Actions of the soy phytoestrogen genistein in models of human chronic disease: potential involvement of transforming growth factor β

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Abstract

The structural similarity, but non-identity, between 17β-oestradiol and the soy phytoestrogen genistein suggests that the two compounds will have actions that may be identical in some target biological systems, but different in others. Epidermal growth factor (EGF)-stimulated proliferation of human mammary epithelial cells (that do not express the oestrogen receptor) was significantly suppressed at genistein concentrations (5–10 μM) that are attainable physiologically. Others have shown previously that transforming growth factor β (TGFβ) has similar growth-inhibitory effects on human cells. Analysis of the conditioned medium of human mammary epithelial cells exposed to genistein plus EGF showed increased levels of TGFβ relative to those in the medium of cells exposed to EGF or genistein alone. Related experiments in a primate model of menopause demonstrated that ingestion of soy containing isoflavones was correlated with the suppression of neurodegeneration-relevant phosphorylation of the microtubule-associated protein tau, while intake of Premarin (a hormone replacement therapy that is commonly prescribed for women) was not correlated. The results discussed here indicate that genistein, and probably other related phytoestrogens, have pleiotropic actions, some of which may involve TGFβ activity.

Introduction

Women living in South-East Asian countries have a lowered incidence of breast cancer compared with those in Western countries [1–3]. Since one of the striking differences between these two populations is diet, much experimentation has addressed the question of whether dietary components that are either enriched in the Asian diet, or missing from the Western diet, could lower the risk of breast cancer. Soy-based foods are an obvious difference between the South-East Asian and the Western diets; people in South-East Asian countries consume probably 10–50-fold more soy protein than those in Western countries [4,5]. Since the initial finding that ingested plant isoflavones might have anti-oestrogenic activity in animals [6], experiments have shown that the isoflavones, the expression of which is essentially restricted to the soybean [7], bind to both oestrogen receptors (ERs), ERα and ERβ [8–10], suggesting that these isoflavones could affect receptor-mediated actions of oestrogen. More recently, isoflavones were shown to inhibit the proliferation of mammalian cells, including human mammary epithelial (HME) cells, suggesting inhibition of cell proliferation as the basis of a
potential chemopreventive action of soy against breast cancer [11,12].

To date, the mechanisms of action of individual isoflavones are poorly understood, in part because of the difficulty intrinsic to the analysis of changes in factors, often expressed at a low copy number, that comprise signalling cascades, but in part because of investigators’ expectations based on existing literature. Since 1987, when Akiyama et al. [14] first demonstrated that genistein has the ability to inhibit epidermal growth factor (EGF) protein tyrosine kinase (PTK) activity in vitro, this suppression of PTK activity has been presumed to be the primary action of genistein where it has biological effects.

The transforming growth factor β (TGFβ) superfamily of ligands comprises a complex family of peptides that have pleiotropic regulatory actions in a number of cell types and organs, including the vasculature, bone, the cardiovascular system and the brain (reviewed in [15–17]). A common effect of TGFβ is to suppress the proliferation of cultured cells, probably by slowing progression through cell cycle checkpoints [17,18]. However, the role of TGFβ in malignancies is not well understood, because there is disagreement as to whether its levels are enhanced or reduced in malignant tissues [17,19]. Furthermore, while oestrogen regulation of osteoblast viability is thought to involve secretion of TGFβ, which then may suppress osteoclast proliferation [20], the relationship between oestrogen-like actions and TGFβ is not fully understood in bone, or in other cell systems that are nonetheless targets of oestrogen action, such as the mammary glands [21–26,60].

Following up initial observations that dietary soy provided significant protection against carcinogen-induced mammary cancer [27], Lamartiniere and Fritz [28] showed that genistein alone in the diet can provide similar protection, even when administered over a narrow window of time prior to the administration of carcinogen. Such experiments provided a strong rationale for consideration of genistein as a chemopreventive agent. However, cell culture experiments demonstrated that its mechanisms involved neither ERs nor significant PTK inhibition [11,29]. In view of the similarity of their effects on cell proliferation, it was not inconceivable that TGFβ might be involved in the mechanism of action of genistein. Thus experiments were carried out on cultured HME cells to determine whether TGFβ synthesis and/or secretion could be considered to be part of the mechanism of action of genistein.

The microtubule-associated protein tau [30] is hyperphosphorylated in neurofibrillary tangles, aberrant structures that are a hallmark of Alzheimer’s disease (AD) [31,32] and at least one other human dementia, frontotemporal dementia with Parkinsonism on chromosome 17 (FTDP-17) [33]. Epidemiological data indicated a strong correlation between oestrogen replacement therapy and a lowered incidence of AD among postmenopausal women [34]. Thus there was the rationale to examine whether there is a relationship between oestrogen replacement therapy, soy isoflavone intake and AD-relevant phosphorylation of tau.

Experimental
HME cell culture experiments
The procedures for maintaining and manipulating the HME and MCF-7 cells for these experiments have been described in detail elsewhere [11]. In brief, HME cells (Clonetics Corp.) were plated and grown in 96-well plates, allowed to become quiescent overnight, and then incubated over a 3-day period with EGF. Cell proliferation was quantified with the sulphorhodamine assay at the end of the experiment [35]. To measure the responses to genistein, this agent was added to the medium at appropriate concentrations for 15 min before the incubation with EGF. For analysis of TGFβ secretion, the cells were grown in 10 cm culture dishes under similar conditions, and the medium was collected at the end of the experiment. Quantification of TGFβ was accomplished using the sandwich ELISA described by Danielpour [36], using antibodies monospecific for TGFβ.

Analysis of tau phosphorylation in primate brains
Aged Macaca fascicularis (cynomolgus monkey) females were ovariectomized and segregated into three dietary groups. One group received intact soy protein (containing the isoflavones), the second group received soy protein that had been depleted of 90% of the isoflavones, and a third received the same depleted soy as the second group and Premarin (conjugated equine oestrogens), a common hormone replacement therapy used in postmenopausal women. At 36 months the animals were killed; the brains were dissected out, sliced into 6 mm slices, and stored at −80 °C.
Pieces of slices of frontal cortex were homogenized and total protein was quantified. Homogenates were analysed for their content of tau phospho-epitopes by Western blot analysis, as previously described [37,38], with monoclonal antibodies that recognize tau phospho-epitopes [30,39] that are stabilized in AD [40–42]. The Western blots were quantified for tau antibody reactivity by routine densitometry and image analysis [29,43].

Results

Elevated TGFβ levels in HME cell conditioned medium following exposure to genistein

Previous experiments showed that growth factor-stimulated proliferation of HME cells was significantly inhibited by genistein at concentrations where EGF receptor tyrosine phosphorylation was not affected [29]. Quantification by sandwich ELISA with specific antibodies revealed that TGFβ levels were elevated nearly 5-fold in the conditioned medium of cells exposed to genistein before the addition of EGF to the medium (Figure 1).

Effects of soy isoflavones, compared with those of oestrogen, on tau phosphorylation in postmenopausal primate brain

The correlation between postmenopausal oestrogen loss and an increased risk of AD provided the rationale for analysing brain tissue from ovarioctomized primates for potential benefits of extended exposure to dietary isoflavones. The goal was to assess the efficacy of dietary soy isoflavones in attenuating postmenopausal neurodegeneration relative to that achievable with Premarin, a standard hormone replacement therapy. The marker chosen for initial analysis of the archived tissues was the selective phosphorylation of tau that has been shown to be enhanced in human neurodegeneration. Although hyperphosphorylation of tau at the sites examined in our initial analysis was demonstrated to be a marker for AD neurofibrillary tangles, and was induced in cultured neuron-like cells exposed to amyloidogenic β-amyloid fragment [44], modulation of this phosphorylation in primate brain in vivo has not been demonstrated. The initial analysis of the brain samples revealed that ingestion of soy protein that contained the full complement of isoflavones attenuated the phosphorylation of tau [45] at sites that were recognized by antibodies whose specificity for these epitopes has been documented previously [30,39,40,42].

Discussion

The chemopreventive actions of the soy isoflavone genistein in mammary gland may involve TGFβ signalling

Genistein binds to ERβ with essentially the same affinity as 17β-oestradiol [8–10], suggesting that this soy isoflavone may modulate ER-mediated events. Moreover, the in vitro ability of genistein to inhibit PTK activity provided the rationale for including this biological activity in the repertoire of probable mechanisms that underlie the actions of genistein. The comparison of genistein with 17β-oestradiol and with the amino acid tyrosine (Figure 2) shows clearly the structural basis for the potential for genistein to modulate either oestrogen action or PTK activity.

Paradoxically, previous experiments by Peterson and Barnes [29] demonstrated that geni-
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steroid clearly had effects on cells that did not express detectable ERs and that did not respond to oestrogen. Moreover, while genistein clearly inhibited the EGF-stimulated proliferation of HME cells, no quantitative changes in EGF receptor tyrosine phosphorylation were detected by Western blotting [29]. These data suggested that other, so far unexamined, activities or cascades of events might explain the actions of genistein. Since genistein and TGF\(\beta\) have similar inhibitory effects on cell growth, it was reasonable to speculate that the actions of one might involve or even require the other. The results described here demonstrate that, indeed, the actions of genistein on human cells probably involve TGF\(\beta\). Follow-up experiments demonstrated, moreover, that the TGF\(\beta\) in the conditioned medium induced by genistein was biologically active (H. Kim, J. Xu, Y. Su, G. Peterson, J. Murphy-Ullrich and S. Barnes, unpublished work). Others have shown that the inhibition of MCF-7 cell proliferation by the anti-oestrogen tamoxifen was correlated with increased TGF\(\beta\) mRNA levels in the cells [46], but an increase in the expression of TGF\(\beta\) was not demonstrated. The results described here (H. Kim, J. Xu, Y. Su, G. Peterson, J. Murphy-Ullrich and S. Barnes, unpublished work) thus extend existing data regarding actions of oestrogens and anti-oestrogens on human cells, and provide a strong rationale for further analysis of the link between genistein and TGF\(\beta\). It remains to be determined, for example, to what extent the Smad proteins, which have been implicated as mediators and regulators of TGF\(\beta\) signalling [17,18], are involved in the actions of genistein.

**Clinical data provide a rationale for a role for TGF\(\beta\) in the actions of genistein**

Because of the indication of beneficial actions of hormone therapy in patients with hereditary haemorrhagic telangiactasia [47], a pre-clinical trial with nine patients was carried out to explore the potential efficacy of dietary soy [48]. This condition, a genetic disorder involving several loci, is usually manifested by severe nosebleeds and internal bleeding, and is only alleviated in some patients by weekly blood transfusions [49]. Not all patients responded, but, in five, nosebleeds were dramatically attenuated, and in some cases abolished, within 1 week of beginning ingestion of the soy protein twice a day (40 g total) [48]. While genetic analysis of this condition is ongoing, several loci have been mapped to this hereditary disorder. It is noteworthy that all the loci that have been mapped thus far encode proteins that are involved in TGF\(\beta\) signalling [50–53]. These data provide compelling support for our hypothesis that the actions of the principal isoflavone in soy, genistein, involve TGF\(\beta\) signalling [54].

**Actions of soy isoflavones in the brain**

The rationale for examining the potential similarity of the actions of the soy isoflavone genistein and of oestrogen in the brain was strong, based on both epidemiological data and experimental evidence. However, an animal model of postmenopausal brain had not been examined previously for the actions of either oestradiol or phytoestrogens on the phosphorylation of tau at various sites, which constitute established markers of AD. The experiments described here [45], while preliminary, nevertheless provide compelling evidence that support a novel twist in our collective thinking of phytoestrogens and oestrogens, i.e. that both categories may have ‘beneficial’ and similar end results in target systems, but through different mechanisms. Hyperphosphorylation of tau at the sites examined in our initial analysis was not.

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**Figure 2**

**Structural comparison of the phytoestrogen genistein (B) with 17\(\beta\)-oestradiol (A) and with the amino acid tyrosine (C)**

The structures of genistein (4',5,7-trihydroxygenistein), 17\(\beta\)-oestradiol and tyrosine are shown, so that the similarities and differences among the three can be seen.
demonstrated in situ in AD neurofibrillary tangles [30,42]. Tau phosphorylation at similar sites was induced in neuron-like cells in culture exposed to amyloidogenic β-amyloid fragment [44]. Finally, oestrogen suppressed β-amyloid formation in cultured neurons [55]. These data, taken together, provide a rationale for the hypothesis that dietary soy containing isoflavones could attenuate AD-relevant tau phosphorylation [45]. The results described here [45] demonstrate that ingestion of soy protein containing isoflavones at dietary levels can inhibit the generation of phospho-epitopes on tau that are markers of AD pathology. Future experiments must follow up these initial data, and determine whether this attenuation of AD-linked phosphorylation is correlated with functional differences in the tau protein, and, even more importantly, with beneficial changes in cognitive function.

It is critical to point out here, as a conclusion to this section, that Pan and colleagues [56,57] have already demonstrated in an animal model of postmenopausal cognitive impairment that dietary isoflavones, given in a non-soy protein background, protected rats from ovariectomy-induced decreases in brain factors important for neuronal viability [56], as well as from ovariectomy-induced cognitive impairment [57]. These data, together with our biochemical data, provide strong support for dietary soy isoflavones having beneficial actions in mammalian brain, particularly postmenopaually. Given the lack of undesirable side effects, the evidence suggests that dietary soy isoflavones might be an attractive alternative to physiological oestrogen for postmenopausal women [58]. The challenge will be to determine the optimal timing of intake, and its duration.

Concluding remarks
Neither the data described here, nor that of others, provide simple conclusions regarding mechanisms of action of the soy phytoestrogen genistein (see [59] for a review). It may be that this phytoestrogen, precisely because of the ambiguity of its structure, can be ‘beneficial’ in more than one way, depending on its target and its concentration. Future experiments that address the question of the mechanism of action of genistein, or any other oestrogen-like compound, must carry out the experiment so as to examine all possibilities of effects, taking into account the complexity of the cellular or tissue milieu. For example, do the actions of dietary soy isoflavones in the brain also

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**Figure 3**

Potential sites of action of genistein in target biological systems that are modulated by oestrogens and/or ERs

Shown is a schematic representation of the ER-mediated pathways that exist in different target systems (not necessarily all in one cell type). E2, 17β-oestradiol; ERE, oestrogen response element. The arrowheads indicate molecular relationships or pathways that may be modulated by genistein, as suggested by the data described in this paper. The two arrowheads pointing to the pathway from 17β-oestradiol to the ER indicates that genistein can either enhance or antagonize 17β-oestradiol–ER interactions, depending on the target tissue and whether ERα or ERβ is involved. The black boxes beyond TGFβ and ERE represent areas that future experimentation must address, to better understand potentially critical actions in signalling pathways downstream of gene expression modulated by ER, that genistein, and other phytooestrogens could affect.
involve TGFβ, as they appear to in other target cells? Finally, the mechanisms must be dissected into those that comprise the biological causative action and those that are ‘real’ but are not essential to the cause of the biological effect of interest. Figure 3 shows schematically our current understanding of genistein action, as it impacts on oestrogen-mediated actions and on TGFβ expression.

Note that a potential inhibition by genistein of PTK activity downstream of ER activation has been indicated in Figure 3. This could result from genistein affecting either ER activity directly, or a particular PTK directly. The net effect of either action would appear to be attenuation of PTK activity, but it is important to keep in mind that this could just as easily arise indirectly from effects on events upstream of the PTK. This distinction could be important in terms of developing preventive strategies, as well as for establishing a clear understanding of the mechanisms involved.

In conclusion, it is hoped that the data and thoughts described in this paper will lay the foundation for further experimentation on soy and other phytoestrogens that will address the many questions that remain unanswered.

References
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Structural and functional alterations in the androgen receptor in spinal bulbar muscular atrophy

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Abstract

The androgen receptor is a member of the nuclear receptor superfamily, and regulates gene expression in response to the steroid hormones testosterone and dihydrotestosterone. Mutations in the receptor have been correlated with a diverse range of clinical conditions, including androgen insensitivity, prostate cancer and spinal bulbar muscular atrophy, a neuromuscular degenerative condition. The latter is caused by expansion of a polyglutamine repeat within the N-terminal domain of the receptor. Thus the androgen receptor provides a unique opportunity to investigate the functional significance of poly(amino acid) repeats in normal and disease states.

Introduction

"O what a tangled web we weave"  
Sir Walter Scott

The androgen receptor (AR) is thought to be the sole mediator of the actions of the steroid hormones testosterone and dihydrotestosterone in male reproductive tissues, such as the testes, epididymis and prostate (reviewed in [1–3]). Mutations in the AR can lead to a disruption of male development (androgen insensitivity) and to a neuromuscular degenerative disorder termed spinal bulbar muscular atrophy (SBMA; also called Kennedy’s disease) (Figure 1A) [2,3]. Mutations have also been identified in patients with prostate cancer, both before and after hormone therapy, which may influence the effectivenes of androgen ablation therapy and play a role in the progression to hormone-refractory disease ([4,5], and references therein).

The AR is a member of the nuclear receptor superfamily, and is organized into discrete struc-