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Review

Phytoestrogens in Cancer Prevention and Therapy – Mechanisms of their Biological Activity

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Abstract. *Numerous epidemiological studies suggest that diets rich in phytoestrogens (PE), particularly soy and unrefined grain products, may be associated with low risk of some cancers, especially steroid hormone-dependent, e.g. breast and prostate cancers. Epidemiological, in vitro, animal and human studies have investigated the mechanisms involved in PE biological actions, including steroid hormone activity, effects on cell growth, antioxidant activities, inhibition of chemical carcinogenesis and influences on modulators of cancer risk. The question of whether PE may be used as an anticancer therapeutic and/or chemopreventive agents remains unanswered. Clearly, much more information is required, especially concerning the safety of their use. It seems extremely difficult to predict the effects of various PE mixtures present in different human diets. Long-term studies (in vitro, animal, clinical and epidemiological) with well standardized PE preparations are necessary to assess the potential beneficial and adverse effects. With our current state of knowledge, we cannot conclude whether consumption of soy, SIF (soy isoflavones)-supplemented food or the use of particular isoflavones as therapeutics will have positive, null or even adverse effects on cancer (particularly, steroid hormone-dependent) risk and treatment. A brief review of the effects (preventive, antitumor as well as carcinogenic and tumor-stimulating) of PE on various tumor types is presented.*

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Phytoestrogens and Breast Cancer

Epidemiological data. Numerous epidemiological studies suggest that diets rich in phytoestrogens (PE), particularly soy and unrefined grain products, may be associated with low risk of some cancers, especially steroid hormone-dependent, *e.g.* breast and prostate cancers (1, 2).

Nevertheless, the association between soy food intake and breast cancer risk is controversial. Although isoflavones, such as those found in soy, have been shown to inhibit breast cancer in laboratory studies, correlations between the consumption of isoflavone-containing foods and breast cancer risk have been inconsistent in epidemiological studies. Several studies have indicated that countries with the highest PE consumption have the lowest rates of breast cancer, but other epidemiological studies suggest the lack of a causative relationship. No studies, however, have found an increased risk of breast cancer with increased soy consumption (1).

A population-based, case-control study of breast cancer among Chinese, Japanese and Filipino women in Los Angeles was undertaken to further investigate the role of soy. The primary objective was to quantify breast cancer risks associated with the intake of soy during adolescence and adult life among Asian-American women. The risk of breast cancer was significantly inversely associated with soy intake during adolescence and adult life. After adjusting for age, specific Asian ethnicity, education, migration history and menstrual and reproductive factors, women who reported soy intake at least once per week during adolescence showed a significantly reduced risk of breast cancer. There was also a significant trend of decreasing risk with increasing soy intake during adult life. When one considers soy intake during both adolescence and adult life, subjects who were high soy consumers during both time-

periods showed the lowest risk compared with those who were low consumers. The risk of breast cancer was intermediate among subjects who were high soy consumers during adolescence and low soy consumers during adult life. Based on a relatively small number of subjects, the risk did not appear to differ between those who were low consumers during adolescence and high consumers during adult life. The results remained similar after adjustment for other potential confounders, including other dietary and non-dietary risk factors for breast cancer. These results show that high soy intake in childhood in Asian-Americans is associated with reduced breast cancer risk. The risk may be further reduced by intake as an adult (3).

An association between high soy intake and a reduced risk of mammographic parenchymal patterns that are correlated with high breast cancer risk has been demonstrated (4). Moreover, after 2 years' consumption of soy foods, equivalent to 50 mg of isoflavones, by premenopausal women, the mean percentage density of mammographic parenchyme had decreased by 2.8 and 4.1% in the intervention and control women, respectively. Women who reported eating more soy during their life had lower percentage densities than women whose diet included little soy. This difference was significant only in Caucasians. Lower soy intake during early life followed by a higher intake during adulthood predicted a greater reduction in the percentage density during the study period (5).

In the study of Yamamoto *et al.* (6), the relationship between isoflavone consumption and breast cancer risk among women in the Japan Public Health Center-Based Prospective Study on Cancer and Cardiovascular Diseases (JPHC Study) was evaluated. Consumption of miso soup and isoflavones, but not of soy foods, was inversely associated with the risk of breast cancer (6). Similarly, the inverse associations between urinary PE and breast cancer risk were described by Dai *et al.* (7).

In vitro and in vivo studies. Evidence from *in vitro* and animal studies suggests that PE may inhibit the development of mammary tumors through their role in regulating the synthesis, metabolism and signal transduction of steroid hormones.

Many *in vitro* experiments detected anticancer effects of PE at high concentrations (but mild stimulatory effects at lower concentrations). Animal studies have revealed both cancer-inhibitory and cancer-promoting effects. It has been shown that genistein (GEN, an isoflavonoid present in soy beans) may inhibit *in vitro* human and mouse mammary cancer cell proliferation and invasion (8-10). However, this effect can be achieved only in high doses, ranging from 10 to 100 μM, while at lower concentrations it may stimulate the proliferation of estrogen-dependent tumor cells (8, 9). Schwarz *et al.* have shown that GEN in doses lower than 1 μM reversed a tamoxifen-induced decrease in estrogen receptor (ER) expression (10). Its estrogenic activity was also shown *in vivo* in ovariectomized athymic mice inoculated subcutaneously with the cells of the human breast cancer cell line MCF-7. Tumors were larger in the genistein-fed group than in the control animals, which indicates that dietary genistein may accelerate MCF-7 tumor growth (8). On the other hand, other authors presented results showing an inhibitory activity of GEN towards both ER-positive and -negative human breast cancer cells xenotransplanted into athymic mice, with an accompanying decrease in vessel density and both the VEGF and the TGF-β1 levels (11). Moreover, PE (GEN and quercetin) are known as ER ligands (12, 13). As a result, these PE induce the down-regulation of ER in the human breast cancer cell line MCF-7 (12), and a daily soy diet in humans reduces the levels of circulating estradiol by 25% and of progesterone by 45% (13).

However, GEN, like other estrogen agonists, may exert its chemopreventive activity by enhancing mammary cell maturation, thus reducing cell proliferation (6, 14-16). To explain this phenomenon, the timing of the estrogen administration should be carefully chosen. When an estrogen, or its agonist, is administered before mammary gland maturation and the initiation of mammary carcinogenesis, the incidence of tumors could be lower due to estrogen-induced mammary gland maturation. However, if the estrogen is administered after the development of an estrogen-dependent tumor, the growth of this tumor could be stimulated (8).

The results from a study of Ju *et al.* (17) raise concerns about the consumption of dietary isoflavone supplements in conjunction with tamoxifen (TAM) therapy in postmenopausal women with E-dependent breast cancer. Dietary genistein negates the inhibitory effect of TAM on the growth of estrogen-dependent human breast cancer (MCF-7) cells implanted in athymic mice (17).

In our own experiments, the growth of subcutaneously (*s.c.*)- or orthotopically (*orth.*)-transplanted 16/C mouse mammary cancer was stimulated by GEN administered from day 4 after tumor cell inoculation (early stage of disease). Such stimulation was not observed when the treatment with genistein was started on day 12 after cell inoculation (advanced stage of disease). In another series of experiments, mice were treated with genistein before tumor cell transplantation. In this case also, no effect on mammary tumor growth was observed. Moreover, the growth of 16/C cancer cells transplanted *s.c.* was stimulated by estrogen administered daily before and after tumor transplantation (18). The stimulation of tumor growth by genistein was markedly higher in mice with *orth.*- than in mice with *s.c.*-transplanted tumors. However, GEN did not affect the expression of ER and PgR receptors in the orthotopic model of 16/C tumor. In the case of *s.c.* tumors (with higher control level of both receptors), a two-fold lower expression of both ER and PgR in tumors of GEN-treated mice was detected (18).

We hypothesized that the low level of hormonal receptors in *orth.* tumors may result from their blocking by endogenous hormones, since the treatment with GEN did not affect the ER and PgR levels in this model, in contrast to the *s.c.* tumor model, where "free" receptors were blocked by GEN with the consequently observed decrease in the levels of both receptors. In favor of such an explanation are our observations that the mean levels of both receptors in the GEN-treated animals were the same in the *orth.* and *s.c.* tumors (18).

After combined treatment with CY (cyclophosphamide)+GEN, an increase in the receptors level in *orth.* and a slight decrease in *s.c.* tumor-transplanted mice were observed. It is known that the ER status reflects the intensity of tumor cell proliferation rather than their metastatic potential. This may be a possible explanation for our observation concerning the effect of GEN on metastatic potential, where a lower number of metastatic colonies after treatment with all 3 protocols (CY, GEN, and CY+GEN) was observed, regardless of the primary tumor localization (orthotopic *versus* ectopic). Our observations indicate that, even when the primary tumor growth was stimulated by GEN, the metastatic potential was reduced (18).

A negative correlation between the cell proliferation rate and ER level was observed and described earlier (19, 20). Thus, in *orth*. tumors with low receptor levels, the proliferation rate and sensitivity to chemotherapy could be higher than in *s.c.* tumors. The higher receptor levels in the *orth.*-growing tumors observed after combined treatment with GEN and CY could be a consequence of the elimination of proliferating cells, which is not the case in *s.c.*-growing tumors (18).

Experimental carcinogenesis. There are conflicting reports on the effect of soy and its components on mammary carcinogenesis, mainly because of different rodent models that are used in chemoprevention studies. A study was undertaken to compare the tumor-preventative effects of soy protein isolate (SPI) and two of its isoflavones [daidzein (DAI) and GEN] in a "standard" model that had been used for the identification of many chemopreventive agents (21). The results show that DAI and SPI (with normal or low levels of isoflavones), but not GEN, are effective inhibitors of DMBA-induced mammary tumors in adult rats (21).

Although exposure of rodents to soy isoflavones (SIF) during the perinatal period appears to reduce mammary cancer formation, exposure *in utero* or during adulthood may increase tumor growth. The mouse mammary tumor virus (MMTV)-neu mouse spontaneously develops mammary tumors due to overexpression of the ErbB-2/neu/HER2 oncogene. This model is comparable with human breast cancer, because overexpression of the neu oncogene occurs in 20-40% of human breast cancers. At seven weeks old, MMTV-neu mice were fed AIN-93G diets containing no isoflavones, 250 mg/kg GEN, 250 mg/kg DAI or an isoflavone mixture. Mammary tumor latency was significantly delayed in mice fed isoflavones compared with the control. Once tumors formed, however, the isoflavones did not reduce the number or size of the tumors, such that at 34 weeks of age, there were no differences in tumor burden among the treatment groups. Hence, in the MMTV-neu mouse, SIF delayed mammary tumorigenesis (22).

A contrary effect of dietary GEN on dimethylbenz[α]anthracene (DMBA)-induced mammary tumor development has been shown in wild-type (ER alpha WT) and estrogen-receptor-alpha knockout (ER alpha KO) mice. ER alpha WT and ER alpha KO mice were fed a casein-based diet containing 0 or 1 g GEN/kg diet from weaning. Tumors were induced by oral administration of DMBA and subscapsular implantation of medroxyprogesterone acetate. No tumors were observed in the ER alpha KO mice. In ER alpha WT mice, the dietary intake of GEN influenced tumor development, enhancing anaplasia of mammary cancer. Mice consuming GEN expressed malignant mammary adenocarcinoma, whereas benign adenomas were observed in mice fed the control diet. Overall, this study found no protective effect of GEN on DMBA-induced mammary tumors in mice and suggests a potential adverse effect on tumor development when high levels of GEN are consumed (23).

The results of Kim *et al.* (24) indicate diet dependency of the chemopreventive activity of genistein. In other terms, whether or not an isoflavonoid compound is chemopreventive may depend on the diet in which the agent is administered (24).

It has been shown that GEN stimulates growth of estrogen-dependent human breast cancer (MCF-7) cells in nude mice. Genistin (GSI) is the glycoside form of GEN and the predominant form found in plants. It is generally believed that GSI is metabolized to the aglycone genistein in the lower gut. However, it is unclear if the rate of metabolism of GSI to GEN is sufficient to produce a level of GEN capable of stimulating estrogen-dependent breast cancer cell growth. The question has been raised as to whether dietary GSI would stimulate tumor growth similarly to that observed with GEN in athymic mice. To answer this, GSI or GEN was fed to athymic mice containing xenografted estrogen-dependent breast tumors (MCF-7). Dietary GSI resulted in increased tumor growth, pS2 expression and cellular proliferation similar to that observed with GEN. When mice were switched to isoflavone-free diets, tumors regressed over a span of 9 weeks. Thus, the glycoside GSI, like the aglycone GEN, can stimulate estrogen-dependent breast cancer cell growth *in vivo*. Removal of GSI or GEN from the diet caused tumors to regress (25).

Molecular mechanisms and interactions. Many flavonoids and polyphenols, including resveratrol (RES) in red wine and epigallocatechin gallate in green tea, are known antioxidants. A yeast-based estrogen receptor (ER) reporter assay has been used to measure the ability of flavonoids to bind to ER and activate estrogen-responsive genes. Recently, estrogenic compounds were also shown to trigger rapid, non-genomic effects. The molecular mechanisms, however, have not been completely detailed and little information exists regarding their relevance to cancer progression. As a preliminary step towards elucidating rapid PE action on breast cancer cells, the effects of 17-beta estradiol, GEN, DAI and RES on the activation status of signaling proteins that regulate cell survival and invasion, the cell properties underlying breast cancer progression, were investigated. The effect of these estrogenic compounds on the activation, *via* phosphorylation, of Akt/protein kinase B (Akt) and FAK were analyzed in ER-positive and -negative human breast cancer cell lines. E2, GEN and DAI increased, whereas RES decreased both Akt and FAK phosphorylation in non-metastatic ER-positive T47D cells. In metastatic ER-negative MDA-MB-231 cells, all estrogenic compounds tested increased Akt and FAK phosphorylation. The inhibitory action of RES on cell survival and proliferation is ER-dependent. Therefore, all estrogenic compounds tested, including RES, may exert supplementary ER-independent non-genomic effects on cell survival and migration in breast cancer cells (26).

GEN has been shown to inhibit the growth of cancer cells through the modulation of genes that are related to the homeostatic control of cell cycle and apoptosis. It has been found that GEN inhibits the activation of the nuclear transcription factor, NF-kappaB and Akt signaling pathways, both of which are known to maintain a balance between cell survival and apoptosis. GEN is known to have antioxidant properties, enabling it to target the estrogen and androgen-mediated signaling pathways in the processes of carcinogenesis. Moreover, GEN was also found to be a potent inhibitor of angiogenesis and metastasis (27, 28).

It has recently been reported that GEN treatment of the immortalized but non-malignant human mammary epithelial cell line MCF-10F resulted in growth arrest in the G2-phase of the cell cycle, a large induction of the Tyr15 phosphorylation of Cdc2 (along with decreased activity of Cdc2), increased expression of p21(waf/cip1) and decreased expression of the cell cycle phosphatase Cdc25C. Moreover, GEN rapidly and significantly activated p38, inactivated ERK1/ERK2 and had no effect on SAPK/JNK activity. It has also been shown that p38 is involved in GEN-induced changes in Cdc2 phosphorylation and that the down-regulation of Cdc25C expression by GEN is through the p38 pathway. Finally, evidence has been provided that the p38 pathway is involved in GEN-inhibited cell proliferation. These data suggest an important interplay between the p38 pathway and G2 cell cycle checkpoint control and provide insights into possible mechanisms whereby this isoflavone may inhibit early events in mammary carcinogenesis (29).

Because most non-malignant cells are tolerant to high micromolar concentrations of GEN, inhibitory or stimulatory effects of this compound have been claimed for a wide variety of biochemical targets that lead to a plethora of potential mechanisms. However, because GEN is present in tissues in the nanomol-per-liter range, most of these mechanisms are unlikely to be relevant *in vivo*. To better identify proteins that are targets of GEN, a GEN-agarose-affinity phase was used. With this method, a p38 protein was recovered from MCF-7 cells. N-terminal chemical sequencing of the first 30 residues of the protein revealed a peptide sequence similar to those that have been discovered in human tissues (a T-cell attractant protein from synovial fluid from patients with osteoarthritis and an analogous human skin fibroblast protein using a hirudin-affinity column) as well as a cotonine-binding protein from rat brain and related proteins in plants. In each case, the corresponding gene has not been found. This means that, although much of the human genome has been sequenced, novel proteins that are not described by genome data remain to be found. The DING protein (N-terminal amino acid sequence Asp-Ile-Asn-Gly), that binds to GEN with high affinity, is one of these (30).

It has been shown that GEN inhibits the growth of MDA-MB-231 breast cancer cells, regulates the expression of apoptosis-related genes and induces apoptosis through a p53-independent pathway. Moreover, these effects of GEN were investigated in the breast cancer cell line MDA-MB-435 and 435.eB cells, that were established by transfecting c-erbB-2 cDNA into MDA-MB-435. In addition, the effect of GEN on matrix metalloproteinase (MMP) secretion has been shown to be affected by erbB-2 transfection. GEN was found to inhibit MDA-MB-435 and 435.eB cell growth. Induction of apoptosis was also observed in these cell lines when treated with GEN, as measured by DNA laddering, poly(ADP-ribose) polymerase (PARP) cleavage, and flow cytometric analysis. An up-regulation of Bax and p21WAF1 expression and down-regulation of Bcl-2 and c-erbB-2 in GEN-treated cells have also been found. Gelatin zymography showed that GEN inhibits the secretion of MMP in breast cancer cells. Thus, GEN inhibits the growth of MDA-MB-435 breast cancer cells, induces apoptosis, regulates the expression of genes, and may inhibit invasion and metastasis (31).

DNA methylation is thought to inhibit transcription of genes by regulating alterations in chromatin structure. Estrogenic compounds have been reported to regulate DNA methylation in a small number of studies. It has been shown that consumption of GEN in the diet was positively correlated with changes in prostate DNA methylation at CpG islands of specific mouse genes. Thus, certain soy PE,

such as GEN, may be involved in preventing the development of certain prostate and mammary cancers, by maintaining a protective DNA methylation profile (32).

Osteolytic bone metastasis is a frequent problem in the treatment of breast cancer. Ipriflavone (IPR), a synthetic isoflavone that inhibits osteoclastic bone resorption, has been used for the treatment of osteoporosis. The effects of IPR on osteolytic bone metastasis of MDA-231 human breast cancer cells injected intracardially into athymic nude mice (ICR-nu/nu) was studied. Daily oral administration of IPR at 12 mg/mouse significantly inhibited the development of new osteolytic bone metastases and the progression of established osteolytic lesions, prolonging the life of tumor-bearing mice. In addition, IPR reduced the number of osteoclasts at the bone-cancer interface with no severe adverse effects on the host. *In vitro*, IPR inhibited the proliferation and DNA synthesis of MDA-231 cells and blocked the ligand-induced phosphorylation of Tyr(845) of the EGFR. IPR did not promote apoptosis of MDA-231 cells. Thus, IPR not only directly inhibits the growth of cancer cells, but also reduces osteoclasts, thus preventing the soft tissue tumor burden and osteolytic bone metastases (33).

Although most animal studies have shown cancer-preventive effects, a few recent studies suggest that soy phytoestrogens may stimulate breast cancer cell growth under certain circumstances. Until safety with respect to breast cancer is established, phytoestrogen supplements should not be recommended, particularly for women at high risk of breast cancer (34).

Phytoestrogens and Lung Cancer

Lung cancer is the leading cause of cancer-related deaths in the world, with increasing incidence in many developed countries. Epidemiological data suggest that consumption of soy products may be associated with a decreased risk of cancer. It has been shown that GEN can inhibit the growth of H460 non-small cell lung cancer (NSCLC) cells *in vitro*. To explore the molecular mechanisms by which GEN inhibits the growth of NSCLC cells, H460 cells, which harbor wild-type p53 and H322 cells, which possess mutated p53, were applied. GEN was found to inhibit H460 and H322 cell growth in a dose-dependent manner and *via* a typical apoptotic pathway. Western blot analysis demonstrated up-regulations of p21WAF1 and Bax by GEN in wild-type and mutant p53 cell lines. Furthermore, cells treated with GEN showed an increased expression of endogenous wild-type p53, while the level of the mutant p53 protein remained unchanged. Thus, GEN induces apoptosis in NSCLC cells through a p53-independent pathway and may be considered as a chemopreventive and antitumor agent in lung cancer (35).

In agreement with these data, the results of Finnish epidemiological studies have shown that high isoflavonoid consumption correlates with lower lung cancer incidence (36).

GEN has been also shown to reveal antitumor and antiangiogenic activity, both alone and in combined therapy with cyclophosphamide, in the Lewis lung carcinoma (LL2) mouse tumor model. The additive antiangiogenic, but not cytostatic, effect of GEN combined with cyclophosphamide (CY) was observed (37).

Furthermore, the influence of the route of tumor cell inoculation on the antitumor and antimetastatic effects of these therapeutics was evaluated. The antitumor effect of GEN was observed in mice with LL2 subcutaneously growing tumors. In addition, its antimetastatic effect (reduction of lung colonies) was observed in mice with LL2 cells injected either intravenously or *s.c.* The synergistic effect of both agents in combined treatment was observed when the cells of LL2 cancer were injected *s.c.* When LL2 cells were injected intravenously, no additive effect of GEN and CY could be detected. Thus, both the antitumor and antimetastatic effects of GEN alone, CY alone and those of combined therapy with GEN and CY were dependent on the implantation route of the tumor cells (38).

Further, the antimetastatic activity of GEN alone or combined with CY has been studied in mice, which before treatment were exposed to surgical excision of the primary tumor. The antimetastatic effect was estimated from the number of lung colonies as compared to the control mice exposed to the *s.c.* tumor extirpation only. Twenty days after surgery, an average of 52 lung tumor colonies per mouse were detected in control mice bearing LL2 cancer. Treatment with GEN resulted in the reduction of the lung colonies to 24 per mouse. Treatment with CY reduced the number of lung colonies to 12 ($p<0.05$) and combined treatment with both agents to 4 ($p<0.05$) (39).

On the other hand, it has been shown that GEN and nonylphenol increase the incidence of lung adenomas and carcinomas in male F344 rats initially treated with five different carcinogens, as compared with control animals. A postulated mechanism of this observation is the stimulation of cell proliferation and DNA damage caused by oxygen radicals (40).

Thus, although in most animal and epidemiological studies lung cancer-preventive and anticancer effects of soy PE have been detected, the above study suggests that they may increase lung carcinogenesis under certain circumstances. Again, as in breast cancer, until safety is established, PE supplements should not be recommended.

Phytoestrogens and Melanoma

The relationship between RES bioavalability and its effect on melanoma growth has been investigated (41). *In vitro*, B16 mouse melanoma cell proliferation and generation of reactive oxygen species (ROS) was inhibited by trans-RES (t-RES) in

a concentration-dependent fashion (100% inhibition of tumor growth was found in the presence of 5 μM t-RES). Addition of 10 μM H_2O_2 to B16 cells, cultured in the presence of 5 μM t-RES, reactivated cell growth. Oral administration of t-RES (20 mg/kg twice per day; or included in drinking water at 23 mg/l) did not inhibit the growth of B16 melanoma inoculated into the footpad of mice. However, oral administration of t-RES (as above) decreased hepatic metastatic invasion of B16 cells inoculated intrasplenically. The antimetastatic mechanism involves a t-RES (1 μM)-induced inhibition of vascular adhesion molecule 1 (VCAM-1) expression in the hepatic sinusoidal endothelium (HSE), which consequently decreased *in vitro* B16 cell adhesion to the endothelium *via* very late activation antigen 4 (VLA-4) (41).

The effect of dietary supplementation with isoflavones on pulmonary metastasis of B16BL6 murine melanoma cells in C57BL/6 mice was also investigated. Mice were fed a basal AIN-93G diet or the basal diet supplemented with GEN and DAI for two weeks before and after the intravenous injection of 0.5 x 10^5 melanoma cells. At necropsy, the number and size of tumors that formed in the lungs were determined. The number of mice that had >15 lung tumors was 17 in the control group, and 16, 15, 13 (statistically significant) and 10 (statistically significant) in the groups fed isoflavones at 113, 225, 450 and 900 μmol/kg, respectively. The median number of tumors in the control group was 67, and those in the isoflavone-supplemented groups were 57, 33, 32 and 17, respectively. The last was significantly different from the control. Dietary supplementation with isoflavones at 225, 450 and 900 μ/kg also significantly decreased tumor size (median cross-sectional area and volume) compared to the control values. Thus, dietary supplementation with isoflavones reduces experimental metastasis of melanoma cells in mice (42).

The antitumor and antiangiogenic effects of GEN *in vivo*, applied alone or in combined therapy with cyclophosphamide, have been shown in the B16 melanoma model (37).

The influence of the route of inoculation of the tumor cells on the antitumor and antimetastatic effects of these therapeutics was also evaluated (38). The antitumor effect of GEN was observed in mice with B16F-10 intradermically (*i.d.*)-growing tumors. In addition, its antimetastatic effect (reduction of lung colonies) was observed in mice inoculated with B16F-10 intravenously (*i.v.*). No lifespan prolongation of mice injected intraperitoneally (*i.p.*) with B16F-10 cells was observed, either after treatment with GEN alone or with CY alone. The synergistic effect of both agents in combined treatment was observed when the cells of B16F-10 melanoma were injected *i.p.*, *i.v.* or *i.d.*. Thus, both the antitumor and antimetastatic effects of GEN alone, CY alone and those of combined therapy with GEN and CY were dependent on the implantation route of the tumor cells (38).

Further, the antitumor activity of GEN alone or combined with CY has been studied in mice which, before this treatment, were exposed to surgical excision of the primary tumor. The antitumor effect was evaluated by the percentage of primary tumor recurrence as compared to the control mice exposed to the *s.c.* tumor extirpation only. The percentage of primary tumor recurrence in the control, GEN-treated, CY-treated and GEN- + CY-treated mice was: 86, 29, 57 and 67%, respectively (39).

Other Tumors

The development of endometrial cancer is largely related to prolonged exposure to unopposed estrogens. The associations between dietary intake of seven specific compounds representing three classes of PE (isoflavones, coumestans and lignans) and the risk of endometrial cancer was evaluated (43). Some phytoestrogenic compounds, at the levels consumed in the typical American-style diet, are associated with reduced risk of endometrial cancer (43).

Newbold *et al.* investigated the incidence of uterine adenocarcinoma in outbred female CD-1 mice treated on days 1-5 with equivalent estrogenic doses of diethylstilbestrol (DES, 0.001 mg/kg/day) or GEN (50 mg/kg/day). At 18 months, the incidence of uterine adenocarcinoma was 35% for GEN and 31% for DES. These data suggest that GEN is carcinogenic if exposure occurs during critical periods of differentiation. Thus, the use of soy-based infant formulas in the absence of medical necessity, and the marketing of soy products designed to appeal to children, should be closely examined (44).

Preclinical studies suggest that GEN may have prostate cancer chemopreventive activity (45). The effects of a low-fat diet or a low-fat diet with the addition of a soy supplement were investigated in a pilot phase II clinical study for asymptomatic, hormonally-naïve prostate cancer patients with rising prostate-specific antigen (PSA) levels. The primary endpoint was PSA reduction by 50%. Secondary endpoints were PSA doubling time and time to progression (TTP). A low-fat diet with the subsequent addition of a soy supplement did not result in a significant decline in PSA levels. The addition of soy protein had a modest effect on TTP. A potentially undesirable effect associated with the administration of soy was an increase in IGF-I serum levels (46). In Finnish epidemiological studies, it has been shown that men with higher myricetin intakes had a lower prostate cancer risk (36).

The molecular mechanism(s) by which GEN elicits its effects on prostate cancer cells has not been fully elucidated. It has been shown that the inhibition of Akt and NF-kappaB activity and their cross-talk provide a novel mechanism by which GEN inhibits cell growth and induces apoptotic processes in tumorigenic, but not in non-tumorigenic, prostate epithelial cells (47).

To better understand the precise molecular mechanism(s) by which GEN exerts its effects on PC3 cells, cDNA microarray interrogating 12558 known genes to determine the gene expression profiles altered by GEN treatment was utilized. A total of 832 genes, that showed a greater than two-fold change after GEN treatment in two independent experiments with a high degree of agreement, was found. Among these genes, 774 genes were down-regulated and 58 genes were up-regulated with GEN treatment. Among them, the down-regulation of 11 genes (MMP-9, protease M, uPAR, VEGF, neuropilin, TSP, BPGF, LPA, TGF-beta2, TSP-1, PAR-2) and the up-regulation of 2 genes (connective tissue growth factor, connective tissue activation peptide) were found, which are related to angiogenesis, tumor cell invasion and metastasis. Cluster analysis showed 9 different types of expression. These genes were also subjected to cluster analysis according to their biological functions. It was found that GEN regulated the expression of genes that are critically involved in the regulation of cell growth, cell cycle, apoptosis, cell signaling transduction, angiogenesis, tumor cell invasion and metastasis. Reverse transcription-polymerase chain reaction (RT-PCR) analysis was used to confirm the results of cDNA microarray, and the results were found to be consistent. Thus, GEN affected the expression of a large number of genes that are related to the control of cell survival and physiological behaviors. The gene expression profiles provide comprehensive molecular mechanism(s) by which GEN exerts its pleiotropic effects on cancer cells (48, 49).

It has been shown that polyphenols from tomatoes and soy (GEN, quercetin, kaempferol, BCA, DAI and rutin) have the ability to modulate insulin-like growth factor-I (IGF-I)-induced *in vitro* proliferation and apoptotic resistance in the AT6.3 rat prostate cancer cell line. IGF-I, at 50 μg/L in serum-free medium, produced maximum proliferation and minimized apoptosis. Polyphenols exhibited different abilities to modulate IGF-I-induced proliferation, cell cycle progression and apoptosis. GEN, quercetin, kaempferol and BCA exhibited dose-dependent inhibition of growth, whereas rutin and DAI were less potent. GEN and kaempferol potently induced G_2/M cell cycle arrest. GEN, quercetin, kaempferol and BCA, but not DAI and rutin, counteracted the antiapoptotic effects of IGF-I. Human prostate epithelial cells grown in growth factor-supplemented medium were also sensitive to growth inhibition by polyphenols. GEN, BCA, quercetin and kaempferol reduced the insulin receptor substrate-1 (IRS-1) content of AT6.3 cells and prevented the down-regulation of IGF-I receptor beta in response to IGF-I binding. IGF-I-stimulated proliferation was dependent on activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) and phosphatidylinositide 3-kinase pathways. Western blotting demonstrated that ERK1/2 was constitutively phosphorylated in AT6.3 cells with no change in response to IGF-I, whereas IRS-1 and AKT were rapidly and sensitively phosphorylated after IGF-I stimulation. Several polyphenols suppressed phosphorylation of AKT and ERK1/2, and more potently inhibited IRS-1 tyrosyl phosphorylation after IGF-I exposure. Thus, polyphenols from soy and tomato products may counteract the ability of IGF-I to stimulate proliferation and prevent apoptosis *via* inhibition of multiple intracellular signaling pathways involving tyrosine kinase activity (50).

Zhou *et al.* (51) tried to identify possible synergistic effects between soy and tea components on prostate tumor progression in a mouse model of orthotopic androgen-sensitive human prostate cancer. A soy phytochemical concentrate (SPC), black tea and green tea were compared with respect to tumorigenicity rate, primary tumor growth, tumor proliferation index and microvessel density, serum androgen level and metastases to lymph nodes. SPC, black tea and green tea significantly reduced tumorigenicity. SPC and black tea also significantly reduced the final tumor weights. Green tea did not reduce the final tumor weight, although it tended to elevate the serum dihydrotestosterone (DHT) concentration. The combination of SPC and black tea synergistically inhibited prostate tumorigenicity, final tumor weight and metastases to lymph nodes *in vivo*. The combination of SPC and green tea synergistically inhibited the final tumor weight and metastasis and significantly reduced serum concentrations of both testosterone and DHT *in vivo*. Inhibition of tumor progression was associated with reduced tumor cell proliferation and tumor angiogenesis. Thus, a soy and tea combination could be an effective nutritional regimen in prostate cancer prevention (51).

The low incidence of colon cancer in Asian countries is associated with consumption of soybean products. A limited number of human and animal studies suggested that soybean consumption might prevent colon cancer; other studies did not support this conclusion. Therefore, it is important to understand the biological effects of soybeans on colon cells. In the study of Zhu *et al.*, the cultures of Caco-2, SW620 and HT-29 colon cancer cells were treated with soybean extract, the soluble fraction of a soybean product containing proteins and many soluble components of soybeans. Exposure to soybean extract affected the morphology (vacuoles formation within the cytoplasm) and survival of colon cancer cells and this effect varied depending on the cell lines examined and the concentration of the soybean extract (52).

It has been shown that GEN has anti-colon cancer effects *in vitro*. These effects are attainable at high concentrations that are difficult to achieve in the serum. Gentile *et al.* (53) tried to enhance the activity of GEN against colon cancer cells by coupling it to monoclonal antibody 17.1A, which recognizes an epithelial membrane antigen that is

overexpressed in colon cancer. The conjugate of GEN and 17.1A induced apoptosis and significantly inhibited colon cancer cell growth *in vitro* and *in vivo*. Thus, conjugating GEN to 17.1A monoclonal antibody enhances its effects against colon cancer cells (53).

The incidence rates of ovarian cancer remain lowest in Asian nations, where diets rich in soy products are consumed, whereas they remain among the highest in the United States and other Western nations, whose peoples consume low amounts of soy foods. It has been shown that the DNA synthesis of Caov-3 and NIH:OVCAR-3, two ovarian cancer cell lines, was significantly inhibited by GEN or DAI at dietary relevant concentrations. Also, the number of viable cells was significantly lower (45-75%) in all isoflavone-treated groups than in the control group. The addition of ICI-182780, an estrogen antagonist, blocked these inhibitory effects. In addition, interleukin-6 synthesis by these two cell lines was inhibited by GEN or DAI; production was decreased by approximately 20% compared with the control group. In contrast, transforming growth factor-beta 1 production in ovarian cancer cells incubated with GEN or DAI was significantly greater, *i.e.*, by approximately 30%, than in the control group. Addition of ICI-182780 also neutralized the effects of isoflavones on the production of these two cytokines by ovarian cancer cells. Thus, GEN and DAI independently modify cytokine production and reduce ovarian cancer cell proliferation *via*, at least in part, an estrogen receptor-dependent pathway (54).

The relationship between the intake of soy products and death from stomach cancer was examined in a community-based prospective study of Japanese men and women. In men, the highest consumption of total soy product was significantly associated with lower death risk from stomach cancer. Decreased hazard ratios for the highest compared to the lowest consumptions of total soy product were observed in women, although this association was of marginal significance. These data suggest that soy intake may reduce the risk of death from stomach cancer (55).

The chemoprotective effects of GEN, BCA, EQU and COU on human pancreatic adenocarcinoma cells *in vitro* have been examined. Two human adenocarcinoma cell lines, HPAF-11 from a male and Su 86.86 from a female, were used. COU and EQU at high concentrations were toxic to the Su 86.86 cells. These agents displayed marked differences between cell lines in inhibition of growth. EQU and COU inhibited the growth of the female pancreatic tumor cells by 95%; however, they stimulated the growth of pancreatic tumor cells from the male. GEN also stimulated growth in the male pancreatic tumor cells, but had little effect on pancreatic tumor cells from the female. BCA inhibited growth of both male and female tumor cells, but to a lesser extent than other agents. This study also indicated a difference in K-ras expression in pancreatic tumors cells treated with these agents. EQU and COU decreased K-ras expression in the female tumor cell line. GEN increased expression of K-ras in both male and female pancreatic tumor cells. GEN also increased expression of the multidrug-resistant (mdr-1) gene in the male tumor cell line, while COU and BCA decreased its expression. EQU had no effect on mdr-1 expression. Thus, the chemoprotective potential of EQU and COU against pancreatic cancer seems to be greater in females than in males (56).

Epidemiological and pathological data suggest that thyroid cancer may well be an estrogen-dependent disease. The relationship between thyroid cancer risk and dietary PE intake has been examined in a multiethnic population-based case-control study of thyroid cancer conducted in the San Francisco Bay area, USA. The consumption of traditional and non-traditional soy-based foods and alfalfa sprouts were associated with reduced risk of thyroid cancer. Consumption of "Western" foods with added soy flour or soy protein did not affect risk. Of the seven specific phytoestrogenic compounds examined, DAI, GEN and the lignan secoisolariciresinol, were most strongly associated with risk reduction. Findings were similar for white and Asian women and for pre- and post-menopausal women. These results suggest that thyroid cancer prevention *via* dietary modification of soy and/or phytoestrogen intake in other forms may be possible (57).

Conclusion

Further epidemiological, *in vitro*, animal and human studies are required to elucidate the mechanisms involved in PE biological actions, including steroid hormone activity, effects on cell growth, antioxidant activities, inhibition of chemical carcinogenesis and influences on modulators of cancer risk. The question of whether PE may be used as an anticancer therapeutic and/or chemopreventive agents remains unanswered. Clearly, much more information is needed, especially concerning the safety of their use. It seems extremely difficult to predict the effects of various PE mixtures present in different human diets. Long-term studies (*in vitro*, animal, clinical and epidemiological) with well standardized PE preparations are necessary to assess the potential beneficial and adverse effects. At the current state of our knowledge, we cannot conclude whether consumption of soy, SIF-supplemented food or use of particular isoflavones as therapeutics will have positive, zero or even adverse effects on cancer (particularly, steroid hormone-dependent) risk and treatment.

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