THE ROLE OF ISOFLAVONES IN CANCER CHEMOPREVENTION

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1. ABSTRACT

Cancer is one of the major health problems around the world. However, it has been estimated that more than two-thirds of human cancers could be prevented by modification of lifestyle including dietary modification. The incidences of hormone-related cancers are much higher in Western countries compared to Asian countries. One of the major differences in diet between these populations is that the Asians consume a traditional diet high in isoflavones. Epidemiologic evidence together with data from animal and *in vitro* studies strongly supports relationship between isoflavones and the lower risk of cancers. Isoflavones have been shown to inhibit carcinogenesis *in vivo* in animal experiments. It has been known that genistein, one of the major isoflavones, inhibits the growth of various cancer cells through the modulation of genes that are related to the control of cell cycle, apoptosis, and cell signaling pathways. Moreover, genistein has been found to be a potent inhibitor of oxidative stress, angiogenesis, and metastasis. Therefore, isoflavones exert beneficial effects on human health and may be promising agents for cancer prevention and/or treatment. However, further in depth experimental investigations along with clinical trials are needed to fully evaluate the value of isoflavones in human cancer prevention and/or treatment.

2. INTRODUCTION

Cancer is one of the major health problems around the world. Approximately, 1, 334, 100 new cases of cancer will be diagnosed and 556, 500 people will die from cancer in 2003 in the United States (1). However, it has been estimated that more than two-thirds of human cancers could be prevented by modification of lifestyle including dietary modification (2). Epidemiological studies have provided convincing evidence that dietary factors are tightly associated with the risk of cancers. The incidences of hormone-related cancers including breast, prostate, endometrium, and ovary cancers are much higher in the United States and European countries compared to Asian countries such as Japan and China. One of the major differences in diet between these populations is that the Japanese and the Chinese consume a traditional diet high in soy products. Therefore, isoflavones that mainly exist in soybean have received much attention as dietary factors having inhibitory effects on cancers. Epidemiologic evidence, together with data from animal and *in vitro* studies, has demonstrated that isoflavones exert their inhibitory effects on the carcinogenesis and cancer cell growth, suggesting that isoflavones may be promising agents for cancer prevention and/or treatment.

3. SOURCES AND METABOLISM OF ISOFLAVONES

3.1. Sources and of isoflavones

Isoflavones are found primarily in members of the Leguminosae family. Foods such as soy, lentil, bean, and chickpea are sources of isoflavones; however, soybean

is the food that contains abundant amounts of isoflavones. Levels of isoflavones in soybean from published literature vary between 560 and 3810 mg/kg, depending on growing conditions (3). Soy proteins isolated from soybean contain 466-615 mg isoflavones/kg (3). Soymilk, bean curds, and bean sprouts contain up to 2030 mg isoflavone/kg, depending on the material and the processing (3). The isoflavones in soybean mainly include genistein, daidzein, and glycitein. Most studies regarding isoflavones have been focused on genistein, which shows significant favorable bioactivity on human health.

3.2. Metabolism of isoflavones

It has been known that isoflavones are mostly present in the inactive form as glycosides in the plants. In intestines, isoflavone glycosides (such as genistin and daidzin) are hydrolyzed by bacterial beta-glucosidases and converted to corresponding bioactive aglycones (such as genistein and daidzein) (4-6). The aglycones are then absorbed from the intestine to blood and conjugated mainly in liver to glucuronides, which are excreted in the urine (7). Genistein and daidzein are the major isoflavones that have been detected in the blood and urine of humans (8). It has been found that the isoflavone aglycones are absorbed faster and in greater amounts than their glycosides in humans (9), therefore, isoflavone aglycone-rich products may be more effective than glycoside-rich products in cancer chemoprevention.

When investigating the effects of isoflavones on cancers, one major concern is the physiologically achievable concentration of isoflavones in human plasma *in vivo*. The physiologic concentration of isoflavones in plasma varies in different populations with different amounts of soy food intake. Dietary intakes of 39.4 and 47.4 mg isoflavone/d in Chinese and Japanese populations, respectively, have been reported while the dietary consumption of isoflavones is <1 mg/d in the general population in the United States (10-12). Plasma concentration of genistein in the nanomolar range has been detected in the American and the European, while 1.4 ± 0.7 to 4.09 ± 0.94 micromole/L of plasma genistein has been found in various population groups consuming foods rich in isoflavones (13-17). Recent report has shown that up to 27.46 ± 15.38 micromole/L of genistein in human plasma can be achieved after receiving genistein supplement at a dose of 16.0 mg/kg (18), suggesting the bioavailability of genistein from supplement for cancer prevention.

4. ISOFLAVONES AND CANCERS

4.1. Isoflavones and breast cancer

The incidence rate of breast cancer has historically been 4-7 times higher in the United States than in China or Japan. Epidemiological studies have suggested a protective role of soy-derived substances against breast cancer. The much higher levels of isoflavones in plasma are found in Asian women with low breast cancer incidence (19). It has been reported that Asian women, who

immigrated from their native countries to the United States and adopted Western lifestyles, typically experience increasing breast cancer incidence (20, 21), suggesting that a high intake of soy food in their native countries may have a role in protecting women against breast cancer. It has been found that isoflavone intake affects estrogen metabolism *in vivo* by altering the steroid hormone concentrations and menstrual cycle length, thereby demonstrating a potential role in reducing the risk for breast carcinoma (22).

In animal experiments, several studies have shown that isoflavones have chemopreventive activity in rat models of carcinogen-induced breast cancer (23-26). It has been found that the timing of the exposure of rats to isoflavones is critical. Rats treated neonatally or prepuberally with genistein have a longer latency before the appearance of carcinogen-induced mammary tumors and a marked reduction in tumor number (23). Isoflavones have also shown the anti-carcinogenic effects in mouse mammary tumor virus-induced breast cancer (27). In addition, treatment of breast cancer cells with genistein before implantation into nude mice has shown to diminish the cells' tumorigenic potential in nude mice (28). Recent *in vivo* animal experiments also demonstrated that isoflavones inhibited mammary adenocarcinoma growth in syngeneic mouse model (29).

It has been well known that isoflavones, in particular genistein, inhibit the growth of breast cancer cells *in vitro* (30). Experiments from our laboratory have shown that genistein inhibited the growth of breast cancer cell lines including MDA-MB-231, MDA-MB-435, MCF-7, and MCF10CA1a, regardless of the status of p53 and ER (31-34). Other investigators have also reported similar results in MDA-MB-468, BT20, and T47D breast cancer cells (35, 36). These results suggest that the inhibitory effects of isoflavones on cell growth may not be merely mediated by estrogen receptor (ER) pathway. Flow cytometric analysis data from our laboratory and other investigators showed that genistein induced a G2/M cell cycle arrest in MCF-7, MDA-MB-231, MCF10CA1a and other breast cancer cells (33, 36-38). These data indicate that genistein inhibits the growth of breast cancer cells through induction of G2/M cell cycle arrest.

4.2. Isoflavones and prostate cancer

Prostate cancer is the most common male malignancy in the Western countries; however, Asian men have much lower incidence of prostate cancer than men in North America and Europe. Epidemiological studies have suggested that soy-rich foods have a protective role against prostate cancer. It has been reported that a reduced risk of prostate cancer is associated with consumption of soy foods and isoflavones in China (39). High consumption of soymilk has also been associated with reduced risk of prostate cancer in the United States (40).

Several animal studies have demonstrated that soy diets inhibit the development of spontaneous and

carcinogen-induced prostate cancers in animal models. It has been reported that the spontaneous development of prostate and seminal vesicle cancers was significantly prevented in Lobund-Wistar (L-W) rats consuming soy diet (41). In a rat carcinogenesis model induced by 3, 2'-dimethyl-4-aminobiphenyl (DMAB), isoflavone supplemented diets have been found to prevent the development of adenocarcinomas in the prostate and seminal vesicles (42). Isoflavones also suppress other chemical induced prostate cancer in L-W rats (43). In addition to the inhibition of carcinogenesis, soy diet also shows to reduce the growth of transplanted prostate adenocarcinomas and inhibit tumor cell proliferation and angiogenesis of transplanted prostate cancer in immunodeficient mice (44, 45). Recent *in vivo* animal experiment also showed that isoflavones inhibit orthotopic growth and metastasis of androgen-sensitive human prostate cancers in mice (46). These *in vivo* animal experiments provide strong evidence to support the role of isoflavones in the inhibition of carcinogenesis and prostate cancer cell growth in animal.

Experimental studies have also demonstrated that isoflavones inhibit the growth of prostate cancer cells *in vitro*. The results from our laboratory have shown that genistein inhibits the growth of PC3 and LNCaP prostate cancer cells, regardless of the status of p53 and AR (47-49). Flow cytometric analysis also showed that genistein induces a G2/M cell cycle arrest in PC3 and LNCaP cells (47). Other investigators have also reported similar results in other prostate cancer cells(50-53).

4.3. Isoflavones and other cancers

In addition to breast and prostate cancer cells, isoflavones also show the inhibitory effects on other hormone-related cancers including endometrial, ovarian, and cervical cancers. It has been reported that isoflavone consumptions are inversely related to the risk of endometrial cancer (54). Animal study has shown that genistein has an inhibitory effect on estrogen-related endometrial carcinogenesis in mice, possibly by suppressing expression of estrogen-induced genes (55). Isoflavones also exhibit inhibitory effects on the growth of HeLa and ME-180 cervical cancer cells and Caov-3 (56) and NIH:OVCAR-3 ovarian cancer cells (57).

Moreover, isoflavones have been found not only to decrease the risk of hormone-related cancers, but also to inhibit hormone independent cancers including leukemia, lymphoma, melanoma, lung, pancreatic, gastric, intestinal, hepatic, urinary, and head and neck cancer cells *in vitro* (58-68). Flow cytometric analysis also showed that genistein induces a G2/M cell cycle arrest in non-small cell lung cancer (61), gastric adenocarcinoma (64), hepatoma (69), and melanoma cells (60). Animal experiments have shown that genistein inhibits the growth of human leukemia cells transplanted into mice (58, 70). A diet rich in soy has been found to inhibit pulmonary metastasis of melanoma cells in C57Bl/6 mice (71). In an orthotopic model of pancreatic cancer, genistein increases apoptosis, almost completely inhibits metastasis, and significantly improves survival (63). These results suggest that isoflavones have inhibitory effects relevant to cancer prevention on both hormone-related and hormone independent cancers.

5. MOLECULAR MECHANISMS OF ACTION OF ISOFLAVONES

5.1. Regulation of genes related to cell cycle

To explore the molecular mechanisms by which genistein induces cell cycle arrest, the expression of cell cycle-related genes including cyclins, CDC2, and cyclin dependent kinase inhibitors (CDKIs) was examined. The results from our laboratory and other investigators showed that the treatment of cells with different concentrations of genistein caused a dose-dependent decrease in the expression of cyclin B_1 (38, 47, 50, 61), corresponding with the G2/M phase cell cycle arrest observed by flow cytometry. Isoflavone caused G2/M arrest has also been associated with inhibition of CDC2 kinase activity (69, 72). A significant dose dependent up-regulation of p21^{WAF1} expression was observed in genistein treated cancer cells compared to control cells (31, 32, 38, 47, 50, 61, 68, 72). Moreover, our microarray data also showed that genistein inhibited cell growth through down-regulation of cell proliferation and cell cycle related genes (cyclin B, CDC25A, TGF-beta, ki67) (73). These results suggest that down-regulation of cyclin B_1, CDC2, CDC25A, TGF-beta, and ki67, and up-regulation of p21^{WAF1} could be one of the mechanisms by which genistein arrests cancer cells in G2/M phase and inhibits cancer cell growth.

5.2. Induction of apoptosis

Induction of apoptosis is an important event when anti-cancer agents exert their effects on cancer cells. By using DNA ladder, poly(ADP-ribose) polymerase (PARP), CPP32, and 7AAD assays, genistein has been found to induce apoptosis in all cancer cells tested in our laboratory (31-34, 47, 48, 61, 62, 68), suggesting that genistein may inhibit cancer cell growth through induction of apoptosis. Other investigations have also observed similar results in genistein or other isoflavone treated cancer cells (52, 69, 74, 75).

To explore the molecular mechanisms by which genistein induces apoptosis, our laboratory have examined the expression of genes that are critically involved in the apoptotic pathways after genistein treatment. The results showed that genistein treatment for 48 hours or longer reduced Bcl-2 protein expression and significantly increased expression of Bax in all cancer cells tested (31, 32, 47, 61, 68, 76). Other investigators also reported that soy isoflavones could induce apoptosis in human hepatoma cells and breast cancer cells through caspase-3 activation and down-regulation of Bcl-2, Bcl-$_{XL}$, and HER-2/neu (69, 74, 77). These results suggest that caspase activation, up-regulation of Bax, and down-regulation of Bcl-2, Bcl-

$_{XL}$, and HER-2/neu may be the molecular mechanisms by which isoflavones induce apoptosis.

Recent study reported by Kazi *et al.* showed that genistein inhibited the proteasomal chymotrypsin-like activity *in vitro* and *in vivo* with accumulation of p27^{KIP1}, I kappa B-alpha, and Bax, and that genistein-mediated proteasome inhibition was accompanied by induction of apoptosis. These results suggest that inhibition of the proteasome activity by genistein may be another molecular mechanism by which genistein induces apoptosis and may contribute to its cancer-preventive properties (78).

It has been reported that p53 down-regulates Bcl-2 which protects cells from apoptosis (79, 80), or induces p21^{WAF1} which inhibits the activity of CDKs, resulting in growth arrest and apoptosis (81-85). The induction of apoptosis by genistein treatment is both p53-dependent and p53-independent. It has been found that genistein down-regulated the expression of dysfunctional p53 in cancer cells (62), while p21^{WAF1} was induced after treatment (31, 32, 47, 61, 68, 76). Microarray analysis showed that genistein regulated the expression of genes that are critically involved in the apoptotic processes (73). These results suggest that isoflavones inhibit cancer cell growth partly through induction of apoptosis.

5.3. Regulation of cell signaling pathways
5.3.1. NF-kappaB pathway

It has been well known that nuclear factor-kappaB (NF-kappaB) pathway plays important roles in the control of cell growth, differentiation, apoptosis, inflammation, stress response, and many other physiological processes in cellular signaling. NF-kappaB has been described as a major culprit and a therapeutic target in cancer (86-90). To investigate whether genistein regulates cell growth and apoptosis through NF-kappaB pathway, our laboratory examined NF-kappaB DNA-binding activity in genistein treated PC3 and LNCaP prostate cancer cells by electrophoresis mobility shift assay (EMSA) (49). The results showed that genistein significantly inhibited the NF-kappaB DNA-binding activity in both cell lines and abrogated the induction of NF-kappaB DNA-binding activity stimulated by either H$_2$O$_2$ or TNF-alpha. These results demonstrated that genistein inhibits NF-kappaB DNA-binding activity in both non-stimulated and stimulated conditions (49). Similar results have been reported by other investigators, showing that NF-kappaB DNA binding and COX-2 promoter activity were enhanced by TNF-alpha, and these effects were inhibited by genistein in human lung epithelial cells (91).

It has been known that NF-kappaB DNA-binding activity could be activated by IkappaB phosphorylation, IkappaB could be phosphorylated by activated IkappaB kinase (IKK), and IKK could be phosphorylated and activated by an upstream kinase, mitogen activated kinase kinase 1 (MEKK1) (92-95). The results from our laboratory showed that genistein treatment inhibited MEKK1 kinase activity and reduced the amount of phosphorylated IkappaB in prostate cancer cells. Cells treated with TNF-alpha or H$_2$O$_2$ showed increased MEKK1 kinase activity and

genistein pre-treatment blocked MEKK1 kinase activity activated by TNF-alpha or H$_2$O$_2$. These results suggested that genistein could inhibit MEKK1 kinase activity, which appears to be responsible for decreasing phosphorylation of IkappaB and, thereby, resulting in the inactivation of NF-kappaB.

5.3.2. Akt pathway

Akt signaling pathway is another important cell signaling pathway and has received much attention in cancer research area (96). Akt is activated by phospholipid binding and phosphorylation at Thr308 by PDK1 or at Ser473 by PDK2 (97). It has been found that activated Akt functions to promote cell survival by inhibiting apoptosis through inactivation of pro-apoptotic factors (98-100). Akt also regulates the NF-kappaB pathway via phosphorylation and activation of molecules in the NF-kappaB pathway, and has been believed to be a therapeutic target in cancer (101, 102). We have conducted *in vitro* experiments to investigate the effects of genistein on Akt pathway in PC3 prostate cancer cells (48). We found that genistein decreased the phosphorylated Akt protein at Ser473 and the Akt kinase activity under non-stimulated condition, and also abrogated Akt activation stimulated by EGF, suggesting the inactivation of Akt kinase under both non-stimulated and stimulated conditions after genistein treatment.

To further explore the inhibitory mechanisms of genistein on Akt and NF-kappaB pathways, Akt expression construct (pLNCX-Akt) was transiently co-transfected with NF-kappaB-Luc reporter construct into PC3 prostate cancer cells (48). Luciferase assay showed an increased luciferase activity in PC3 cells co-transfected with pLNCX-Akt and NF-kappaB-Luc. However, genistein inhibited the luciferase activity in PC3 cells co-transfected with pLNCX-Akt and NF-kappaB-Luc, and abrogated the activation of Akt stimulated by EGF. EMSA for NF-kappaB DNA-binding activity in transfected cells also showed similar results. These results suggest that genistein exerts its inhibitory effects on NF-kappaB pathway through Akt signaling pathway. Down-regulation of NF-kappaB and Akt signaling pathways by genistein may be one of the major molecular mechanisms by which genistein inhibits cancer cell growth and induces apoptosis. Recent report by other investigators also demonstrated that genistein could inhibit Akt activation induced by estradiol in MCF-7 cells (103), collectively providing molecular evidence for the role of Akt and NF-kappaB in mediating the anti-cancer effects of isoflavone genistein.

5.3.3. AR and ER pathways

Androgen receptor (AR) signaling pathway has been known involved in the development and progression of prostate cancer through regulation of transcription of androgen-responsive genes (104) such as prostate specific antigen (PSA) (105, 106). We have investigated the effects of genistein on the expression of PSA through androgen regulation (107). We found that genistein at low concentration (<10 micromole/L) transcriptionally down-

regulated AR, decreased nuclear protein binding to ARE and, thereby, inhibited the transcription and protein expression of PSA in androgen-sensitive LNCaP cells. However, higher concentrations (10 to 50 micromole/L) of genistein were needed to significantly inhibit PSA secretion in VeCaP cells which are androgen-insensitive, and no alternation in the AR expression or ARE binding activity was observed. Furthermore, we transiently transfected PSA promoter-reporter construct into LNCaP and VeCaP cells followed by treatment with or without genistein (0.5-50 micromole/L) in the presence of media with or without R1881, a synthetic androgen. The results showed that genistein inhibited PSA synthesis in prostate cancer cells through both androgen-dependent and androgen-independent pathway .

Isoflavones have a close similarity in structure to estrogens, and have been known as phytoestrogens. Because of the structural similarity to estrogen, isoflavones have been believed to exert their effects through ER signaling pathway. However, experimental study has found that isoflavones at different concentration may exhibit different effects. Genistein might either induce breast cancer cell proliferation by estrogenic agonistic properties (at concentrations =1 micromole/L) or prevent hormone-dependent growth of breast cancer cells by potential estrogen-antagonistic activity (at concentrations =5 micromole/L) dependent on its concentrations (108). Experimental studies also showed that isoflavones exert their inhibitory effects on hormone independent cancers. These results suggest that isoflavones may be powerful chemopreventive and/or therapeutic agents for cancers, irrespective of hormone responsiveness.

5.4. Regulation of genes related to angiogenesis and metastasis

In addition to the inhibition of cancer cell growth and induction of apoptosis, genistein has been shown to reduce the angiogenic and metastatic potential of cancers (71, 109). We have investigated the inhibitory effect of genistein on the expression of MMPs in MDA-MB-435 breast cancer cells transfected with *c-erb*B-2 (32), which has been shown to promote secretion of MMPs and subsequent metastasis in experimental models (110). We found that the expression of *c-erb*B-2, MMP-2, and MMP-9 in MDA-MB-435 cells stably transfected with *c-erb*B-2, was much higher than that in parental MDA-MB-435 cells. However, the high expression of *c-erb*B-2, MMP-2, and MMP-9 in 435 transfectants was significantly down-regulated by genistein treatment (32). These results suggest that genistein may inhibit the expression of *c-erb*B-2 and subsequently decrease the secretion of MMPs in breast cancer cells.

To further investigate molecular mechanisms by which genistein exerts its anti-angiogenic and anti-metastatic effects on PC3 cells, we have utilized microarray to determine the gene expression profile altered by genistein treatment (111). We found that genistein

down-regulated the expression of MMP-9, protease M, uPAR, VEGF, neuropilin, TSP, BPGF, LPA, TGF-beta, TSP-1, and PAR-2, and up-regulated the expression of connective tissue growth factor and connective tissue activation peptide (111). All of these genes are related to angiogenesis and metastasis. The microarray data were confirmed by RT-PCR, Western Blot, and zymographic analysis in mRNA and protein levels. Our results demonstrated that genistein regulated the transcription and translation of genes critically involved in the control of angiogenesis, tumor cell invasion and metastasis, suggesting that genistein may be used for treatment of metastatic prostate cancer. The inhibitory effect of isoflavones on metastasis has been confirmed by *in vivo* animal experiments demonstrating that isoflavones inhibited bone metastasis of human breast cancer cells in a nude mouse model, and metastasis of androgen-sensitive human prostate tumors in mice (46, 112).

5.5. Regulation of oxidative stress

Isoflavones have been known to function as antioxidants, and increasing oxidative stress has been related to carcinogenesis. It has been reported that isoflavone reduces hydrogen peroxide-induced DNA damage in sperm (113), and that genistein inhibits tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced hydrogen peroxide production in human polymorphonuclear leukocytes and HL-60 cells (114), suggesting that the inhibitory effect of isoflavones on carcinogenesis could be attributed to its anti-oxidant properties. Genistein has also been shown to stimulate anti-oxidant gene expression in colon cancer cells (115), and inhibit UV irradiation-induced oxidative stress in epidermal carcinoma (116).

Because oxidative stress activates NF-kappaB DNA binding activity (117, 118), we investigated whether the effect of isoflavone supplementation could inactivate NF-kappaB *in vivo* and reduce oxidative damage in lymphocytes in human subjects (119). The lymphocytes from healthy male subjects were harvested from peripheral blood and cultured for 24 hours in the absence and presence of genistein. We found that genistein treatment inhibited basal levels of NF-kappaB DNA binding activity by 56% and abrogated TNF-alpha induced NF-kappaB activity by 50% (119). Furthermore, when human subjects received 50 mg of isoflavone supplements Novasoy[TM] (Archer Daniels Midland Company, Decatur, IL, USA; containing genistein, daidzein, and glycitein) twice daily for three weeks, TNF-alpha failed to activate NF-kappaB activity in lymphocytes harvested from these volunteers, while lymphocytes from these subjects collected prior to isoflavone intervention showed activation of NF-kappaB DNA binding activity upon TNF-alpha treatment (119). These results suggest that isoflavone supplementation has a protective effect against TNF-alpha induced NF-kappaB activation in humans both *in vitro* and *in vivo*.

We further investigated the effect of isoflavone supplementation on oxidative DNA damage by measuring

the levels of 5-OHmdU, that represents the endogenous status of cellular oxidative stress, in the peripheral blood lymphocytes of normal human subjects before and after supplementation with Novasoy ™. The results showed that 5-OHmdU was significantly decreased after three weeks of isoflavone supplementation (119). These results demonstrate that isoflavones may exert their chemopreventive effects through regulation of oxidative stress.

6. SUMMARY AND PERSPECTIVE

The data from epidemiological studies, *in vivo* human and animal studies, and *in vitro* experiments clearly indicate that isoflavones exert inhibitory effects on carcinogenesis and cancer cell growth. These effects may be mediated by pleiotropic molecular mechanisms through regulation of cell proliferation, cell cycle, apoptosis, cell signaling pathways, cellular oxidative stress, and cell physiological behaviors. Therefore, isoflavones may be promising preventive and/or therapeutic agents against various cancers. However, further in depth experimental investigations along with clinical trials are needed to fully evaluate the value of isoflavones in human cancer prevention and/or treatment.

7. ACKNOWLEDGMENTS

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