetime dietary exposure, or exposure during critical stages in development, to therapeutic efficacy in high-risk cohorts, for example, older American men at high risk for prostate cancer. In this regard, it is important to note that the current study, which focuses on invasion, as well as prior studies by us focusing on genistein-mediated effects on adhesion (13), both show increased efficacy with increased genistein concentration. This finding raises the notion that it may be possible to "make up" for a lack of lifetime dietary exposure by administration of higher amounts of genistein. This concept is currently being tested by us in a clinical trial wherein men with localized prostate cancer are treated with genistein in amounts that are twice those associated with dietary consumption. End points are biomarkers related to adhesion and invasion.

Although we could have chosen to focus invasion studies on any of a relatively wide array of extracellular matrix proteins or complex protein mixtures, we felt gelatin would be optimal. Because gelatin was used in zymogram assays, this would provide a direct comparison of activities. More importantly, gelatin is denatured collagen, and collagen represents a major extracellular matrix protein for human prostate (47).

To our knowledge, we show for the first time in any cell type that p38 MAPK is necessary for TGF-β-mediated increases in MMP-2 activity as well as cell invasion. This represents an extension of our previous work in which we showed that p38 MAPK is necessary for TGF-β-mediated increases in prostate cell adhesion (27). It is not clear if the primary effect of genistein is on p38 MAPK or on another enzyme that in turn regulates p38 MAPK activation. More importantly, however, we have previously shown that in an older cohort of men with prostate cancer, oral genistein will alter levels of protein-tyrosine phosphorylation in peripheral blood mononuclear cells (5). Thus, genistein alters cell signaling pathways in a clinical setting, directly supporting current findings.

Figure 4. p38 MAPK regulates MMP-2 and cell invasion in human prostate. A, PC3 and PC3-M cells were treated for 24 hours with different concentrations of the p38 MAPK inhibitor, SB203580, or the inactive chemical analog, SB202474, and cell invasion measured. *, P < 0.05, values significantly below inactive chemical control (at corresponding concentrations); two-sided t test. Columns, mean of a single experiment (n = 4); bars, SE. Similar results seen with a replicate experiment, done at a different time (also n = 4). B, cells were pretreated (or not) for 1 hour with 10 μmol/L SB203580 or SB202474, before treatment with TGF-β X 24 hours, and MMP-2 activity measured by zymography. C, cells were pretreated (or not) with SB203580 for 1 hour, and then with TGF-β (or not) for an additional hour, and levels of phospho-p38 MAPK (pp38), total p38 MAPK, and β-tubulin measured in equal amounts of cell protein by Western blot. Individual bands were quantitated, and the level of pp38 to β-tubulin depicted graphically. With zymography and Western blot experiments, similar results were seen with multiple repeats done at separate times.
We go on to show for the first time that genistein is blocking TGF-β-mediated activation of p38 MAPK as well as dependent activation of MMP-2 and cell invasion. Concomitant phosphorylation on both threonine and tyrosine on p38 MAPK is associated with activation of its kinase activity (48). Because genistein has a tyrosine-like moiety, it is known to inhibit protein-tyrosine kinase activity (49), and has been shown by us to alter protein-tyrosine phosphorylation in humans after p.o. dosing with dietary amounts of genistein (5), it is possible that genistein may be inhibiting the enzyme(s) directly upstream of p38 MAPK. Additional investigations will be required, however, before this can be determined.

Because MMPs are so important in regulating metastatic behavior, they are an obvious target for drug development. However, when tested clinically, MMP inhibitors have been disappointing (50). Lack of clinical activity is believed to stem from the fact that inhibitors used in the clinic directly targeted the active site of MMPs, which do not vary significantly among the over 20 different isoforms. Thus, it has not been possible to achieve specificity. Genistein works by targeting an upstream activator of MMP-2, thus avoiding problems with active site inhibitors. Given that genistein seemed to preferentially inhibit MMP-2, as compared with MMP-9, the current study supports the notion of specificity. However, additional studies will be required in order to more fully characterize genistein’s spectrum of activity with respect to the different MMP isotypes.

In summary, we show for the first time that p38 MAPK is necessary for TGF-β-mediated activation of MMP-2 and cell invasion, and that genistein inhibits p38 MAPK activation. We also show for the first time that genistein is a broadly active inhibitor of

Figure 5. Dominant-negative p38 MAPK inhibits cell invasion. A, PC3 and PC3-M cells were transfected with wild-type (WT), dominant-negative (DN) p38 MAPK, or vector only control (VC). Phospho-p38 MAPK (pp38) and total p38 MAPK (p38) protein levels were then measured by Western blot. Individual bands were quantitated, and the level of pp38 to p38 depicted in the graph. Equal protein loading was confirmed by staining the blot with Ponceau S. B, photomicrograph of cells cotransfected with WT p38 MAPK and constitutively active β-gal vectors, which were allowed to invade through a gelatin-coated polycarbonate membrane that contained 8-μm pores. Transfected cells stain blue, nontransfected cells are pink, invading cells are in the focal plane, and noninvading cells are not in the focal plane. C, cells were transfected with either WT or dominant-negative p38 MAPK and treated with 2 ng/mL TGF-β, 50 μmol/L genistein, 10 μmol/L SB203580, or SB202474 (or not) and cell invasion measured. *, P < 0.05, values significantly different from WT cells not treated with TGF-β (two-sided t-test). NS, values (indicated by arrows) that do not differ significantly. Invasion experiments were done in replicates of four. Columns, mean of a single experiment, with multiple replicates done at separate times yielding similar results; bars, SE. All other experiments were repeated at least once at a separate time, with similar results.
MMP-2 in human prostate cancer and that it will inhibit MMP-2 activation and invasion at the low nanomolar concentrations attained in the blood with dietary soy consumption. If genistein were exerting this activity in humans, it would support a causal relationship to epidemiologic findings. This possibility is being investigated in a phase 2 clinical study we are currently conducting in men with prostate cancer.

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References

9. Crowell JA, Holmes CJ. Agent identification and


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