Mechanisms of action of the soy isoflavone genistein: emerging role for its effects via transforming growth factor β signaling pathways

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ABSTRACT The soy isoflavone genistein attenuates growth factor- and cytokine-stimulated proliferation of both normal and cancer cells. This article reviews our current understanding of the potential mechanisms of action of genistein. In membrane preparations from mammalian cells, genistein is a potent and specific inhibitor of tyrosine autophosphorylation of the epidermal growth factor (EGF) receptor. However, in several cell systems in which it inhibits growth, genistein does not alter tyrosine phosphorylation of the EGF receptor or other tyrosine kinase substrates thought to be involved in signal transduction pathways, suggesting that other mechanisms may be responsible for its action. Alternatives include inhibition of DNA topoisomerase II activity, regulation of cell cycle checkpoints, and antiangiogenic and antioxidant activity. Experiments in our laboratory suggest a new concept, that genistein may inhibit cell growth by modulating transforming growth factor (TGF) β1 signaling pathways. Such a link between genistein action and TGFβ1 function is supported by preliminary results of studies in patients with hereditary hemorrhagic telangiectasia (a genetic disorder involving mutations in proteins that regulate TGFβ receptor complex formation and signaling) in which several patients had dramatic attenuation of their symptoms after 1 wk of ingesting soy-based beverages. These preclinical studies in combination with our cell culture data suggest that the mechanism of genistein involves, if not requires, TGFβ1-signaling. Am J Clin Nutr 1998;68(suppl):1418S–25S.

KEY WORDS Soy, isoflavone, genistein, chemoprevention, estrogen, breast cancer, tyrosine kinase inhibitor, transforming growth factor β1, cell cycle checkpoints, growth factors, hemorrhagic telangiectasia

INTRODUCTION

The extensive research on genistein (4′,5,7-trihydroxyisoflavone) over the past 10 y has occurred because of 2 sets of important observations, one resulting from investigations on the factors responsible for the lower incidence of chronic diseases in Southeast Asia and the other from the search for pharmacologic agents that interfere with growth factor signaling pathways in cells. The incidence of and deaths from the hormone-dependent cancers of the breast and prostate are significantly lower in Asia than in Western populations (1–3). However, these rates increase when Asians emigrate to Western countries (4). A similar pattern has also been observed for cardiovascular disease. Interest has focused in recent years on the question of whether there are components in the Asian diet that protect against these chronic diseases. Because a striking dietary difference is that Asians consume 20–50 times more soy-based food per capita than Americans do, it has been suggested that components in soybeans might be chemoprotective (5). Indeed, rats maintained on a soy-based diet were significantly protected against mammary tumor induction by the carcinogens dimethylbenz[a]anthracene (DMBA) (6, 7) and N-methyl-N-nitrosourea (6, 8).

Soybeans and most soy products contain large amounts of the isoflavones genistein and daidzein (9–11). Because of the structural similarity of these isoflavones to naturally occurring estrogens (Figure 1), it was initially suggested that these isoflavones might prevent hormone-dependent breast and prostate cancers by virtue of their potential estrogen-antagonist activity (6, 12, 13). Purified genistein by itself significantly protects rats against DMBA-induced mammary cancer when it is administered to neonatal rats (14) or over a brief, 4-d prepubertal window of time (15). This anticancer effectiveness falls off, however, with increasing age of first exposure; rats given genistein daily from age 35 d (before administration of DMBA a 50 d) and throughout the remainder of a 6-mo experimental period had only a 27% reduction in tumor multiplicity and no reduction in incidence (16). In neonatal and prepubertal rats, genistein causes precocious cell differentiation in the breast (15, 17), making the organ resistant to the effects of carcinogens. In this respect genistein is similar to a combination of estrogen and progesterin that, when administered prepuber-
stimulated. In the presence of $17\beta$-estradiol, the growth of these cells was fully eradicated, suggesting that the apparent growth stimulation by estrogen was an artifact of the experimental system caused by the low-estrogen concentration of the media, not its antiestrogen, properties. Human breast cancer (MCF-7) cells, when placed ectopically in ovariectomized adult rats, grew at a faster rate in rats fed genistein-containing diets than in those fed control diets (19), yet another indication of the estrogenic activity of genistein.

Genistein cannot, therefore, be viewed simply as either an agonist or antagonist of estrogen. Understanding the biological effects of genistein in vivo also requires an appreciation of all of its mechanisms of action. This review focuses on the current state of knowledge of the mechanisms of action of genistein in biological systems. It extends previous reviews of this subject (20–22) and suggests a provocative new concept for the action of genistein as an enhancer of transforming growth factor (TFG) β action.

**FIGURE 1.** Similarities in the chemical structures of the isoflavone genistein, the physiologic estrogen $17\beta$-estradiol, and the estrogen antagonist tamoxifen. Et, ethyl group; Me, methyl group.

### GENISTEIN AS AN ESTROGEN

In cell culture, genistein inhibited proliferation of human breast and prostate cancer cells stimulated by epidermal growth factor (EGF) independently of whether the cells expressed estrogen (23, 24) or androgen receptors (25), respectively. When all the estrogens in cell culture media were removed, genistein, at concentrations < 1 μmol/L, acted as a growth stimulant to estrogen-dependent breast cancer cells (26, 27). However, the basal growth of these estrogen-dependent breast cancer cells was markedly slowed in estrogen-free media, suggesting that the apparent growth stimulation by estrogen was an artifact of the experimental system caused by the lowered growth rate of the control cells not exposed to estrogen. When $17\beta$-estradiol (0.3 nmol/L, a physiologic concentration) was added to the media, the growth of these cells was fully stimulated. In the presence of $17\beta$-estradiol, genistein induced no additional proliferation at any concentration examined (27, 28). Instead, at concentrations > 5 μmol/L, it caused a dose-dependent reduction in $17\beta$-estradiol–stimulated cell proliferation (24, 27). These data indicated that genistein may inhibit cell proliferation by mechanisms other than the classical estrogen-receptor-mediated pathway.

### IS GENISTEIN A TYROSINE KINASE INHIBITOR?

In 1987 genistein was identified as a protein-tyrosine kinase (PTK; EC 2.7.1.112) inhibitor because it inhibited the EGF receptor (EGF-R) PTK activity in vitro (29). This unleashed great interest in the compound, and there are now nearly 2000 published articles on the subject.

Genistein’s capacity to inhibit the mitogen-stimulated growth of mammalian cells in culture has been presumed by most investigators to be due to the inhibition of tyrosine kinase activities associated with critical growth factor receptors. However, experiments in our laboratories with human breast (24) and prostate cancer (25) cell lines showed that neither EGF-stimulated EGF-R tyrosine autophosphorylation nor EGF-stimulated tyrosine phosphorylation of intracellular target proteins such as mitogen-activated protein kinase and phospholipase C is affected at concentrations of genistein that inhibit proliferation of these cells. Furthermore, normal human mammary epithelial (HME) cells (which do not express estrogen receptor) stimulated by EGF are exquisitely sensitive to growth inhibition by genistein without any inhibition of EGF-R tyrosine autophosphorylation; the concentration at which cell growth is inhibited by 50% (IC50) is 1 μmol/L (30). These data strongly suggest that genistein affects these cells (and therefore other systems) by mechanisms other than inhibition of PTK activity. Nonetheless, individual polypeptides have been identified that have altered concentrations of tyrosine phosphorylation after exposure to genistein (31). Given the totality of evidence, however, these altered tyrosine phosphorylations are probably not due to genistein’s direct effects on a tyrosine kinase, but rather to indirect effects mediated by other mechanisms.

### DOES GENISTEIN UNCOUPLE KINASE-INDEPENDENT SIGNALING OF EGF?

Wright et al (32) found that a subset of protein-tyrosine phosphorylation could still be induced by EGF-stimulation of tyrosine-kinase-defective EGF-Rs in cultured cells. This suggests that there are critical tyrosine kinase events downstream of EGF-R and regulated by EGF that are independent of the PTK capacity of the EGF-R itself. Is genistein uncoupling 2 subsets of activities of EGF-R? In a glioblastoma model in vitro, both invasiveness and proliferation of glioblastoma cells were dependent on EGF, but the invasiveness was blocked by genistein at concentrations that did not affect proliferation or EGF-R autophosphorylation (33). These data provided compelling evidence of 2 subsets of activities modulated by EGF-R, only one of which is affected by genistein. In this context, experiments with estrogen receptor knockout mice showed that although normal amounts of EGF-R were expressed in the uterus and some variables downstream from EGF-R remained normally modulated, the estrogen-like effects of EGF required expression of the estrogen receptor. This suggests that there is cross-talk between the EGF-R and estrogen receptor-signaling pathways (34) as well as subsets of events catalyzed by EGF-R.
ALTERNATIVE MECHANISMS OF ACTION OF GENISTEIN

In addition to its PTK-inhibitory activity, genistein has also been shown to inhibit DNA topoisomerase II, cell cycle progression, angiogenesis, and oxidation reactions (20–22). Most of these activities, however, have been shown only in vitro and at genistein concentrations in excess of those attainable physiologically in humans (10 μmol/L) (35). Thus, the relevance of these varied activities of genistein in vivo remains to be ascertained.

At physiologically attainable concentrations, however, Peterson et al (30) showed (as noted above) that genistein inhibits the EGF-stimulated proliferation of normal HME cells in culture. Because this effect was not correlated with inhibition of EGF-R autophosphorylation, additional experiments were carried out to determine whether other as yet unidentified mechanisms might underlie the inhibitory action of genistein on the proliferation of these cells.

INHIBITION OF CELL PROLIFERATION BY GENISTEIN INVOLVES TGFβ1 SIGNALING

Genistein’s effects on cells, including inhibition of proliferation, induction of differentiation (20, 21, 36, 37), apoptosis (38), and arrest of cells at cell cycle checkpoints (39, 40) are reminiscent of those of TGFβ1. This peptide growth factor was identified as a major factor that regulates eu karyotic cell proliferation by attenuating passage through cell cycle checkpoints (41, 42).

TGFβ1, a member of the TGF superfamily of growth factors (43), is a homodimeric protein of 25000 Da that binds to plasma membrane–associated receptors that then phosphorylate intracellular protein substrates on serine and threonine residues (44). TGFβ1 is highly pleiotropic; it was shown to both stimulate and inhibit cell proliferation in a cell-type-specific manner (41). TGFβ1 attenuates passage through cell cycle checkpoints, predominantly G1/S, via transcriptional regulation of selected proteins, which in turn have roles in preventing the cells from going through the G1/S transition (42, 44). The dominant events involved in the TGFβ1-mediated inhibition of cell proliferation are shown schematically in Figure 2.

To determine whether the actions of genistein involved or affected TGFβ1 activity, antibodies to TGFβ1 were added to the culture medium of HME cells, which blocked the inhibition of genistein on EGF-stimulated cell growth (45). Moreover, in a sandwich, enzyme-linked immunosorbent assay (46) with monospecific TGFβ1 antibodies, HME cells incubated with genistein and EGF secreted nearly a 5-fold higher amount of TGFβ1 than did cells not incubated with genistein (45). Thus, exposure to genistein caused either synthesis and secretion of TGFβ1 by the HME cells or secretion of a preexisting intracellular pool of TGFβ1. Although Peterson et al (47) showed previously that genistein is taken up and concentrated 20-fold above the medium concentration within HME cells, the more recent results with the TGFβ1 antibodies indicate that the growth-inhibitory effect of genistein requires molecular events that occur outside the cell and that involve TGFβ1.

A full discussion of the TGFβ1 signaling pathway is beyond the scope of this review. However, the somewhat surprising evidence of a direct link between genistein and TGFβ1 warrants discussion both of other recently described experimental paradigms in which genistein action has been shown and of the implications for interactions between known signal transduction pathways.

ATTENUATION OF CHRONIC BLEEDING BY CONSUMPTION OF A SOY BEVERAGE: AN INDICATION OF A LINK BETWEEN GENISTEIN AND TGFβ1

Hereditary hemorrhagic telangiectasia (HHT) is a genetic disorder characterized by an inappropriate proliferation of blood vessels at the capillary arteriovenous junction that results in chronic bleeding in the nose and in the gastrointestinal tract and migraine headaches (48). Because genistein had been shown to inhibit angiogenesis (49, 50), experiments were conducted to determine whether a soy-based beverage could be therapeutic for patients with HHT. After 2 wk of ingesting a soy beverage twice daily, 3 of 5 patients experienced complete remission of symptoms (51).

The genes in which HHT mutations have been mapped encode the following plasma membrane–associated proteins that are involved in TGFβ signaling: endoglin (a protein expressed in endothelial cells) (52), TGFβ receptor type 2 (53), and activin-like kinase (54). All identified endoglin mutations encode truncated proteins that lack the transmembrane domain (55). Thus, the protein may no longer be anchored in the membrane but may be free in solution, where it can interact with TGFβ1 and prevent it from binding to its membrane-associated receptor.

The specific HHT genetic mutations of the patients involved in the study by Korzenik et al (51) have not been identified, but on the basis of the results of our study indicating that genistein mimics and enhances TGFβ1 signaling in cultured cells (45), we predict that the HHT patients who responded to the soy beverage have mutations that inhibit the normal binding of TGFβ1 to the
plasma membrane. However, it remains to be seen if pure genistein is as efficacious in attenuating HHT as was the unfraccionated soy-based beverage.

**GENISTEIN AND TGFβ1 ACTION IN OTHER BIOLOGICAL SYSTEMS**

**Tamoxifen- and vitamin E–regulated cell growth**

In light of the structural similarity of genistein to 17β-estradiol, it is interesting that the growth inhibitory effects of both the estrogen antagonist tamoxifen (56) and vitamin E (57) were nearly identical to those of genistein involving TGFβ1 on the growth of human breast cancer MCF-7 cells. For both compounds, the inhibition of cell proliferation was correlated with increased amounts of TGFβ1 in the culture medium and was blocked by antibodies against TGFβ1 added exogenously to the culture medium.

**Osteoporosis**

Bone loss after an ovariectomy is linked to estrogen deprivation because the reduction in bone is prevented by the administration of the hormone. Recent experiments showed that a genistein-containing, soy-based diet ameliorated the loss of bone density in ovariectomized rats (58); moreover, cell culture experiments with osteoclasts indicated that pure genistein inhibits osteoclast function (59). Accordingly, the attenuation of bone loss by soy or genistein could be considered an estrogenic mode of action for genistein. However, the regulation of bone growth and repair is complex, involving signaling events in both osteoblasts and osteoclasts (60); there are multiple possibilities for genistein’s effects. However, both estrogen and tamoxifen stimulate the production of TGFβ in osteoclasts and precipitate apoptosis in these cells (61); consistent with this, ovariectomy down-regulates TGFβ1-transcription in bone (62). Thus, in bone as in HME cells, enhancement of TGFβ1 activity could be the basis for the action of genistein.

Cell culture experiments have shown that TGFβ1 reduces intracellular concentrations of PTK pp60src (63), which is required by osteoclasts for bone resorption in vivo (64). In vitro experiments with osteoclast membranes enriched in PTK pp60src showed that genistein inhibits their acid transport (65); thus, inhibition of PTK pp60src in osteoclasts could be a mechanism for the prevention of bone loss by genistein and soy. Although the role of PTK pp60src in the TGFβ1-signaling cascade is not completely understood, activation of mitogenic signals was shown to occur downstream of tyrosine phosphorylation of protein substrates containing src homology domains (66); the latter apparently then allows the association of src homology domains with growth factor receptor–bound protein 2 (67). Although this link between TGFβ1 action and PTK activity suggests that in osteoclasts genistein could enhance TGFβ1 signaling by directly inhibiting pp60src, further experiments are required to determine whether this occurs in vivo.

**ANGIOGENESIS**

The discovery of mutated genes in HHT patients suggests that TGFβ signaling is an important feature in the regulation of angiogenesis in HHT as well as other pathologies involving angiogenesis. Treatment of HHT patients with 17β-estradiol has only been moderately successful (48), implying that there is an unidentified, underlying mechanism for its effect. The naturally occurring estrogen metabolite 2-methoxyestradiol has antiangiogenic activity on breast cancer cells (68), whereas the parent molecule 17β-estradiol (which markedly reduces TGFβ expression in many cells outside of bone) enhances angiogenesis in human endometrium (69). However, the 2-methoxyestradiol effect was observed only in the presence of fibroblast growth factor, indicating that it had an indirect effect on a signaling pathway mediated by the growth factor (68).

In other biological systems, TGFβ1 enhances angiogenesis. This may occur indirectly, such as by modulation of vascular endothelial growth factor gene expression (70). In these other systems, as opposed to the bone modeling system, both estrogen and TGFβ1 enhance angiogenesis rather than having opposing effects. Thus, it is not clear whether the effects of genistein-containing soy on HHT patients are due to the action of a genistein metabolite similar to 2-methoxyestradiol on the aberrant regulation of angiogenesis in the system or to an estrogenic effect of genistein that modulates events downstream in the TGFβ1-signaling pathway. Note that Peterson et al (47, 71) recently detected a hydroxylated and methylated metabolite of genistein that is formed in the breast cancer cells most sensitive to growth inhibition by genistein.

**Atherosclerosis and cardiovascular disease**

Antiatherogenic effects of dietary soy protein have been recognized for several decades (72) but only recently have been brought to the attention of the medical community (73). In 1984, the concept that the phytoestrogens in soy are responsible for the cardioprotective effects of soy emerged (12).

Recent studies in rhesus monkeys showed that soy-protein preparations containing isoflavones significantly improved cardiovascular risk factors, specifically plasma lipid and lipoprotein concentrations, but soy-protein preparations without isoflavones did not (74). However, experiments with genistein and other soy isoflavones verifying that they are responsible for the antiatherogenic effect of soy have yet to be reported.

Nonetheless, evidence suggesting that genistein has antiatherogenic properties has come from the observation that female macaques fed soy with its isoflavones had a vasodilative response to acetylcholine in their carotid arteries (75). Removal of the isoflavones (by washing the soy-protein preparation with alcohol) caused a vasoconstrictive response, which also occurred in animals consuming the control (casein) diet. When genistein was infused into animals consuming the alcohol-extracted soy protein, vasodilation occurred in response to acetylcholine, suggesting that genistein is the compound responsible for the cardioprotective effects of soy (75). These data are consistent again with an estrogen-like mechanism of action for genistein (76). Moreover, there is greater promise in these findings than in conventional estrogens in the treatment and prevention of cardiovascular disease because, unlike the true estrogens, the soy phytoestrogens (fed in the diet) did not adversely affect the reproductive system of the monkeys (74).

Although the mechanisms that underlie the efficacy of soy and genistein in altering cardiovascular risk factors are unknown, it is noteworthy that the serum concentrations of active TGFβ in patients with advanced atherosclerosis is one-fifth that of patients with normal coronary arteries (77). Moreover, in mice fed a high-fat diet, administration of tamoxifen at doses similar
to those used in breast cancer treatment substantially reduced aortic lipid lesions and enhanced aortic concentrations of TGFβ (78). These data are consistent with the ability of tamoxifen to induce TGFβ1 in MCF-7 cells (79, 80) and in breast cancer in vivo (81). These data strongly suggest that the pathology in atherosclerosis arises from abnormally low TGFβ1 concentrations in the affected vasculature and that the cardioprotection afforded by genistein-containing soy in the studies by Anthony et al (74, 75) involves an induction of TGFβ1 similar to that observed in the experiments with HME cells discussed earlier (45).

**GENISTEIN: ANTIOXIDANT OR TRANSCRIPTION FACTOR?**

Gut ileitis, or inflammatory bowel disease, is a chronic inflammatory condition thought to result from up-regulation of the gene for inducible nitric oxide synthase (82). As a follow-up to results showing an antioxidant capacity of genistein (83), experiments testing the antioxidant efficacy of genistein in a guinea pig model system of inflammatory bowel disease showed that genistein ameliorated symptoms of the disorder, concomitant with an attenuation of inducible nitric oxide synthase expression (84). Because inducible nitric oxide synthase gene transcription was shown to be regulated by TGFβ1 (85), these studies again link genistein action with TGFβ1-signaling pathways.

Although preliminary studies have indicated an antioxidant capacity of genistein in inhibiting lipid oxidation in vitro (86, 87), much of the data reviewed here indicate that genistein’s pleiotropic effects in cells go beyond directly chemical ones. In its enhancement of TGFβ1 activity, genistein appears to have effects at the level of gene transcription. For example, transcriptional regulation of TGFβ genes by the environmental toxin 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was attenuated by genistein (88). Gel mobility shift assays did not reveal a quantitative effect on transcription factor DNA complex formation, suggesting the possibility of a more complex situation involving tyrosine phosphorylation as a potential modulator of transcription factor DNA interaction (86). Recent studies on the pathogenesis of cystic fibrosis lung disease in human bronchial epithelial cells showed that increased transcriptional activation of the mucin gene (MUC2) by *Pseudomonas aeruginosa* lipopolysaccharide could be blocked by genistein and the tyrosine kinase inhibitor tyrphostin AG126 (89). Because tyrosine phosphorylation of p42MAPK is activated by lipopolysaccharide (90), the effects of the tyrosine kinase inhibitors implicate a role for tyrosine phosphorylation of p42MAPK in the transcriptional regulation of MUC2 (89). It remains to be determined, however, whether p42MAPK is aberrantly phosphorylated in cystic fibrosis and whether this is ameliorated by genistein. In another example of an effect of genistein on transcription, activation of double-stranded-RNA–activated factors that bind to the response elements in interferon-stimulated genes is inhibited by genistein as indicated by gel-mobility assays (91).

The idea of a role for genistein in regulation of transcription is not new. In the soybean plant itself, genistein that is secreted through the roots into the soil is a chemoattractant for *Bradyrhizobium* sp, in which nodulation genes are up-regulated in response to the genistein, resulting in the production of lipochitin polysaccharides. These in turn induce root nodules on the soy plant that house the bacteria. These root nodules convert atmospheric nitrogen to ammonia, which is taken up by the soybean plant (92), thus defining a symbiotic relation. In this plant-microbial environment, uptake of genistein results in transcriptional up-regulation. An elucidation of the molecular events involved in this system might contribute to our understanding of the mechanism of action of this important soy isoflavone in mammalian cells.

**FUTURE DIRECTION FOR RESEARCH INTO THE MECHANISMS OF ACTION OF GENISTEIN**

The estrogenicity of genistein in several models has caused concern because of the potential toxicities of estrogens. However, the work with the estrogen receptor knockout mouse model showed that estrogens are essential for health, development, and behavior in both male and female mice (93–95). Additionally, the health characteristics of human populations exposed to dietary genistein are the opposite of what would occur if health risks were associated with estrogen.

Women have an advantage over men with respect to cardiovascular disease risk (96). In postmenopausal women, however, this advantage disappears unless they receive hormone replacement therapy (97). Moreover, estrogen therapy has been found to delay the onset of Alzheimer disease in women (98). Thus, there is impetus to identify or develop estrogen-like compounds that provide beneficial effects without toxicity.

To better understand the physiologic action of genistein and other isoflavones, study of estrogen receptor–mediated processes and signal-transduction-pathway cross-talk at the molecular level is required. The recent discovery of a second type of estrogen receptor (the β estrogen receptor) (99) suggests new pathways in which estrogen-like compounds such as genistein may have differential tissue-specific effects. The β estrogen receptor has a different ligand specificity from the classical (α) estrogen receptor (100) and a different distribution, not only from tissue to tissue but also within a tissue, ie, the hypothalamus (101). Finally, it should be realized that many of the estrogen receptors expressed in human breast tumors contain mutated sequences, some of which lead to estrogen-induced inhibition rather than stimulation of cell growth (102). The study of genistein and related phytoestrogens in the context of its potential estrogenicity, therefore, must address these new issues.

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