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THE BIOLOGICAL AND BIOCHEMICAL ROLES OF GENISTEIN AND ITS METABOLITE, DIHYDROGENISTEIN, IN HUMAN BREAST CARCINOGENESIS

presented by

Ching-Yi Hsieh

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Bill Helferich
Major professor

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THE BIOLOGICAL AND BIOCHEMICAL ROLES OF GENISTEIN AND ITS METABOLITE, DIHYDROGENISTEIN, IN HUMAN BREAST CARCINOGENESIS

By

Ching-Yi Hsieh

A DISSERTATION

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Department of Food Science and Human Nutrition
and
The Institute for Environmental Toxicology

1997

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ABSTRACT

THE BIOLOGICAL AND BIOCHEMICAL ROLES OF GENISTEIN AND ITS METABOLITE, DIHYDROGENISTEIN, IN HUMAN BREAST CARCINOGENESIS

By

Ching-Yi Hsieh

Genistein (GEN) is one of the isoflavones found in soy products. Several biochemical effects, including estrogenic effects of GEN have been reported, i.e. inhibition of protein tyrosine kinase, DNA topoisomerase and antioxidant activity. Three objectives of the present study aim to evaluate both the potential beneficial and detrimental effects of GEN related to breast cancer development. First, dietary GEN was found to enhance mammary gland development, and to show uterotrophic effects and tumor growth of implanted MCF-7 cells [estrogen receptor (ER)-positive human breast cancer (HBC)] in ovariectomized athymic mice. GEN acts as an estrogen agonist resulting in the proliferation of MCF-7 cells and in the induction of the expression of an estrogen responsive gene, pS2, in MCF-7 cells in vitro. GEN produces a dose-dependent stimulatory effect on growth of MCF-7 cells at concentrations from 1 nM to 1 μM and a growth inhibitory effect at levels greater than 10 μM in both MCF-7 and MDA-mB-231 (ER- negative HBC) cells. The effect of GEN on cell growth at lower concentration appears to be mediated by an ER-mediated pathway and the effect of GEN at higher concentration could be mediated by a different mechanism (e.g. tyrosine kinase inhibition). Second, dihydrogenistein (DHG) is the only metabolite of GEN found in the urine of humans consuming soy. DHG increased cell growth in a dose-dependent manner in ER-positive MCF-7 cells, but not in ER-negative MDA-mB-231 cells. DHG differs from GEN in failure to inhibit cell growth at higher concentration (above 25 μM)

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in either ER-positive or ER-negative HBC cells. pS2 mRNA expression was stimulated by DHG. Additionally, we demonstrated that the ER antagonist, ICI 164,384, is able to block the stimulation of pS2 expression and cell growth induced by DHG. DHG competed with [^{3}H]estradiol for binding to the ER. The level of p21 $^{WAF1/CIP1}$ protein increased after treatment with GEN, but not with DHG. Lastly, we studied the ability of genistein to inhibit the growth of two types of normal human breast epithelial (HBEC) cells (i.e. Type I cells with luminal and stem cell characteristics, and Type II cells with basal epithelial cell phenotypes) and to induce the differentiation of Type I cells to Type II cells. The results show that GEN, at concentrations lower than 1 μM, significantly increased the differentiation of Type I HBEC to Type II cells in one of two primary cultures derived from different human subjects. Significantly, GEN completely arrested cell growth of Type I HBEC at concentrations higher than 5 μM and Type II HBEC at concentrations higher than 50 μM in all of the six independent primary cultures examined. Flow cytometric analysis revealed that GEN was able to arrest cell cycle progression of both Type I and Type II HBEC at both G1/S and G2/M checkpoints. Western blot analysis showed that the level of p21$^{WAF1/CIP1}$, which negatively regulates the G1/S transition and cdc2 protein, which positively regulates the G2/M transition, are significantly enhanced and decreased respectively by GEN in both Type I and Type II HBEC. Since Type I HBEC have been shown to be more susceptible to neoplastic transformation, the inhibition of Type I cell growth by genistein at physiological dose could reduce the number of target cells for carcinogenesis, thereby providing a mechanism for chemoprevention of breast cancer. Depending on the dosage of GEN and the target cell type, our results provide evidence for both beneficial and detrimental effects of GEN on breast carcinogenesis.

To Wen-Jen, Kevin and Karen

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BPE	Bovir
CDK	Cyclin
DHG	Dihyd
DMBA	Dime
DMSO	Dimet
DTT	Dithio
E	Estrog
E2	17β-Es
EGF	Epider
EGF-R	Epider
ER	Estrog
EDTA	Ethyle
FACS	Flow a
FBS	Fetal b
GEN	Geniste
HBC	Human
HBEC	Human
HME	Human
PBS	Phospha

LIST OF ABBREVIATIONS

AIG	Anchorage independent growth
BPE	Bovine pituitary extract
CDK	Cyclin dependent kinase
DHG	Dihydrogenistein
DMBA	Dimethylbenz[a]nthracene
DMSO	Dimethylsulphoxide
DTT	Dithiothreitol
E	Estrogen
E2	17β-Estradiol
EGF	Epidermal growth factor
EGF-R	Epidermal growth factor receptor
ER	Estrogen receptor
EDTA	Ethylenediamine tetraacetic acid
FACS	Flow activated cell sorter
FBS	Fetal bovine serum
GEN	Genistein
HBC	Human breast cancer
HBEC	Human breast epithelial cell
HME	Human mammary epithelium
PBS	Phosphate buffered saline

PCNA	Proli
PDGF	Plate
PDGF-R	Plate
PKC	Prot
P-Tyr	Phos
SV40	Simi

PCNA	Proliferating cell nuclear antigen
PDGF	Platelet-derived growth factor
PDGF-R	Platelet-derived growth factor receptor
PKC	Protein kinase C
P-Tyr	Phosphorylated tyrosine
SV40	Simian virus 40

CHAPTER 1

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CHAPTER 1

INTRODUCTION AND OBJECTIVES

Epidemiological data show that American women have higher breast cancer incidences than oriental women (Messina et al., 1994). Dietary components are believed to be a major causal factor. Diets rich in fruits, vegetables, and grains have been associated with the prevention of cancer. Fruits and vegetables contain non-nutritive phytochemicals which possess biological activities that may be involved in cancer chemoprevention. A traditional diet consumed by an oriental female is high in soy products. Soy contains several biologically active phytochemicals including phytosterols, fiber, phytate, isoflavones and flavones. One of the principle isoflavones is genistein which has been hypothesized to be a chemopreventive agent against breast cancer. However, in some cases these phytochemicals may possess biological activities that may promote or suppress cancer growth. Genistein is one such phytochemical that possesses both potential beneficial and detrimental effects with regard to breast cancer. Specifically, genistein has been reported to have estrogenic effect, protein tyrosine kinase inhibition, DNA topoisomerase inhibition and antioxidant activities. It is conceivable that genistein may possess beneficial biological effects such as inhibition or prevention of cancer cell growth and some detrimental biological effects such as estrogenic stimulation of estrogen-dependent cancer cell growth.

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This dissertation is composed of six individual chapters. The first two chapters (1 and 2) are the introduction and literature review for all of the studies completed for my dissertation. Chapters 3, 4 & 5 contain original new data and are written in the form of three manuscripts with their own introduction, materials and methods, results and discussion. The last section (Chapter 6) contains the conclusions and implications of the studies and a list of references for the entire dissertation. A brief discussion of the content and hypotheses of chapters 3, 4, and 5 is presented below.

Studies presented in chapter 3 were designed to evaluate the potential detrimental estrogenic effects of genistein to women at risk of estrogen-dependent breast cancer. Postmenopausal women with low levels of circulating estrogen and with estrogen receptor positive breast cancer are potentially at high risk for exposure to genistein. Here, I utilize a unique animal model which mimics the hormonal condition of postmenopausal women to test the effect of genistein on growth of human breast cancer cells in vivo. It is my hypothesis that genistein will stimulate the growth of existing estrogen-dependent tumors in ovarectomized athymic mice. The results addressed the question whether the estrogenic activity of genistein was able to promote the growth of estrogen receptor positive breast cancer.

Studies presented in chapter 4 were designed to evaluate the biochemical and biological effects of dihydrogenistein, the only one metabolite of genistein found in human urine samples in persons consuming soy, on growth of cultured human breast cancer cells. Genistein is known to promote or to inhibit cancer cell growth at low and

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In summary

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high concentrations respectively, possibly through different functions of the chemical. The studies here will test whether minor modification in the chemical structure of genistein will affect these functions. The results will help to characterize the biological and biochemical nature of this chemical.

Studies presented in chapter 5 were designed to investigate a possible mechanism of beneficial effects of genistein to prevent human breast cancer. The hypothesis to be tested here is that genistein could decrease the number of target cells for neoplastic transformation either by inhibiting the growth of target cells or promoting the differentiation of target cells. A recently developed normal human breast epithelial cell type with estrogen receptor expression and stem cell characteristics was used in this study.

In summary, the three objectives of my dissertation research are as follows:

1. To confirm the estrogenic effect of genistein in: (i) promoting cell proliferation of MCF-7 cells in vitro, and (ii) activating the expression of estrogen responsive gene, pS2, in MCF-7 cells in vitro ; and to examine the effects of dietary genistein on: (i) the growth of MCF-7 cells implanted into ovariectomized athymic nude mice which had elevated blood genistein level, and (ii) the stimulation of mammary gland growth in vivo.
2. To test the biological and biochemical effects of dihydrogenistein in breast cancer cells mediated by: (i) the estrogen receptor (ER); i.e. competitive ER binding, and induction of cell proliferation, and induction of pS2 gene expression, (ii) the protein

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tyrosine kinase; i.e. changes in total protein tyrosine phosphorylation analyzed by Western blots, and (iii) the cell cycle regulating genes, especially the expression of the p21 protein.

3. To test the hypothesis that genistein could induce the differentiation and/or suppress the proliferation of human breast epithelial cell type with stem cell characteristics, thereby reducing the number of target cells for neoplastic transformation and thus to provide a mechanism of chemoprevention for breast cancer. The experiments include: (i) the studies of cell proliferation and differentiation, (ii) flow cytometric analysis, and (iii) Western blot analysis of the expression of two major cell cycle checkpoint regulating proteins, i.e. p21 and cdc2 kinase.

The results of these studies will help to elucidate the potential beneficial and detrimental effects of dietary genistein concerning the prevention and development of breast cancer.

CHAPTER 2

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CHAPTER 2

Literature Review

I. Breast Cancer Epidemiology and Etiology

Breast cancer is one of the most common cancers among women in the United States. It is estimated that there were 180,200 cases of female breast cancer diagnosed and 43,900 deaths from this disease in 1997 (Cancer Facts and Figures, 1997). The incidence of breast cancer is 1 in 9 women by the age of 85 in the United States (Marshall et al., 1993). The rising incidence of breast cancer could be due to early screening or due to the exposure to increasing number of risk factors. Many factors for breast cancer in females have been documented (Marshall et al., 1993). These are old age, early menarche, late menopause, late first full-term pregnancy, country of birth (North America, Europe vs. Asia, Africa), oophorectomy, obesity after menopause, family history of premenopausal bilateral breast cancer, history of fibrocystic disease and history of primary cancer in ovary or endometrium. The correlation of higher breast cancer incidence with early menarche and taller stature is widely invoked as reflecting the influence of nutrition during childhood and adolescence (Pollner, 1993). Many environmental agents might be associated with breast cancer by functioning as xenoestrogens (e.g. op'-DDT, PCBs, heptachlor and other pesticides) or as estrogenic potentiating factors (e.g. alcohol, low fiber, fat/total calories) (Davis et al., 1993). While substantial evidence for these environmental agents are not available, ionizing

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radiation is perhaps the best established exogenous carcinogen of breast cancer. The evidence primarily came from studies with atomic-bomb survivors in Japan and in women treated with radiation therapy (Land et al., 1991; Pollner et al., 1993; Hildreth et al., 1989; Shore et al., 1977). However, the dietary risk and protective factors of breast cancer are getting more attention in current breast cancer research.

II. Soybeans and Breast Cancer

A Soy diet is one of the dietary factors suspected to contribute to a lower breast cancer risk among Asian and US women. It was reported that there is an inverse correlation between soy intake and the risk of breast cancer among 142,857 women in Japan over a period of 17 year (Messina et al., 1994). In rats, soybean diets reduced mammary tumor occurrence induced by irradiation (Troll et al., 1980) and the chemical carcinogen, N-methyl-N-nitrosourea (MNU) (Barnes et al., 1988). These data suggest that certain components present in soy could play an important role in the prevention of mammary cancer.

Isoflavonoids, some of which function as phytoestrogens, and which are abundant in soybeans, could be candidates of chemopreventive agents to prevent breast cancer. Studies with chimpanzees have shown that the animals were highly resistant to breast cancer and that their urine contained high amounts of phytoestrogens and their metabolites (Aldercreutz et al. 1986[a]). Similar results were observed for human vegetarians that consume a lot of phytoestrogen-containing foods (Aldercreutz et al.

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1986[b]). In contrast, a lower amount of lignans, phytoestrogens and their metabolites were found in the urine from breast cancer patients (Aldercreutz et al. 1986[b]). Studies of breast cancer cells in vitro, have shown that breast cancer cell growth was inhibited by phytoestrogens at concentrations higher than 10 μM (Barnes, 1995).

III. Genistein Contents in Soybeans and Soy Products

Genistein, present in high concentration in soy and soy products is a diphenolic planar molecule with a molecular weight of 270.2, similar to that of the steroidal estrogens (Figure 6). Genistein, one of the principal soy isoflavones, has been shown to inhibit the growth of a number of tumor cell lines in vitro (Barnes, 1995) and therefore it is one of the most promising candidates among the soy isoflavonoids to prevent the breast cancer. Genistein (4',5,7-trihydroxyisoflavone) is found naturally in soy as the β-glucoside, genistin, and other more complex glycosidic conjugates (Barnes et al. 1994). Microflora in the intestine are able to hydrolyze the glucoside to the aglycone, genistein.

Genistin and its aglycone, genistein, account for the majority of the isoflavones found in soybeans which contain 2-3 mg/g of these two compounds (Eldridge et al., 1983). Genistin, the glycone, is the major form present in soybeans (99%) with less than 1% for the aglycone, genistein (Naim et al., 1974). There are six major isoflavones in soybeans which are genistin, malonyl genistin, daidzin, malonyl daidzin, glycitin and malonyl glycitin. The total percentage of isoflavones in soybeans is about 0.25%, of which 59% was genistin and malonyl genistin, 37% was daidzin and malonyl daidzin and

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4% was glycitin and malonyl glycitin (Wang and Murphy, 1994). The isoflavone content in soybeans is distributed to the hypocotyl and the hull which contained 90% and 1 % of the total isoflavones, respectively (Eldridge et al., 1983). Therefore, dehulled soybeans still retain most of the total isoflavonoid content. Wang and Murphy, 1994, compared their results with the published data and concluded that there was a decrease in genistin content in processed soybean food products. Generally, the total isoflavone content in soy food products is lower than soybean itself.

IV. Biochemical Effects of Genistein

Estrogen Receptor Mediated Genistein Effect

At low concentrations (100-200 nM) and in the absence of estrogens, genistein has been reported to increase the serum-stimulated growth of cultured human breast cancer, estrogen receptor-positive, MCF-7 cells (Martin et al. 1978 and Wang et al. 1996). The affinity of genistein to the estrogen receptor is approximately 100 time less than that of 17-β estradiol. Genistein competes with [^{3}H] 17-β estradiol binding to the human estrogen receptors from a partially purified cytosol fraction of MCF-7 cells, with 50% inhibition at 5 x 10^{-7} M and the non-radioactive 17-β estradiol competes with [^{3}H] 17-β estradiol binding to the human estrogen receptors from a partially purified cytosol fraction of MCF-7 cells, with 50% inhibition at 5 x 10^{-9} M (Wang et al., 1996). Using rat uterine cytosol fraction, genistein also showed a relative binding affinity to the estrogen receptor to be approximately 1% of that by 17-β estradiol (Santell et al., 1997).

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It has been reported that genistein can elevate the estrogen responsive pS2 gene expression as well as c-fos expression, both genes contain an ERE (estrogen response element) in the promoter region. In MCF-7 cells, genistein increased pS2 gene expression at doses from 10^{-8} M to 10^{-5} M and this effect could be blocked by tamoxifen, an estrogen receptor anatogonist (Wang et al., 1996). Similarly, dietary genistein (750 μg/g) induced the uterine expression of c-fos in rats and the presence of total genistein (conjugated plus free) at 2.2 μM and 0.4 μM free genistein in serum (Santell et al., 1997). Furthermore, a low concentration of genistein was also found to stimulate the expression of a luciferase reporter gene under the control of an ERE in MVLN cells (Dees et al., 1997).

A recent report demonstrated that low concentration of genistein can stimulate MCF-7 cells to enter the cell cycle similar to estradiol that stimulates human breast cancer cells to enter the cell cycle (Dees et al., 1997). In the same report, a low concentration of genistein was found to increase the activity of cyclin dependent kinase 2 (Cdk2) and cyclin D-1 synthesis and to stimulate the hyperphosphorylation of the retinoblastoma susceptibility gene product, pRb, in MCF-7 cells. The steroidal antiestrogen, ICI 182,780, suppressed this genistein mediated activation of Cdk2 activity confirming that the estrogenic effect of low concentrations of genistein is mediated by the estrogen receptor.

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Effect of Genistein on Protein Tyrosine Kinase and Signal Transduction

Exogenous factors may function as ligands to interact with receptor tyrosine kinases such as the epidermal growth factor receptor (EGF-R) and the platelet-derived growth factor receptor (PDGF-R) in the cell membrane to trigger signal transduction such as the mitogen-activated protein [MAP] kinase cascade, resulting in gene expression and cell proliferation/ differentiation.

Genistein has been reported to exhibit specific inhibitory activity against tyrosine kinases of the epidermal growth factor receptor (EGF-R), $pp60^{v\text{-}src}$ and $pp^{110gag\text{-}fes}$ (Akiyama et al.,1987). The inhibition is competitive with respect to ATP and non competitive with the phosphate acceptor. Treatment of A431 cells with high doses of genistein (IC_{50} =120 μM) prevented the autophosphorylation of EGF-receptor mediated by EGF (Akiyama et al.,1987). Tyrosine protein kinase activities are known to be associated with oncogene products of the retroviral "src" gene family (Hunter and Cooper, 1985) and with several cellular growth factor receptors such as EGF(Ushiro and Cohen, 1980), platelet-derived growth factor (PDGF) (EK et al., 1982), insulin (Kasuga et al., 1982) and insulin-like growth factors (Rubin et al., 1983). Tyrosine protein kinases seem to play a key role in tumorogenesis. Therefore, inhibitors of protein tyrosine kinase activity might represent a new class of antitumor agents (Levitzki and Gazit, 1995).

Genistein at 100 μM also inhibited nerve growth factor (NGF)-, fibroblast growth

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factor (FGF)- and insulin- induced Ras : GTP complex formation in rat pheochromocytoma cells pc-12 (Nakafutu et al., 1992). Genistein at 100-200 μM inhibited PDGF-induced DNA synthesis and also inhibited phosphatidylinositol phosphate (PIP-2) hydrolysis which increases intracellular Ca^{2+}, PDGF-R autophosphorylation on tyrosine residues, and PKC dependent phosphorylation of an 80 kd protein (Hill et al., 1990). However, the results reported by Sit et al., 1997, showed that genistein at 500 μM inhibited DNA sythesis but had no effect on levels of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). It is likely that the mitogenic signaling function of phosphoinositol second messengers is not always associated with protein tyrosine kinase activity.

Genistein at 224 μM also decreased Shc tyrosine phosphorylation, and Shc's association with Grb-2 and MAP kinase activity in both MCF-7 and SKBR3 cells. The latter cells overexpress c-erbB-2 with low levels of the EGF receptor and are estrogen receptor-negative. (Clark et al., 1996) Shc, which is tyrosine-phosphorylated by src and activated by growth factor receptors, and Grb-2, mediate signal transduction through ras and MAP kinases.

Genistein blocked EGF and insulin induced DNA synthesis in mouse fibroblast and NIH-3T3 cells, at an IC_{50} of 12 μM and 19 μM respectively. At 12 to 40 μM, genistein inhibited cell growth. S6 kinase activity was also inhibited by genistein at 6 μM with the maximal effect at 15 μM. (Linassier et al., 1990) However, at these concentrations, genistein did not inhibit EGF-, or PDGF- stimulated receptor phosphorylation or other

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protein tyrosine kinases that are involved in these pathways. These data imply that the activity of S6 kinase is specifically decreased in response to low concentrations of genistein.

Effect of Genistein on DNA Topoisomerases II

Genistein inhibits topoisomerase II (topoII) activity by stabilizing the DNA-topoII complex, called the cleavable complex (Yamashita et al., 1991). It does not intercalate, rather it is thought to interfere with the binding of ATP to topoII by inhibiting topoII's catalytic activity. Stabilization of this complex in tumor cells leads to double and single strand breaks in cellular DNA, leading to growth inhibition or cell death (Gewirtz 1991). The resulting fragmentation of DNA may be the result of the genistein-induced apoptosis (programed cell death) (Robinson et al., 1993). Genistein inhibits the activity of topoII in vitro with IC_{50} values around 111 μM (Constantinou et al., 1990 and Markovitz et al., 1989). Slight inhibition occurs at genistein concentrations as low as 11.1 μM; complete inhibition requires concentration higher than 185 μM. However at IC_{50} values for growth inhibition, little DNA damage is observed, suggesting that genistein does not inhibit cell growth through inhibition of topoII activity. On the other hand, topoII inhibitors are known to result in cellular differentiation. It has been reported that genistein at around 20 to 40 μM can induce tumor cell differentiation (Constantinou et al., 1990 and Krygier et al., 1997). Differentiation may change the ability of tumor cells to grow. Therefore, inducing tumor cell differentiation could be one mechanism by which genistein prevents cancer cell growth.

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Genistein As an Antioxidant

Production of reactive oxygen species (ROS), especially by activated cells of the immune system, has been postulated to play a role in carcinogenesis, particularly in tumor promotion. Genistein has been shown to decrease production of ROS by tumor cells and cells of the immune system (Akimura et al., 1992, Tanimura et al., 1992, Wei et al., 1993). Genistein can inhibit both the priming events necessary for high level ROS production or can directly inhibit agonist-stimulated ROS production, with IC_{50} values from 1.8 to 29.6 μM (Akimura et al., 1992, Tanimura et al., 1992, Wei et al., 1993). Genistein inhibits the production of hydrogen peroxide in response to the phorbol ester TPA in HL-60 cells, human polymorphonuclear cells and in a mouse skin tumorigenesis model (Wei et al., 1995). Genistein also inhibits the expression of the immediate early gene c-fos in this model. Another report showed that genistein can reduce oxidative DNA damage and lipid peroxidation and quench free radicals in rat liver (Cai et al., 1997).

Genistein and Cell Cycle Regulation

The cell cycle is defined as the interval during which cells duplicate and divide into two daughter cells. A complete cell cycle is composed of four phases: G1 (gap 1), S (DNA synthesis), G2 (gap 2) and M (mitosis) phases. Cell cycle is regulated at two checkpoints for the G1/S transition and the G2/M transition. The major components

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that control cell cycle are cyclin dependent kinases (cdks), cyclins which are positive regulators and cyclin dependent kinase inhibitors (CKIs) which are negative regulators. In G1/S checkpoint, the pRb, which is a protein encoded by the retinoblastoma tumor suppressor gene, plays a critical role in cell cycle regulation. pRb in turn is controlled through phosphorylation by the cyclin/cdk complex. Once pRb is phosphorylated, E2F is released to transactivate several critical genes including cdc2, thymidine kinase, myc, dihydrofolate reductase, Cyclin E required for progression from G1 to S phase. On the other hand, the G1/S progression may be blocked by CKIs such as p21 and p16. In G2/M checkpoint, cdc2 kinase and cyclin A or B complex play an important role to control cells entering either M phase or apoptosis. (Murakami et al., 1995 and King and Cidlowski, 1995)

Genistein has been shown to arrest a human gastric cancer cell line (HGC-27) at G2/M checkpoint at a concentration of 60 μM (Matsukawa et al., 1993) and also to arrest the MCF-7 (Pagliacci et al., 1994) and MDA-mB-231 cell lines (Santell, 1997) at G2/M checkpoint at concentrations equal to or greater than 50 μM. Genistein could function in arresting the cell cycle by suppressing or activating important regulatory proteins which are involved in the critical stages of the cell cycle either by inhibiting their tyrosine kinase activity or by altering the level of protein expression. Currently, there is no report studying the effect of genistein on cell cycle regulation or expression of cell cycle regulating genes in normal human breast epithelial cells (HBEC).

V. Biological Effects of Genistein

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Genistein is a phytoestrogen with low affinity for the estrogen receptor. It has been shown that genistein competes with 17 β-estradiol in receptor binding assays (Shutt and Cox 1972, Mathieson and Kitts 1980) and has estrogenic properties in cell culture and uterine weight assays (Cheng et al. 1980). Moreover, as a phytoestrogen, genistein is known to reduce reproductive performance of sheep grazing on subterranean clover, rabbits fed with soybean hay, captive cheetah fed a diet containing soybean protein and desert quail feeding on desert brush (Setchell et al. 1984 and Leopold et al. 1976). A decrease in reproductive performance was observed in female rats fed either a soy based diet or a diet supplemented with genistein (Carter et al. 1955). Estrogenic activity from components in these diets may act to prevent normal estrus in these animals and is a likely mechanism by which these diets act to alter reproduction. Human diets, containing 60 g of soy products providing 45 mg of isoflavones, increase the length of menstrual cycle in women (Cassidy et al. 1994), further suggesting that dietary phytoestrogens produce a biological response to altering the reproductive hormone balance in humans.

On the other hand, in two-thirds of studies on the effect of genistein-containing soy materials on animal models of cancer study, the risk of cancer was significantly reduced (Barnes, 1995). For example, genistein has been found to suppress mammary cancer which is induced by dimethylbenz[a]anthracene (DMBA) and to enhance mammary gland differentiation in female Sprague-Dawley SD rats which were exposed to genistein at prepubertal stage (Murrill et al. 1996 and Lamartiniere et al. 1995). Although these

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animal models have their limitations, it does indicate the possibility that genistein could be a chemopreventive agent in human subjects.

Dietary genistein has been found to induce prolactin secretion and increase uterine weight and mammary gland maturation in rats (Santell et al., 1997). Genistein also can be used as an inducer of tumor cell differentiation to slow down the proliferation of tumor cells (Constantinou et al., 1990 and Krygier et al., 1997). Genistein acts as an estrogen agonist to stimulate growth of cultured human breast cancer (MCF-7) cells at concentrations as low as 200 nM (Martin et al. 1978). Genistein can also stimulate MCF-7 cells to enter the cell cycle (pass through the restriction point in G1/S transition) (Dees et al., 1997). Genistein can inhibit the proliferation of several different cancer cell lines in vitro (Barnes, 1995). The chemotherapeutic effect of genistein on tumor growth in vivo is still unclear. No systematic study of genistein on normal HBEC has been done. Further investigations are reguired.

VI. Concentration of Genistein in Individuals Consuming Soy Beans and Soy Products

It is important for the evaluation of the significance of the cell culture or animal model studies to know the concentrations of genistein present in individuals consuming a soy-containing diet. Urinary excretion of genistein in Japanese men and women consuming a traditional Japanese diet was as high as 15.5 μM per day with a mean value of 6 μM per day (Adlercreutz et al., 1991). In a different study, Adlercreutz et al., (1993) reported that plasma total genistein concentrations can be as high as 0.1 μM in some

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vegetarian women. Later, Adlercreutz et al., (1995) has suggested that the plasma level of genistein in people on a high-soy-containing diet was 1-4 μM . Nonetheless, reasonable estimates for the plasma level of genistein can be inferred from consideration of dietary intake and rates of metabolism and excretion (Barnes, 1995). A person consuming 35 g/day of soybeans (the average amount consumed by Taiwanese) (Soyatech Survey, 1991) has an intake of around 50 μg of genistein which is mostly in the glycoside form. Maximum plasma levels, 23 μM, would be attained if genistein was fully absorbed from the diet and not metabolized or excreted. But, in reality, the equilibrium plasma concentration would be 3.3 μM if one assumes that genistein equilibrates with total body water. Based on the above estimates, genistein would have less than a 20% inhibitory effect on cellular processes with IC_{50} values of greater than 13.2 μM (Barnes, 1995). It also has been suggested that the physiological serum concentrations of genistein are less than 18.5 μM (Peterson, 1995).

VII. Metabolism of Genistein

Liver and the lower gut are two of the major metabolic sites of the human body for genistein metabolism. Dihydrogenistein was the only metabolite of genistein detected in human urine samples (Joannou, G.E. et al. 1995). It is a metabolite found in the lower gut and is generated by the metabolism of gut flora. Most oxidation and conjugation reactions are carried out by the liver. For example, the liver can form genistein-sulfate, genistein-glucuronide and so on. *p*-Ethyl phenol was detected as a metabolite of genistein in the urine of rumenants (Batterham et al., 1965, Batterham et al.,

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1971, Shutt et al., 1971). However, it was not detected in human urine. Furthermore, Peterson et al., (1996) conducted a mtabolism experiment with [4-^{14}C] genistein using MCF-7 and normal human mammary epithelial cells, and demonstrated that they produce several metabolites such as genistein 7-sulfate and hydroxylated and methylated form of genistein sulfate. However, the metabolism studies of genistein in humans showed a great variation in the production of metabolites. The reports in this area are limited. Further investigation is needed.

VIII. Dihydrogenistein (DHG)

Dihydrogenistein is the only metabolite of genistein found in the urine of human volunteers (Joannou, G.E. et al. 1995). When volunteers consumed soy (Kelly, G.E. et al. 1993), urinary dihydrogenistein was detected and identified by profile capillary gas chromatography (GC) and electron ionization mass spectrometry (GC-EIMS) analysis of the trimethylsilyl ether (TMS) derivatives. However, no quantitation of the concentration of dihydrogenistein in urine was reported. In another study, dihydrogenistein was found when pure genistein were fermented with human fecal bacteria under anaerobic conditions (Chang, Yu-Chen and Nair, M.G. 1995a). Dihydrogenistein is a reduction product of genistein by hydrogenation at the C2 and C3 positions. Chang and Nair (1995b) synthesized dihydrogenistein from genistein by hydrogenation to provide sufficient quantities for in vitro studies. Additionally, Chang and Nair (1995a) conducted fungal, bacterial, yeast and mosquito bioassays with dihydrogenistein to evaluate the antifungal, antibacterial, mosquitocidal , nematocidal

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and topoisomerase effects. To date, no other characterization of dihydrogenistein has been conducted in human cells. Therefore, it is important to further investigate differences in the biologic effects between genistein and dihydrogenistein in human cells. Furthermore, dihydrogenistein, formed after bacterial biotransformation, is likely to have different biological effects from those of genistein.

IX. Experimental Evidence for the Role of Genistein in Chemoprevention of Breast Cancer

The ability of chemicals to transform human breast epithelial cells appears to be dependent on host developmental conditions, such as age at first pregnancy and history of pregnancy at the time of exposure to a carcinogen. Therefore, the timing of exposure is considered to be an important factor in chemoprevention of breast cancer. Pregnancy induces full differentiation of the mammary gland, with reduction in the number of terminal end buds, resulting in refractoriness of the mammary gland to carcinogenesis (Russo et al. 1990a). A placental hormone such as human chorionic gonadotropin (hCG) administered produces a degree of differentiation in the rat mammary gland similar to that induced by pregnancy (Russo et al. 1990b). This observation indicates that gland differentiation is important in prevention of chemically-induced carcinogenesis. The mammary gland differentiation can be induced by means other than pregnancy such as hormone and hormone-like agents as an approach for mammary cancer reduction (Russo et al. 1988). Hence, a hormone-like compound such as genistein could be administrated at different time points during tumor initiation or promotion to study their

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In 17 of 26 studies on the effect of genistein-containing soy materials on animal models of cancer, the risk of cancer was significantly reduced and none showed that soy increased cancer risk (Messina et al., 1994 and Barnes, 1995). For example, genistein and other genistein-containing soy materials, such as soy protein isolates, whole soy beans and Miso (fermented soy paste), have been found to protect dimethylbenz[a]anthracene (DMBA) and N-methyl-N-nitrosourea (MNU) and x-ray irradiation induced mammary cancer in female Sprague-Dawley SD rats (Sharma et al., 1992, Barnes et al., 1990, Baggott et al., 1990, Hawrylewicz et al., 1991 and Troll et al., 1980). Genistein also has been found to suppress mammary cancer which is induced by DMBA and to enhance mammary gland differentiation in female SD rats which were exposed to genistein at prepubertal stage (Murrill et al. 1996 and Lamartiniere et al. 1995). The above reports indicate that genistein can alter the development of the mammary gland during the early prepubertal stage, and support the idea that genistein may decrease the number of target cells for neoplastic transformation by inducing the differentiation of target cells. However, this hypothesis needs to be substantiated by more evidence.

X. Anatomy and Component Cells of The Mammary Gland

The basic architecture of the mammary gland is a complex structure composed of parenchyma and stroma. The parenchyma consists of one or two major lactiferous ducts

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that grow from the nipple into the surrounding fat pad. The terminal end buds (TEBs) which are the portion of the gland opposite to the nipple contains the most actively growing terminal ductal structures. TEBs are believed to contain mammary epithelial stem cells, especially at the tip (cap cells) which differentiate into luminal and basal mammary epithelial cells that form the mammary gland. TEBs are also believed to be the target for mammary carcinogenesis. (Russo and Russo, 1996)

The human breast contains a variety of cell types, including luminal and basal epithelial cells that line the interior and exterior of the ductal tree, respectively. Two types of morphologically distinguishable normal human breast epithelial cells (HBEC) have been derived from reduction mammoplasty and characterized (Kao et al., 1995). Type I cells expressed ER (Kang et al., 1997) and show luminal cell markers [i.e. epithelial membrane antigen (EMA), and cytokeratin-18] and stem cell characteristics. The morphology of Type I cell colonies are morphologically distingnishable from that of Type II cells (Kao et al., 1995). Type II cells express the basal cell markers such as α-6 integrin, cytokeratin-14 and the expression of gap junction genes, connexins 43 and 26 (Kao et al., 1995).

XI. Role of Stem Cell and Differentiation in Carcinogenesis

Tumor cells are considered to be less differentiated from normal cells as a result of dedifferentiation or blocked differentiation in stem cells which give rise to cancer cells (Varmur and Weinberg, 1993). Furthermore, cancer has been described as a disease of

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differentiation (Markert, 1968) or oncogeny as blocked ontogeny (Potter, 1978). As mentioned before, early full-term pregnancy decreases risk for breast cancer. This has been explained by John Cairns (1975) as related to stem cell multiplication that occurs commencing at the time of puperty and during each ovarian cycle until, but not after, the first pregnancy or by Russo at al. (1990) as inducing full differentiation of the mammary gland by pregnancy.

Type I HBEC have stem cell characteristics (i.e. ability to differentiate into Type II HBEC and to form budding / ductal structures on Matrigel) and were more susceptible to neoplastic transformation (i.e. SV40 large T-antigen induced anchorage independent growth and spontaneous immortalization of SV40 transformed HBEC at high frequency in Type I cells but not Type II cells) (Kao et al., 1995; Chang et al., 1996). Furthermore, breast cancer cells share many phenotypes of Type I HBEC such as deficiency in gap junctional intercellular communication, expression of luminal epithelial cell markers, estrogen receptors and telomerase (Kao et al., 1995; Kang et al., 1997), supporting the oncogeny as blocked or partially blocked ontogeny theory (Potter, 1968).

CHAPTER 3

Estrogenic Effects of

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ABSTRACT

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CHAPTER 3

Estrogenic Effects of Genistein on Growth of Estrogen Receptor Positive Human Breast Cancer (MCF-7) cells In Vivo

ABSTRACT

Genistein, found in soy products, is a phytochemical with several biological activities. In the current study, our research focused on the estrogenic and proliferative activity of genistein. We have demonstrated that genistein enhanced the proliferation of estrogen-dependent human breast cancer (MCF-7) cells in vitro at concentrations as low as 10 nM with 100 nM producing similar proliferative effects as estradiol at 1 nM. Expression of the estrogen-responsive gene, pS2, was also induced in MCF-7 cells in response to treatment with a concentration of genistein as low as 1 nM. At higher concentrations (above 20 μM), genistein inhibited MCF-7 cell growth. In vivo, we have shown that dietary treatment with genistein (750 ppm) for 5 days enhanced mammary gland growth in 28 day old ovariectomized athymic mice indicating that genistein acts as an estrogen in normal mammary tissue. To evaluate whether the estrogenic effects observed in vitro with MCF-7 cells could be reproduced in vivo, MCF-7 cells were implanted subcutaneously in ovariectomized athymic mice and the growth of the estrogen-dependent tumors was measured weekly. Negative control animals received AIN-93G diet only, the positive control group received a new subcutaneous estradiol (2 mg) pellet plus AIN-93G diet, and the third group received genistein at 750 ppm in the AIN-93G diet. Tumors were larger in the 750 ppm genistein treated group compared to the negative

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control group, demonstrating that dietary genistein was able to enhance the growth of MCF-7 cell tumors in vivo. Increased uterine weights were also observed in the genistein treated groups. In summary, genistein can act as an estrogen agonist in vivo and in vitro resulting in proliferation of cultured human breast cancer cells (MCF-7), induction of pS2 gene expression and stimulation of normal murine mammary gland growth.

INTRODUCTION

The phytoestrogen, genistein, is present at high concentrations in plant foods such as soybeans. Genistein and other isoflavones are known to reduce reproductive performance of sheep grazing on subterranean clover, rabbits fed soybean hay, captive cheetah fed diets containing soybean protein and desert quail feeding on desert brush (Setchell et al., 1984 and Leopold et al., 1976). Additionally, a decrease in reproductive performance was observed in female rats fed either a soybean based diet or a diet supplemented with genistein (Carter et al., 1955), and has been attributed to an estrogenic effect produced by dietary phytoestrogens. In vitro, genistein at low concentrations (200nM) stimulates the growth of cultured human breast cancer (MCF-7) cells (Martin et al., 1978) and enhances expression of the estrogen-responsive pS2 gene (Wang et al., 1996). These effects can be inhibited by tamoxifen (Wang et al., 1996) further confirming that these effects are mediated via the estrogen receptor. Conversely, high concentrations of genistein (above 10 μM) inhibit growth of estrogen-dependent human breast cancer cells in vitro. Gensitein blocks the cell cycle at G2/M and this inhibitory effect is likely mediated via inhibition of tyrosine phosphorylation (Pagliacci et al., 1994).

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It is well documented that estrogens act to stimulate growth of estrogen-dependent mammary tumors in vivo. However, there are numerous reports which suggest that estrogen and estrogen agonists, such as genistein, can act as chemopreventative agents to inhibit the development of carcinogen-induced mammary tumors in laboratory animals (Setchell et al., 1984, Adlercreutz et al., 1990 and Barnes et al., 1990). When genistein was administered subcutaneously to neonatal or pubertal Sprague-Dawley rats followed by initiation of mammary tumors with DMBA (dimethylbenz[a]anthracene) a reduction in tumor incidence was observed when compared to control rats. The authors concluded that genistein, like other estrogen agonists, may exert its chemopreventative action by enhancing cell maturation, thus reducing cell proliferation in the mammary gland and subsequent initiation by DMBA. This presents a paradox since it is also well documented that estrogens stimulate growth of estrogen-dependent tumors (Henderson et al., 1988). The resolution of this paradox is in the timing of the estrogen administration. If an estrogen is administered prior to mammary gland maturation and initiation with a mammary carcinogen then the number of tumors will likely be reduced due to the effect of estrogen to cause mammary gland maturation. However, if the estrogen is administered to an animal after the development with an estrogen-dependent tumor the growth of this tumor will be stimulated. The present study was undertaken to test the hypothesis that dietary genistein will act as an estrogen agonist to stimulate growth of MCF-7 cells in vivo, in athymic mice. Thus, in the present study, we examined the effects of dietary genistein on: (i) the growth of MCF-7 cells implanted into ovariectomized athymic nude mice, and also on (ii) blood genistein levels, and (iii) stimulation of mammary gland growth. We present the novel finding that dietary genistein stimulates mammary gland development and enhances the growth of MCF-7 cell tumors in ovariectomixed athymic mice.

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MATERIALS AND METHODS

In Vitro studies

Cell Proliferation Study. MCF-7 cells were maintained in IMEM media (Biofluids Inc., Rockville, MD) containing 5% fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (100 μg/ml). Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were harvested, and then plated at $1.5x10^4$ cells per well in 24 well tissue culture polystyrene plates in media containing 5% FBS-IMEM media for twenty-four hours. Media was removed, cells were washed with 150 mM phosphate buffered saline (PBS) and then incubated with media containing 5% charcoal dextran-treated fetal bovine serum (Hyclone Inc., Logan, UT) in phenol-red free media for an additional forty-eight hours. The media was replaced with the same media containing genistein. Genistein was synthesized as described by Chang, et al. 1994. Concentrations of genistein utilized were at 1 nM, 10 nM, 100 nM, 200 nM, 500 nM, 1 μM or 10 μM. Proliferation was assessed by the increase in DNA content daily for 96 hours. To determine DNA content, cells were lysed in situ with 10 mM EDTA (pH 12.3) at 37°C for 30 minutes, neutralized with KH_2PO_4 and then Hoechst reagent 33258 was added. Fluorescence was measured by excitation at 350 nm and emission at 455 nm to determine DNA content when compare to a standard of salmon sperm DNA (West et al., 1985 and Labarca et al., 1980).

Cell Culture and Treatment for Expression of pS2 mRNA. MCF-7 cells were cultured in 100 mm x 20 mm polystyrene tissue culture plates with the growth conditions and growth media described above. One week prior to the initiation of experimental treatments, cells were switched to medium supplemented with 5% charcoal-dextran-stripped fetal bovine serum. Cells were grown

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to confluence and removed from the tissue culture plate using trypsin-EDTA (5 mg/ml trysin and 2 mg/ml EDTA) and replated at a density of $1x10^6$ cells/100mm plate. Due to its potential estrogenic effect, phenol-red free media was utilized twenty-four hours prior to treatments, the cells were switched to medium supplemented with phenol-red free IMEM containing penicillin (100 units/ml), streptomycin (100 μg/ml) and 5% charcoal-dextran-stripped fetal bovine serum. Twenty-four hours after initial plating of the MCF-7 cells, estradiol or genistein was added. The final concentrations of estradiol in the culture media were 200 pM, 1 nM and the final concentrations of genistein were 1 nM, 10 nM, 100 nM, 1 μM. Both estradiol and genistein were dissolved in DMSO; the final concentration of DMSO in the medium was 0.1%. The growth medium was changed every 36 hours after initial plating, and fresh estradiol and genistein were added with each media change.

RNA Isolation. RNA was isolated 72 hours after addition of estradiol or genistein using the method of Chomczynski et al. (Chomczynski et al., 1987) as modified by Xie and Rothblum (Xie et al., 1991). Briefly, the cells were lysed with 1.7 ml of lysis butter which contained 4 M quanidium thiocyanate, 25 mM sodium citrate, 100 mM 2-mercaptoethanol and 0.5% sodium sarcosyl (pH 7.0). The cell lysate was transferred from the tissue culture plate to a 15 ml Corex tube, and mixed with water-saturated phenol containing 0.04% (w/w) hydroxyquinoline and 2 M sodium acetate (pH 5.0) in a ratio of 10:10:1 followed by the addition of 0.36 ml of chloroform:isoamyl alcohol (24:1). Tubes were vortexed for 10-20 seconds, placed on ice for 30 minutes, and then centrifuged at 12,000 x g for 30 minutes at 4°C. The upper layer containing the RNA was then transferred to a new tube containing an equal volume of isopropanol at -20°C. Samples were vortexed and RNA was precipitated by placing the mixture at -20°C for at least two hours. The RNA precipitate was recovered by centrifugation at 12,000 x g for 30 minutes. The

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resultant pellet was washed twice with 70% ethanol, and dried under vacuum. RNA was resuspended in 100 µl diethyl pyrocarbonate-treated (DEPC) water. Ten ug of RNA were used for Northern blot analysis.

Probe for pS2. A plasmid containing a 559 base pair cDNA fragment encoding full length pS2 (Jakowlew et al., 1984) was obtained from the American Type Culture Collection. PstI restriction endonuclease digestion of the plasmid (Jakowlew et al., 1984) produced DNA fragments of 4.4, 0.32 and 0.24 kb. These fragments were isolated from the agarose gel slice using the methods adapted from Moore, Chory and Ribaudo (Moore et al., 1995). Twenty-five ng of pS2 cDNA (0.32 and 0.24 kb) and 50 µCi [α-^{32}P]dCTP were used to label the DNA by the random primed labeling method using a commercial kit and following the manufacturer's instructions (Gibco BRL, Gaithersburg, MD).

Northern Blot Hybridization and Detection. For detection of pS2 expression, 10 µg of total RNA were separated on 1.2% formaldehyde agarose gels and transferred to a Hybond-N Nylon membrane (Amersham, Arlington Heights, IL). The RNA was cross-linked onto the membrane using UV light for 3 minutes by a 25 watt transilluminator (Hoefer Scientific Instruments, San Francisco, CA) wavelength 312 nM. The membrane was then hybridized with the ^{32}P-labeled DNA probe and hybridizing RNA molecules detected by performing autoradiography. Northern blots were also probed with a human glucose-3-phosphopate-dehydrogenase (G3PDH) cDNA (Clontech Laboratories, Inc., Palo Alto, CA) as a house keeping gene, to confirm equal loading of RNA among treatment and control groups.

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Diet Selection. Diet selection was based upon recommendations of the American Institute of Nutrition (AIN) (Reeves et al., 1993). The AIN93G Semi-Purified Diet has been established as meeting all the nutritional requirements of mice. One concern about the use of a commercial diet is the presence of phytoestrogens from plant sources such as soybean meal. Protein in the AIN-93G diet is derived from casein; thus, the potential interference of phytoestrogens in soy based protein supplements is eliminated.

Estrogen Pellet Preparation. Each pellet of estradiol contained 2 mg of 17β-estradiol mixed with 18mg of cholesterol as a carrier. The 20 mg mixture containing estradiol and cholesterol were placed into the pellet mold and pressed into a compact pellet approximately 4.5 mm in diameter and approximately 2.5 mm in depth. These pellets then were placed subcutaneously in the interscapular region of mice as previously described (McManus et al., 1981).

Analysis of Uterine Weight and Mammary Gland Growth in Ovariectomized Athymic Female Mice Fed Genistein. Athymic nude mice (Harland, Indianapolis, IN) were ovariectomized at 21 days of age. Dietary treatments were initiated at 28 days of age for a period up to 5 days. Mice were fed by the American Institute if Nutrition (AIN) 93G semi-purified diet containing estradiol at 1 ppm or genistein at 750 ppm. Control animals received only AIN-93G diet. Mice were killed and the uteri were removed for assessing wet weights and mammary gland growth. Mammary glands were removed, fixed and stained as whole mounts (Banerjee et al., 1976). Mammary gland growth was assessed by determining the size and number of endbuds and the extent of ductal development as viewed under a dissecting microscope (Haslam, 1988).

Analysis of Tumor Growth in Athymic Nude Mice Fed Genistein. Female athymic nude mice,

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purchased from a commercial source at 21 days of age (Harland, Indianapolis, IN), were allowed to acclimate for one week. Mice were ovariectomized at 28 days of age, 7 days prior to any treatment. Estrogen-dependent human breast cancer (MCF-7) cells were grown to confluence and collected using trypsin-EDTA. Cells were counted using a hemacytometer (Fisher Scientific, Pittsburgh, PA) and diluted in culture media to a concentration of 1 X 10^7 cells per ml. 100 µl aliquots of cell suspension were injected into four sites in the flanks of five week old female athymic mice. MCF-7 cells will not produce tumors in ovariectomized athymic nude mice unless the mice are supplemented with estrogen. Estradiol was administered in pellets containing 2 mg of estradiol (McManus et al., 1981 and Gottardis et al., 1989) implanted subcutaneously in the interscapular region of mice. Tumors were allowed to develop for 5-6 weeks, and were measured weekly. The cross sectional areas were determined using the formula [length/2 * width/2 * π] as previously described (Gottardis et al., 1989). When tumors reached an average cross-sectional area of 35-40 mm^2, animals were divided into treatment groups with each group normalized for tumor number, tumor size and animal number. Mice were divided into three groups and assigned to treatments (4-6 animals/ group). Estradiol pellets were removed from all animals. Positive controls were re-implanted with a fresh estradiol pellet, negative control mice received AIN-93G diet, and dietary treatments initiated with 750 ppm genistein. Tumor areas were measured weekly. At the completion of the study mice were killed and the uteri were removed for assessing wet weights. Blood was collected from animals by cardiac puncture for analysis of genistein content.

Plasma Genistein Concentration Analysis in Athymic Nude Mice Fed Genistein. Blood from cardiac puncture was placed into EDTA containing tubes and centrifuged at 500 x g for 10 minutes. Plasma was separated and stored at -20°C until analysis. A known amount of daidzein, which

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RESULTS

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differs from genistein in chemical structure by one hydroxyl group, was added to each sample to serve as an internal standard. Daidzein and genistein have similar chromatographic properties. Therefore, the isolation and quantification of daidzein provided a recovery standard which could be used for analyzing the effectiverous in recovery of genistein. Each sample was split into 2 aliquots. One aliquot was incubated with β-glucuronidase/sulfatase for 24 hours at 37°C to liberate free genistein from its conjugated form. The other aliquot was handled in a similar manner without β-glucuronidase/sulfatase. Samples were applied to a C_{18} sep-pak column, washed with 10 ml of H_2O (HPLC grade), and eluted with 10 ml of 85% MeOH. The samples were dried in a vacuum spin dryer (Savant, Farmingdale, NY) and reconstituted in 100 µl 85% MeOH. Twenty microliters were utilized for HPLC analysis using a reverse phase C_{18} column. The mobile phase consisted of 50% methanol, 50% water and 0.1% acetic acid, with a flow rate of 1 ml/min (Santell et al., 1997). The absorbance in samples was monitored using a spectrophotometer at 260 nM.

Statistical analysis. Tumor area data were analyzed statistically using a repeated-measures protocol in a split-plot design (Carmer et al., 1973) and the General Liner Models procedure (GLM) in the SAS program (SAS Institute Inc. SAS User Guide, 1985). Independent variables were treatments (estrogen, genistein, or control), tumor nested within treatments, week, and the treatment*week interaction. Treatment means for each week were compared using the Least Significant Difference (LSD) method.

RESULTS

Effect of Genistein on MCF-7 Cell Proliferation in Vitro. In order to determine the minimum

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concentration of genistein which will stimulate or inhibit MCF-7 cell growth we conducted a cell proliferation dose response study. MCF-7 cell DNA content was measured in response to estradiol (1 nM) and various concentrations of genistein ranging from 0.01 μM to 100 μM. (Figure 1 expressed as percentage of the control cell cultures). These levels were chosen because genistein blood levels reported in animals and humans consuming diets high in genistein (such as soy containing diets) range from 0.1 to 6 μM (Xu et al., 1995). Estradiol (1 nM) stimulated cell proliferation 2.4 fold over the control MCF-7 cells. Genistein increased cell growth in a dose-dependent manner in the range of 0.01 μM to 1 μM. Maximal growth stimulation (approximately 3 fold over control) was observed at 1 μM and was sustained at this level of stimulation dose up to 10 μM. In contrast, higher concentrations of (25-100 μM) genistein produced a dose-dependent decrease in cell growth when compared to nontreated controls.

Effect of Genistein on Estrogen-dependent pS2 mRNA Expression in MCF-7 Cells. To evaluate the potential of genistein to enhance expression of an estrogen-repsonsive gene, we conducted Northern blot analysis using RNA isolated from genistein-treated MCF-7 cells and probing with a human pS2 cDNA probe (Jakowlew et al., 1984). The cell proliferation studies (Figure 1) suggested that genistein acts via the estrogen receptor to enhance cell proliferation at low concentrations. Estradiol at concentrations of 0.2 nM and 1 nM was also observed to induce pS2 gene expression (Figure 2). Stimulation of pS2 mRNA expression by genistein occurred in a concentration-dependent fashion. The stimulatory effect of genistein on pS2 gene expression was observed at concentrations as low as 0.01 μM (Figure 2). Genistein stimulated pS2 gene expression at concentrations ranging from 0.01 μM to 50 μM. These data indicate that genistein can act as a week estrogen agonist in vitro as measure by induction of estrogen-dependent gene expression.

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Effect of Genistein on Uterine Weight and Mammary Gland and End Bud Development in Ovariectomized Athymic Female Mice. Mammary gland whole mounts, were obtained from ovariectomized mice consuming either estradiol (1 ppm) or genistein (750 ppm) in AIN-93G diet or controls fed AIN-93G diet only. In pubertal mice, mammary gland growth occurs via ductal elongation with the end buds as the major growth points (Lyons et al., 1958). Increases in both the numbers and size of end buds was observed in animals consuming estradiol and genistein (Figure 3). The average end bud number was 2.9± 0.85 in the ovariectomized control group, 9.1±0.67 in the estradiol group and 6.4± 0.89 in genistein treated groups (Figure 4). Mammary gland growth was significantly greater ($P<0.05$) in mice fed genistein or estradiol when compared to ovariectomized controls. Genistein and estradiol treated animals were not significantly different from each other ($P>0.05$). The uterine weight of the genistein treatment group was also increased in comparison with control group. These data suggest that dietary genistein has the potential to stimulate estrogenic responses in vivo.

Effect of Genistein on Tumor Growth in Athymic Nude Mice. Since genistein stimulated both mammary gland and uterine growth, it was of interest to determine the effects of an estradiol (2 mg) pellet or dietary genistein (750 ppm) on MCF-7 cell tumor growth. Ovariectomized athymic mice, implanted with MCF-7 cell tumors, were divided into three treatment groups after tumors reached an average cross-sectional area of approximate 55 mm^2. The three treatment groups were 1) negative controls (without estradiol pellets or genistein treatments), 2) positive controls (estradiol pellets) and 3) dietary genistein (750 ppm) treatment groups. Tumor growth was monitored weekly.

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Tumors in the positive control group grew rapidly and after an additional two weeks on estrogen treatment, the average tumor cross-sectional area had reached 120 mm^2(Figure 5). After two weeks of estradiol treatment the average tumor weight was 750 mg and total tumor weight per mouse was approximately 3 gram (approximately 10% of the body weight). Therefore, mice were killed after two additional weeks on estrogen treatment due to size of the tumors. After the estrogen pellets were removed in the negative control group, tumor growth stopped and tumor size was maintained at an average cross-sectional area of approximately 58 mm^2 for an additional 12 weeks (Figure 5). In the dietary genistein treatment group, after the estrogen pellets were removed and mice were switched to 750 ppm genistein diets, tumor growth increased. The average tumor cross-sectional area in dietary genistein treatment group increased from 55 mm^2 to 120 mm^2 after 12 additional weeks on genistein treatment. These MCF-7 cell tumors were similar in size to tumors after two weeks on estradiol treatment. These results indicated that dietary genistein acts as an estrogen agonist in vivo to stimulate growth of estrogen-dependent MCF-7 tumor cells implanted into athymic mice.

Plasma Genistein Concentration Analysis in Athymic Nude Mice Fed Genistein. Plasma genistein levels were measured by high performance liquid chromatography (HPLC). The concentration of free genistein in plasma was 0.24 ± 0.08 μM. The concentration of total genistein including free and conjugated forms in plasma was 2.1 ± 0.14 μM. These concentrations are within the range of concentrations which stimulated pS2 mRNA expression (Figure 2) and the growth of MCF-7 cells (Figure 1).

DISCUSSION

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Genistein elicits a concentration-dependent dual threshold effect with regard to growth stimulation or inhibition on cultured estrogen-dependent human breast cancer (MCF-7) cells in vitro. At low concentrations of genistein, in dextran charcoal-strip fetal bovine serum, we observed a dose-dependent increase in MCF-7 cell proliferation from 0.01-1 μM (Figure 1). Maximal growth stimulation was observed at 1 μM and this level of growth was sustained up to 10 μM. At higher concentrations, we observed a dose-dependent inhibition in cell growth when concentrations were 25 μM-100 μM. These results are consistent with those reported by Martin et al. (1978), in which growth stimulation in MCF-7 cells was observed a concentration of 200 nM. Wang et al. (1996) observed a concentration-dependent growth stimulation effect between 10 nM and 1 μM. Wang et al. (1996) also observed that growth inhibition at concentrations higher than 10 μM. These results suggest that there are at least two dose-dependent mechanisms by which genistein can alter cell proliferation. One mechanism stimulates proliferation at low concentrations of genistein (10 nM to 1 μM) and is likely mediated via the estrogen receptor. The other mechanism, which is anti-proliferative, is active at high concentrations of genistein (25-100 μM) and is likely mediated via anti-tyrosine phosphorylation and inhibition of cell cycle progression (Pagliacci et al., 1994). The threshold for the stimulation of proliferation was in the range of concentrations required to stimulate expression of pS2 mRNA (Figure 2). Additionally, pS2 gene expression was enhanced at concentrations up to 50 μM. However, cell proliferation was inhibited at concentrations above 25 μM. These observations suggest that the phytoestrogen estrogen receptor-mediated mechanism is active at the higher concentrations however the growth inhibitory mechanism associated with genistein overrides the growth stimulatory effects from genistein.

Genistein binds to the estrogen receptor with an affinity approximately 100-fold less than that of estradiol (Wang et al., 1996 and Santell et al., 1997). To further confirm whether genistein is acting via an estrogen receptor mechanism, we evaluated expression of the pS2 gene in cultured MCF-7 cells. The human pS2 gene was initially characterized as a gene whose expression is specifically controlled by estrogen in the breast cancer cell line MCF-7 (Brown et al., 1984). The increase in pS2 mRNA after addition of estradiol to the culture medium is a primary transcriptional event, suggesting that control of pS2 gene promoter activity by the estrogen receptor is mediated by a cis-acting estrogen-responsive element that could be located in the 5'-flanking region of the pS2 gene (Jakowlew et al., 1984). pS2 stimulation by genistein (Figure 2) is consistent with the results observed on cell proliferation in that 10 nM (Figure 1) was effective of increasing proliferation and pS2 gene expression. Additionally, Wang et al. reported that tamoxifen will inhibit genistein-stimulated pS2 gene expression in MCF-7 cells. These data further suggest that genistein can act via the estrogen receptor mediated mechanism.

Here we report that dietary genistein (750 ppm) fed to 28 day athymic ovairectomized mice will stimulate mammary gland growth (Figure 3, 4). This is the first report in which dietary genistein has been shown to enhance mammary gland growth in vivo. Recent reports (Lamartiniere et al., 1995 and Murrill er al., 1996) in which high dosages of genistein were administered subcutaneously have been reported to stimulate mammary gland differentiation, promoting lobuloalveolar development in immature Sprague-Dawley rats. No studies have been reported in which the effect of genistein was evaluated for its potential to stimulate growth of estrogen-dependent tumors in vivo. The studies presented here provide compelling evidence that MCF-7 estrogen-dependent tumors can be stimulated to grow in ovariectomized athymic

host mice supplemented with dietary genistein (750 ppm). It is important to point out that both estrogen and genistein have been shown to be chemopreventive in the rat DMBA animal model (Lamartiniere et al., 1995, Murrill er al., 1996 and Nagasawa et al., 1974). Estrogen administered to neonatal rats has been shown to reduce the incidence of DMBA-induced mammary tumors (Nagasawa et al., 1974). In the studies with genistein, three subcutaneous injections of genistein were given to young rats which were protective against DMBA initiated carcinogenesis. The authors suggest that genistein is acting as an estrogen agonist to stimulate mammary gland differentiation. This enhanced mammary gland differentiation may be the mechanism responsible for the chemopreventive effects of genistein in the rat DMBA mammary carcinogenesis model. Thus, there appears to be a paradox with regard to the action of genistein on tumor growth in rodent models. Our results suggest in the ovariectomized athymic mouse that genistein stimulates mouse mammary gland growth without maturation which is different than the effect of genistein in the immature rat in which genistein stimulate prolactin secretion (Santell et al., 1997) and ultimately results in lobuloalveolar development and mammary gland maturation (Lyons et al., 1958). Genistein may act similarly to estradiol because estradiol is known to reduce tumor incidence in some instances (Miller, 1990) whereas there are numerous reports (Henderson et al., 1988) that indicate that estrogen act as tumor promoters. Thus, whether genistein acts as a chemopreventative agent or as a tumor promoter will likely depend on the timing of administration of the genistein.

The present study focused on the estrogenic effect of genistein in vitro and in vivo systems. Genistein binds to the estrogen receptor, with an affinity approximately 100-fold lower than estradiol, resulting in enhanced proliferative activity. In culture cells, genistein stimulates MCF-

7 cell growth at concentrations as low as 100 nM achieving similar effects to that of estradiol at 1 nM (Figure 1). We observed a 240% increase in growth at 100 nM genistein treatment compared to control. Expression of the estrogen-responsive gene, pS2 was also induced in response to treatment with genistein at concentrations higher than 100 nM (Figure 2). In vivo, using ovariectomized athymic nude mice implanted with estrogen-responsive MCF-7 cells, we have demonstrated that treatment with 750 ppm dietary genistein was able to stimulate the growth of these estrogen-responsive MCF-7 cell tumors in the absence of estrogen (Figure 5). Plasma genistein was 240 nM (free form) which is above the concentration required for MCF-7 cell growth stimulation observed in vitro. From this concentration, one can predict from the in vitro cell growth studies that at this dosage of genistein will be stimulatory to implanted MCF-7 cell tumors. Thus, these results are consistent with in vitro cell culture studies. At high concentrations (above 25 μM) in vitro, a dose-dependent decrease in MCF-7 cell growth is observed, however, it is unlikely that the concentrations required to inhibit MCF-7 cell growth can be achieved in vivo.

In summary, genistein can act as an estrogen agonist resulting in proliferation of human breast cancer cells (MCF-7) in vitro and enhances growth of MCF-7 cell tumors implanted into ovariectomized athymic nude mice in vivo. Thus, there is the potential of dietary genistein to stimulate growth of estrogen-dependent tumors in women with low circulating endogenous estrogen levels such as in postmenopausal women.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Y.C. Chang and Dr. M.G. Nair for providing genistein. Thanks are also due to Dr. P. Dickerson-Weber for her technical assistance and Dr. L. Bourquin for his statistical consultant. Sincere appreciation is extended to Dr. J. Linz for his critical review of the manuscript.

Figure 1. Effects of estradiol and genistein on the growth of estrogen responsive MCF-7 cells. MCF-7 cells were cultured in the presence of various concentrations of genistein (10 nM-100 uM) or control media for 96 hours, in IMEM media containing 5% fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (100 ug/ml) at 37°C in a humidified atmosphere of 5% CO_2 in air. Proliferation was assessed by DNA content as measured using HOEST reagent and fluorometric analysis (expressed as percent of control). Fluorescence was measured by excitation at 350 nm and emission at 455 nm and was used to determine DNA content. The results (mean, n=8) are expressed relative to cells grown without genistein. C represents the vehicle control and E represented as treatment with 1 nM estrogen in media.

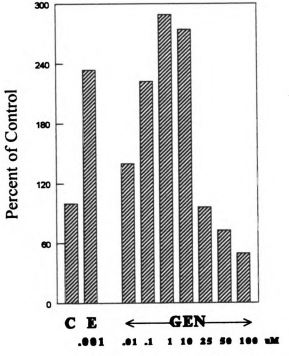
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Figure 2. Concentration-dependent stimulation of pS2 gene expression by genistein. MCF-7 cells were cultured in IMEM or MEM media in the presence of various concentrations of genistein (1 nM-50 μM). RNA was isolated after 72 hours. Northern blot analysis and detection for pS2 was performed using standard as described in methods.

Con
E2 1nM
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Gen 100nM
Gen 500nM

Con
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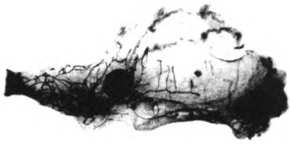
E2 200pM
Gen 1uM
Gen 10uM
Gen 25uM
Gen 50uM

Figure 3. Mammary whole gland mounts from ovariectomized athymic mice fed various dietary treatments. Mice were ovariectomized on day 28, and treatments begun on day 35. Mice were fed A, control (AIN 93-G), B, genistein (750 ppm) or C, estradiol (1 ppm) containing diets. Mice were killed on day 40 and mammary glands were removed for whole gland mount preparation as described in Material and Methods. Note the increase in size and numbers of end buds indicated by arrows. Numerical data are presented in Figure 4.

A



Control (AIN-93G)

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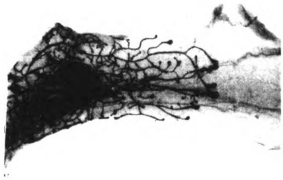

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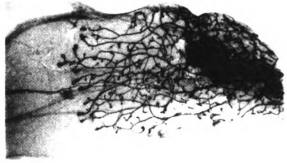

Estradiol (1 ppm)

Figure 4. Effect of genistein on end bud development in ovariectomized athymic female mice. Mice were ovariectomized on day 28, and treatments initiated on day 35. Mice were fed control (AIN 93-G), genistein (750 ppm) or estradiol (1 ppm) containing diets. Mice were killed on day 40 and mammary glands were removed for whole gland mount preparation as described in Material and Methods. Each bar represents the Mean±SEM of four mice per experimental group. The probability (*P=0.05) that estrogen and genistein treated groups had higher numbers of end buds than controls was determined by ANOVA.

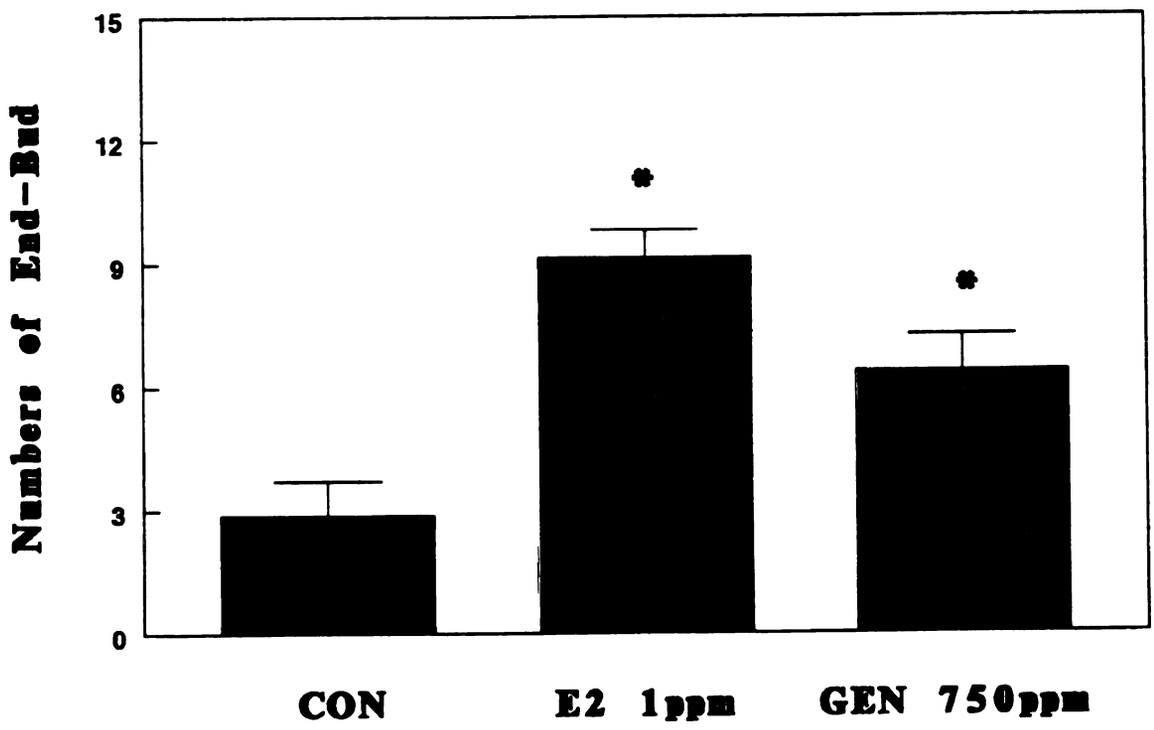
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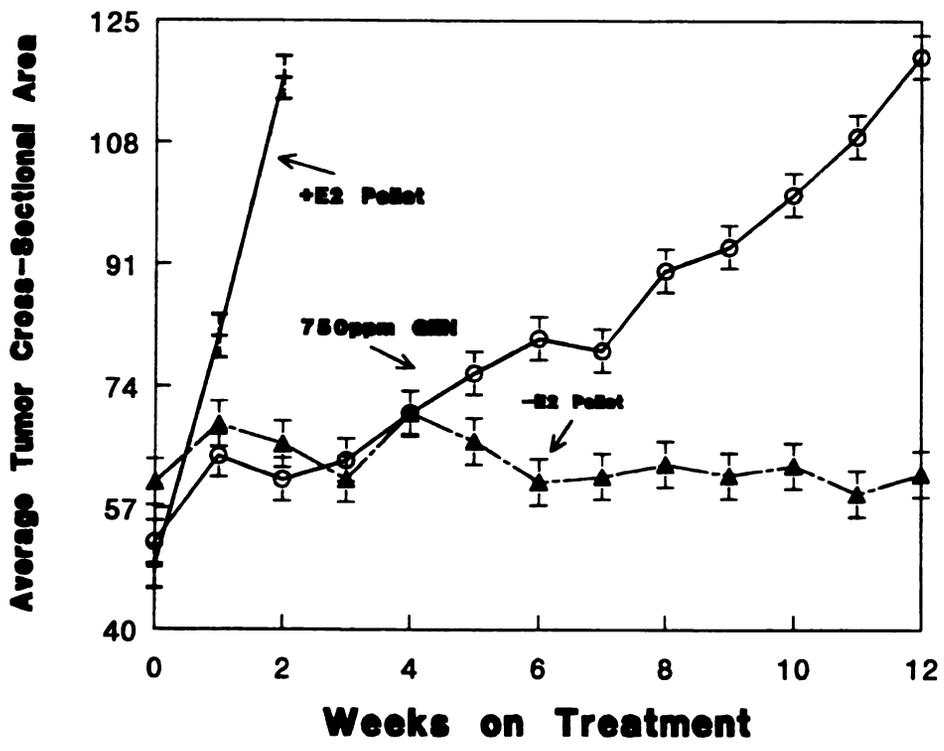
Figure 5. The effect of estrogen pellet (2 mg) and dietary genistein (750 ppm) on MCF-7 tumor growth in athymic nude mice. MCF-7 human breast cancer cells were injected subcutaneously into four sites on the flanks of mice at 1 x 10^6 cells per site. After tumors had formed, the mice were grouped to equalize tumor area and dietary treatment initiated. Experimental groups included negative control AIN-93G (5 mice, 15 tumors=n), positive control implanted 2 mg estrogen pellet (5 mice, 17 tumors=n) and AIN-93G + genistein 750 ppm (5 mice, 17 tumors=n). Data are expressed as the change in tumor areas for each week of measurement. The treatment*week interaction is statistically significant ($P<0.0001$). Treatment means for each week are compared using the Least Significant Difference method.


Average Tumor Cross-Sectional Area
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57
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6
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10
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750ppm GEN
-E2 Pellet

CHAPTER 4

Effects of Dihydrogenistein, a Metabolite of Genistein, on Human Breast Cancer Cells In Vitro

ABSTRACT

Dihydrogenistein (DHG) is the only metabolite of genistein (GEN) found in the urine of human volunteers consuming soy. DHG differs from GEN by the saturation of one double bond (at C2 and C3). In previous studies from our laboratory, we have shown that GEN produces a dose-dependent stimulatory effect on growth of MCF-7 cells [an estrogen receptor (ER)-positive human breast cancer (HBC) cell line] at concentrations from 10 nM to 1 μM and a growth inhibitory effect at levels greater than 10 uM in both MCF-7 and MDA-mB-231 (ER- negative HBC) cells. The effect of GEN on cell growth at lower concentrations is likely mediated by an ER-mediated pathway and the effect of GEN at higher concentration is mediated by a different mechanism. We evaluated the potential of DHG to act by similar mechanisms to GEN on growth of HBC cells. We observed that DHG increased cell growth in a dose-dependent manner from 1 nM to 80 μM in MCF-7 cells, but not in MDA-mB-231 cells. However, DHG did not inhibit cell growth at higher concentrations (above 25 μM) in either ER-positive or ER-negative HBC cells. Furthermore, stimulation of pS2 mRNA expression by DHG occurred in a dose-dependent fashion from 10 nM to 80 μM in MCF-7 cells. Experiments were carried out to confirm that DHG acts as an estrogen (E) agonist and

elicits estrogenic effect by ER-mediated mechanism. The results showed that the ER antagonist, ICI 164,384 (100nM), blocks the stimulation of pS2 expression and cell growth induced by DHG. Furthermore, DHG was found to compete with [^{3}H]estradiol for binding to the estrogen receptor with 50% inhibition at 0.6 μM. Although we failed to detect a difference between GEN and DHG treatment in protein tyrosine phosphorylation as originally suspected, we did find that the level of p21 $^{CIP1/WAF1}$ protein was increased after treatment with GEN (50 μM), but not with DHG (50 μM). The level of p53 protein expression, however, was not changed by GEN treatment, indicating that p21 $^{CIP1/WAF1}$ could be induced by GEN by a p53-independent mechanism. These results suggest that minor modification in the chemical structure of GEN results in the loss of its growth inhibitory effect at high (>25uM) concentrations with no significant change in estrogenic activity.

INTRODUCTION

Dihydrogenistein is the only metabolite of genistein found in the urine of human volunteers (Joannou, G.E. et al. 1995). When volunteers were fed with soy (Kelly, G.E. et al. 1993), urinary dihydrogenistein was detected and identified by profile capillary gas chromatography (GC) and electron ionization mass spectrometry (GC-EIMS) analysis of the trimethylsilyl ether (TMS) derivatives. However, no quantitation was reported in this study for the concentration of dihydrogenistein in urine. Additionally, dihydrogenistein was found when pure genistein was fermented with human fecal bacteria under anaerobic conditions (Chang Yu-Chen and Nair M.G. 1995a).

Dihydrogenistein is a reduction product of genistein by hydrogenation at the C2 and C3 position. Chang and Nair (1995a) were able to synthesize dihydrogenistein from genistein by hydrogenation to provide sufficient quantities for in vitro studies. (Chang Yu-Chen and Nair M.G. 1995b). Chang and Nair (1995a) conducted various bioassays with dihydrogenistein to evaluate the antifungal, antibacterial, mosquitocidal , nematocidal and topoisomerase effects. To date, no other characterization of dihydrogenistein has been conducted in human cells. Furthermore, it is not clear whether dihydrogenistein, formed after bacterial biotransformation, has a different biological effect from that of genistein.

The major known effects of genistein in human breast cancer cells are ER-mediated estrogenic effect (Wang, T.Y. et al. 1996) and non-ER-mediated protein tyrosine kinase inhibition effect (Akiyama, T. et al. 1987). We have shown that genistein produces a concentration-dependent effect on growth stimulation of MCF-7 cells at lower concentrations (10 nM to 1 μM). Additionally, we have shown that, at higher concentrations (>10 μM), genistein inhibited cell growth (see chapter 3). The effect of genistein on cell growth at lower concentration appears to be mediated by an estrogen receptor pathway, while the effects at higher concentrations were independent of estrogen receptor since these inhibitory effects were observed in both estrogen receptor positive and estrogen receptor negative cells. Hence, the effect of genistein at higher concentrations is likely to be mediated by the non-ER-mediated protein tyrosine kinase inhibition effect (Akiyama, T. et al. 1987). Genistein is known to inhibit the tyrosine kinase activity of a number of kinases including both receptor and cytosolic forms

(Akiyama, T. et al. 1987, Nakafutu et al. 1992). Genistein at 100 μM inhibited epidermal growth factor (EGF)-, nerve growth factor (NGF)-, fibroblast growth factor (FGF)- and insulin-induced Ras:GTP complex formation in rat pheochromocytoma PC-12 cells (Nakafutu et al. 1992), and erythropoietin-induced Ras:GTP formation in human erythroleukemia cells (Torti et al. 1992). Furthermore, genistein at >500 μM significantly inhibits the total protein tyrosine phosphorylation (Koroma and DE Juan, 1994). Since DHG has been found in human urine, it is important to evaluate the potential estrogenic and growth inhibitory effects of dihydrogenistein in human breast cancer cells.

Ogawara et al. (1989) reported that a slight change in the structure of genistein could cause a significant decrease in its ability to inhibit protein tyrosine kinase activity. In order to clarify the structure-activity relationship, the tyrosine kinase inhibitory activities of synthetic flavonoids, isoflavonoids and genistein derivatives (PKI-1 to PKI-24) were investigated. The results indicates that a hydroxyl group at position C5 was essential for inhibitory activity and that hydroxyl group at C7 and C4' positions was necessary for full expression of the inhibitory activity (fig. 6). Results from this study show that dihydrogenistein does not inhibit cell proliferation in MCF-7 or MDA-231 cells. The results of Akiyama et al. (1989) and our studies suggest that the hydrogenation at C2 and C3 position of dihydrogenistein may cause the loss of tyrosine kinase inhibitory activity as compared to its parent compound, genistein. This needs to be further examined.

$p21^{CIP1/WAF1}$, a 21 kDa protein, is a major inhibitor of cyclin/CDK catalytic activity. Each member of the cyclin kinase family is inhibited by $p21^{CIP1/WAF1}$, but their relative affinities vary with each enzyme (Gu et al., 1993, Xiong et al., 1993 and Harper et al., 1993). As a potent inhibitor of G1 cyclin-dependent kinase, $p21^{CIP1/WAF1}$ has been found to cause cell cycle arrest specifically in G1/S transition (Harper et al., 1993). On the other hand, $p21^{CIP1/WAF1}$ also has been reported to induce differentiation in a number of cell types in vitro, such as the myelomonocytic cell line U937 (Liu et al., 1996). In this regard, GEN has been well documented as both a cell cycle inhibitor and a differentiation inducer in several cancer cell lines(Constantinou and Huberman 1995 and Pagliacci et al., 1994). A model has been presented by Liu et al., (1996) depicting a key role for $p21^{CIP1/WAF1}$ in facilitating the differentiation of cancer cells in response to inducers such as hormonal ligands in a p53-independent manner. Accordingly, it seems possible that $p21^{CIP1/WAF1}$ could be one of the target proteins of GEN to induce cell growth arrest and/or differentiation in cancer cells.

In summary, the biological effect and mechanisms of function of DHG are largely unknown. There are three major potential mechanisms of DHG which were tested in this paper. First, the estrogen receptor (ER)-mediated pathway was analyzed by cell proliferation, ER competitive binding assay and ER-dependent pS2 gene expression. Second, the protein tyrosine kinase-mediated pathway was studied by comparing the total protein tyrosine phosphorylation level in western blots. Thirdly, the $p21^{CIP1/WAF1}$ protein mediated pathway was analyzed by western blots to detect changes in p21

$^{CIP1/WAF1}$ protein expression.

MATERIALS AND METHODS

Chemicals. Genistein and dihydrogenistein were synthesized by Chang and Nair (Chang et al., 1994 and Chang and Nair, 1995b). ICI 164,384 was a gift from Dr. T. Zacharewski of the Department of Biochemistry, Michigan State University. The other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

The effect of dihydrogenistein on the proliferation of estrogen receptor positive MCF-7 and estrogen receptor negative MDA-mB-231 human breast cancer cells in culture. MCF-7 (estrogen receptor positive) and MDA-mB-231(estrogen receptor negative) cells (American Type Culture Collection) were maintained in the IMEM medium (Biofluids Inc., Rockville, MD) containing 5% fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (100 μg/ml). Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. MCF-7 and MDA-mB-231 cells (American Type Culture Collection) were harvested, and then were plated at $1.5x10^3$ cells per well in 24 well tissue culture polystyrene plates in medium containing 5% FBS for one day. After removing the medium, cells were washed with phosphate buffered saline (PBS) and then were incubated with medium containing 5% charcoal dextran-treated fetal bovine serum (Hyclone Inc., Logan, UT) in phenol-red free media for an additional two days. The medium was renewed using the same medium mentioned above plus dihydrogenistein at 1 nM, 10 nM, 100 nM, 200 nM, 500 nM, 1 μM, 10 μM, 25 μM, 50

μM, 80 μM or estrogen at 1 nM. Proliferation was assessed by fluorometric analysis of DNA content (as an indicator of cell number) daily for 4 days (West et al. 1985, Labarca, C. & K. Paigen.1980). Cells were lysed in situ with 10 mM EDTA, pH 12.3 at 37°C for 30 minutes and neutralized with KH_2PO_4 and the resulting DNA stained by using Hoechst 33258 reagent. Fluorescence was measured by excitation at 350 nm and emission at 455 nm and compared to standard salmon sperm DNA to determine DNA content.

The effect of dihydrogenistein on the expression of the estrogen responsive pS2 gene in estrogen receptor positive MCF-7 human breast cancer cells in vitro. MCF-7 cells were cultured in 100 mm x 20 mm polystyrene tissue culture plates under the condition described above. Twenty-four hours after initial plating of the MCF-7 cells, estradiol or dihydrogenistein were added. The final concentration of estradiol was 1 nM and the final concentrations of dihydrogenistein were 1 nM, 10 nM, 100 nM, 1 μM or 10 μM. Total RNA were isolated 72 hours after the addition of estradiol or dihydrogenistein using the method of Chomczynski and Sacchi (1987) as modified by Xie and Rothblum (1991). Briefly, the cells were lysed with 1.7 ml of lysis buffer which contained 4 M guanidium thiocyanate, 25 mM sodium citrate, 100 mM 2-mercaptoethanol and 0.5% sodium sarcosyl (pH 7.0). The cell lysate was transferred from the tissue culture plate to a 15 ml Corex tube, and mixed with water-saturated phenol containing 0.04% (w/w) hydroxyquinoline and 2 M sodium acetate (pH 5.0) in a ratio of 10:10:1. This was followed by the addition of 0.36 ml of chloroform:isoamyl alcohol (24:1). Tubes were vortexed for 10-20 seconds, placed on ice for 30 minutes, and then centrifuged at 12,000 x g for 30 minutes at 4°C. The upper layer containing

the RNA was then transferred to a new tube containing an equal volume of isopropanol at -20°C. Samples were vortexed and RNA was precipitated by placing the mixture at -20°C for at least two hours. The RNA precipitate was recovered by centrifugation at 12,000 x g for 30 minutes. The resultant pellet was washed twice with 70% ethanol, and dried under vacuum. RNA was resuspended in 100 µl diethyl pyrocarbonate-treated (DEPC) water. Ten µg of RNA were used for Northern blot analysis. Northern blot analysis was performed to determine pS2 mRNA content using pS2 cDNA probe (Jakowlew et al. 1984). The detailed procedure is described below.

Probing for pS2. A plasmid containing a 559 base pair cDNA fragment encoding the full length pS2 (Jakowlew et al. 1984) was obtained from the American Type Culture Collection. PstI restriction endonuclease digestion of the plasmid (Jakowlew et al. 1984) produced DNA fragments of 4.4, 0.32 and 0.24 kb. These fragments were isolated from the agarose gel slice using the methods adapted from Moore et al. (1995). Twenty-five ng of pS2 cDNA (0.32 and 0.24 kb) and 50 µCi [α -^{32}P]dCTP were used to label the DNA by the random primed labeling method using a commercial kit and following the manufacturer's instructions (Gibco BRL, Gaithersburg, MD).

Northern Blot Hybridization and Detection. For detection of pS2 expression, 10 µg of total RNA were separated on 1.2% formaldehyde agarose gels and transferred to a Hybond-N Nylon membrane (Amersham, Arlington Heights, IL). The RNA was cross-linked onto the membrane by UV light for 3 minutes using a 25 watt transilluminator (Hoefer Scientific Instruments, San Francisco, CA). The RNA on the membrane was then hybridized with the ^{32}P-labeled pS2 DNA probe and detected by autoradiography. Northern blots were also probed for human glucose-3-phosphopate-dehydrogenase

(G3PDH) cDNA (Clontech Laboratories, Inc., Palo Alto, CA), a house keeping gene, to confirm equal loading of RNA among treatment and control groups.

The effect of the pure estrogen receptor antagonist (ICI 164,384) on dihydrogenistein stimulated cell growth and pS2 expression in estrogen receptor positive MCF-7 human breast cancer cells cultured in vitro. MCF-7 cells were treated with ICI 164,384 at100 nM which has been reported as a maximal inhibition concentration (Colin et al. 1994) in conjunction with various concentrations of dihydrogenistein. As mentioned above, proliferation was assessed by fluorometric analysis of DNA content daily for 4 days (West et al. 1985, Labarca, C. & K. Paigen.1980). Hoechst 33258 reagent was added and fluorescence was measured by excitation at 350 nm and emission at 455 nm to determine the DNA content. Total RNA was isolated 3 days after the addition of ICI 164,384 and dihydrogenistein using the method of Chomczynski et al. (1989) as modified by Xie and Rothblum (1991). Northern blot analysis was performed as described above.

Determination of receptor binding affinity of dihydrogenistein to human estrogen receptor (hER) isolated from estrogen receptor positive MCF-7 human breast cancer cells. Competitive binding assays were conducted using a modification of the hydroxyapatite (HAP) binding procedure of Murdoch et al, 1990. A human breast cancer cell line, MCF-7, was the source of human estrogen receptor (hER) for the binding assays. Four days before harvest, the medium was changed to 5% DCC-FBS medium in which the serum supplement was treated with dextran-coated charcoal

(Hyclone Laboratories) to remove steroid hormones. Twenty four hours before harvesting, the 5% DCC-FBS medium was replaced with serum-free medium to minimize the level of 17 β-estradiol in the cells and to improve the recovery of unliganded hER. Cells at near confluence were gently suspended by a 30 min incubation in 1 mM EDTA in Ca- Mg- free phosphate buffered saline at 37°C. Cell suspensions were combined and centrifuged at 800 g for 10 min at room temperature to pellet cells. The cells were washed once with homogenization buffer [10 mM Tris HCl pH 7.5, 1.5 mM Na_2EDTA, 1 mM dithiothreitol, 1 mM sodium molybdate, and 10% (v/v) glycerol], resuspended in 2 ml ice cold homogenization buffer, disrupted with 60 passes of a Dounce homogenizer on ice, and centrifuged at 800 xg, at 4°C for 10 min to pellet cellular debris. The hER preparation was obtained by centrifugation of the supernatant at 100,000 xg at 4°C for 30 min. The hER preparation was stored in 200 µl aliquots at -80°C until use. Protein was determined using the Bradford dye binding assay in 1 ml homogenization buffer (Bradford, 1976).

Inhibition of 10 nM [^{3}H]-17β-estradiol (^{3}H-E2) binding to hER was measured by incubation at 4 C for 2 hr of 40 pM hER in 1 ml Assay Buffer [10 mM Tris HCl pH 7.5, 1.5 mM Na_2EDTA, 1 mM dithiothreitol, and 10% (v/v) glycerol]. Triplicate analyses were conducted at concentrations ranging from 0.001 to 1 µM for genistein and dihydrogenistein. Total binding of ^{3}H-E2 was estimated in the absence of competitor and was used to define the 100% binding level. Following a two hr incubation at 4°C, 0.5 ml of HAP was added. Samples were incubated at 4°C for 15 min to allow proteins in the supernatant to bind the HAP and were mixed at five min intervals during this

incubation. Samples were then centrifuged at 3,000 xg in a Beckman swinging bucket centrifuge for five min at 4°C. The supernatant was removed, and pellets were rinsed three times with 2.5 ml aliquots of ice-cold assay buffer. After the third rinse, the pellets were transferred to scintillation vials with 4 ml Safety Solve per vial (of a liquid scintillation fluid). The radioactivities were then measured using a Beckman LS-100 liquid scintillation counter (Beckman Instruments, Indianapolis, IN). Tritium efficiency standards were prepared fresh each time the assay was performed, and were used to determine the disintegrations per min (DPM) from the counts per min (CPM) reported by the detector. The resulting data was utilized to determine the IC50 of genistein and dihydrogenistein.

Determination of the effect of dihydrogenistein on overall protein tyrosine phosphorylation of human breast cancer cells in culture. Total protein tyrosine phosphorylation content was assessed on the control, positive control (stimulated with 100 nM EGF) and treatment (genistein 300 uM or dihydrogenistein 300 uM alone or plus 100 nM EGF) cultures in both MCF-7 and MDA-mB-231 cells which were plated in 60 mm dishes. There are three steps included in this method: protein isolation, SDS-PAGE and electroblotting, hybridization and autoradiography (Kang et al., 1996).

First, for protein isolation, 300 µl lysis buffer were added to a cell monolayer after discarding the culture medium. The lysis buffer was made up of 20% SDS lysis solution containing several protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 µM antipain, 0.1 µM anprotinin, 1 µM leupeptin, 0.1 uM sodium orthovanadate and 5 mM sodium fluoride). The lysate was carefully

harvested by scraping into 1.5 ml microfuge tubes. Cell debris were removed by centrifugation at 800 xg, and then equal volumes of 2 x sample loading buffer (160 mM Tris-HCl, pH 6.8, 4% SDS, 30% glycerol, 5% β-mercaptoethanol, 10 mM dithiothreitol, 0.01% bromophenol blue) were added to the lysate which was boiled for 5 min at 100°C. Protein concentrations were determined by the DC protein assay kit (Bio-Rad Co., Richmond, CA) and samples were stored at -20°C, until ready for electrophoretic analysis.

For SDS-PAGE, equal amounts of protein samples were run at constant voltage (200 V) for 45 min on 12%, 1 mm thick, precast, polyacrylamide minigels (Bio-Rad Laboratories, Richmond, CA), using a miniprotean apparatus (Bio-Rad). Broad range, prestained (10 to 220 kDa) molecular weight protein markers (GIBCO) were run simultaneously with the samples.

For electroblotting, hybridization and autoradiography, gels were equilibrated in transfer buffer and then electroblotted onto 0.4 um Immobilon-P membranes (Millipore, Bedford, MA) using the trans-Blot SD (Bio-Rad) apparatus. Membranes were blocked in 5% nonfat dried skim milk in PBS containing 0.1% Tween 20 for 1 hr and probed with either antiphosphotyrosine antibody (Oncogene Science, Cambridge, MA) at a 1:1000 dilution or anti-EGF receptor antibody (Oncogene Science, Cambridge, MA) at a 1:50 dilution in the blocking solution, for a minimum of 1 hr at room temperature. Blots were washed in PBS and 0.1% Tween 20 and incubated for 1 hr at room temperature in a double secondary antibody matrix composed of IgG-HRP (horseradish peroxidase) for detecting protein samples (diluted 1:1000) in the blocking solution. Blots were washed (three times, 5 min each), immersed in chemoluminescent (ECL) immunodetection

reagents, and then exposed to Kodak X-ray film from 15 sec to 5 min, as described in Amersham ECL protocols (Amersham Co., Arlington Heights, IL).

Determination of the effect of dihydrogenistein on the level of p21 $^{CIP1/WAF1}$ and p53 protein expression in MCF-7 cells. MCF-7 cells were plated in 60 mm dishes, in the absence or presence of genistein and dihydrogenistein at the concentration of 50 μM. The methods of protein isolation, SDS-PAGE and electroblotting, hybridization and autoradiography which are used for sample analysis were described as above. The only difference is that membranes were blocked in 5% nonfat dried skim milk in PBS containing 0.1% Tween 20 for 1 hr and probed with different primary antibodies which were anti-p21 $^{CIP1/WAF1}$ polyclonal antibody (Oncogene Science, Cambridge, MA) at a 1:500 dilution, anti-p53 monoclonal antibody (Oncogene Science, Cambridge, MA) at a 1:1000 dilution, or anti-actin polyclonal antibody (Oncogene Science, Cambridge, MA) at a 1:1000 dilution in the blocking solution, for a minimum of 1 hr at room temperature. The expression of actin served as an internal control of total protein levels in samples.

RESULTS

The effect of dihydrogenistein on the proliferation of estrogen receptor positive MCF-7 and estrogen receptor negative MDA-mB-231 human breast cancer cells in culture. Dihydrogenistein increased MCF-7 cell (ER positive) growth from 1 nM up to 25 μM in a dose-dependent manner, then reached a maximum effect which is

approximately 2 times higher than the control. Estrogen treatment at concentrations of 1 nM also showed a 2 fold stimulatory effect on MCF-7 cell growth. Although the cell growth showed a slight decrease at dihydrogenistein concentrations higher than 25 μM, it was not significantly different to the effect of genistein treatment at the same concentration. (Fig. 7) In contrast, dihydrogenistein did not stimulate or inhibit cell growth in MDA-mB-231 (ER negative) cells at both low and high concentrations (Fig. 8). Thus, ER appears to play a key role to cause this different response to dihydrogenistein between MCF-7 and MDA-mB-231 cells. Dihydrogenistein, however, did not inhibit cell proliferation at higher concentrations (about 25 uM) as shown by genistein in both MCF-7 and MDA-mB-231 cells.

The effect of dihydrogenistein on the expression of estrogen responsive pS2 gene expression in estrogen receptor positive MCF-7 human breast cancer cells in vitro. As has been noted above, dihydrogenistein can be an estrogen agonist and elicits estrogenic effect by an estrogen receptor-mediated mechanism. This is supported by the stimulation of pS2 mRNA expression by dihydrogenistein which occurred in a concentration-dependent fashion from 10 nM to 80 μM in MCF-7 cells (Fig. 9). The ER-mediated estrogenic effect of dihydrogenistein is thus confirmed by these experiments.

The effect of the pure estrogen receptor antagonist (ICI 164,384) on dihydrogenistein-stimulated cell growth and pS2 expression in estrogen receptor positive MCF-7 human breast cancer cells cultured in vitro. As it has been noted

above, dihydrogenistein might act as an estrogen agonist and elicits estrogenic effect through an estrogen receptor-mediated mechanism. If so, the enhanced pS2 mRNA expression and cell proliferation induced by dihydrogenistein are expected to be inhibited by ICI 164,384, the estrogen receptor antagonist, at 100 nM. Indeed, the prediction is confirmed by results presented in Figs. 10 and 11. As shown, ICI 164,384 blocked the cell proliferation effect of dihydrogenistein at a concentration of 1 μM as well as estrogen at a concentration of 1 nM and genistein at a concentration of 1 μM in MCF-7 cells (Fig. 10). ICI 164,384 also blocked the effect of dihydrogenistein in the stimulation of pS2 expression at a concentration of 1 μM in MCF-7 cells (Fig. 11).

Determination of the receptor binding affinity of dihydrogenistein to the human estrogen receptor (hER) isolated from estrogen receptor positive MCF-7 human breast cancer cells. To further characterize the interaction between dihydrogenistein and the ER pathway, we examined the ability of dihydrogenistein to compete with [^{3}H]estradiol (1 nM) for binding to the ER. Wong et al. (1996) showed that genistein competed with ^{3}H-E2 for binding to the estrogen receptor with 50% inhibition at 500 nM. Dihydrogenistein competed with ^{3}H-E2 for binding to the estrogen receptor with 50% inhibition at 600 nM (Fig. 12). Here, we clearly show that the estrogen receptor binding affinity for dihydrogenistein is similar to the estrogen receptor binding affinity for genistein. These results, together with the results from the cell proliferation, pS2 gene expression assays and studies of estrogen antagonist, ICI 164,384, supported the interpretation that dihydrogenistein induces an ER-dependent estrogenic response.

The effect of dihydrogenistein on overall protein tyrosine phosphorylation of human breast cancer cells in culture. In order to investigate the effect of DHG on overall protein tyrosine phosphorylation, we evaluated the effect of DHG and GEN at various dosages on protein tyrosine phophorylation in MCF-7 cells and MDA-mB-231 cells. The results failed to show a significant difference in protein tyrosine phosphorylation among control, EGF (100 nM), genistein (300 μM) and dihydrogenistein (300 μM) treated MCF-7 cells (fig. 13). One explanation could account for these results. In MCF-7 cells (the transformed tumor cells), the receptor tyrosine kinases or cellular tyrosine kinases might be already overexpressed. Therefore, any effect of weak stimulators (EGF) or inhibitors (genistein) may not be detected. In contrast, EGF (100 nM) pre-treated cells showed an increase in number and intensity of tyrosine phosphorylated proteins compared with those (control) treated with the solvent DMSO in MDA-mB-231 cells. Notably, genistein at 300 μM plus EGF (100 nM) in pre-treated cells inhibited the expression of a major phosphotyrosine protein (180 kDa) compared with the positive control, i.e. EGF pre-treated MDA-mB-231 cells. Similarly, cells pre-treated with dihydrogenistein (300 uM) plus EGF (100 nM) also showed the same inhibition of the expression of this protein band. (Fig. 14b) The same western blot reprobed with anti-EGF receptor antibody showed a single band at the same position where the major band was located. (Fig. 14a) The data indicate that this 180 kDa protein could be the EGF receptor. However, this study failed to show any differences in protein tyrosine phosphorylation between genistein (300 μM) and dihydrogenistein (300 μM) treatment in MDA-mB-231 cells.

The effect of dihydrogenistein on the level of p21 $^{CIP1/WAF1}$ and p53 protein expression in MCF-7 cells. We have observed that the level of p21 $^{CIP1/WAF1}$ protein increased after treatment with genistein at 50 μM, but not with dihydrogenistein at 50 μM. The relative ratio of p21 $^{CIP1/WAF1}$ protein levels are 1: 2.5: 1, for control: genistein (50 μM): dihydrogenistein (50 μM), respectively. There are no significant differences in the level of p53 protein expression among these treatments. (Fig. 15) These data indicate that the mechanism of p21 $^{CIP1/WAF1}$ induction could be p53-independent. It is known that p21 $^{CIP1/WAF1}$ is a very important cell cycle regulatory protein especially at the G1/S checkpoint. Thus, our results showed that genistein, at a concentration of 50 μM, which is a cytostatic, but not cytotoxic concentration, can increase the p21 $^{CIP1/WAF1}$ protein expression level to cause cell cycle arrest. However, dihydrogenistein at 50 μM does not have this effect. The differential effect on the level of p21 $^{CIP1/WAF1}$ protein expression between dihydrogenistein and genistein could be the major reason that dihydrogenistein failed to inhibit cell growth at concentrations higher than 25 μM compared to the effect of genistein at the same concentrations. Our data showing that the induction of p21 $^{CIP1/WAF1}$ by genistein could be p53-independent imply that the effect of genistein might not be due to its effect on DNA damage in MCF-7 cells (El-Deiry et al., 1995).

DISCUSSION

Genistein is a principle isoflavone present in soy beans and soy products and is

suspected to be a chemoprevective agent for breast cancer. Dihydrogenistein is the only metabolite of genistein found in the urine of human volunteers consuming the soy (Joannou, G.E. et al. 1995). The major objectives of this study were to investigate the biological effect of DHG and its biochemical mechanisms of action in human breast cancer cells. Our data revealed that there are at least two important properties of dihydrogenistein in MCF-7 cells. First, dihydrogenistein is able to bind to the estrogen receptor as a ligand, and induces several estrogenic responses which include the increase in proliferation of MCF-7 cells and the induction of pS2 gene expression. Second, unlike genistein, dihydrogenistein appears to lose its ability to induce the expression of $p21^{CIP1/WAF1}$ at concentrations higher than 25 μM. This may explain its inability to inhibit cell proliferation at concentrations higher than 25 μM as compared to genistein at the same concentration.

Akiyama, et al. (1987) showed that genistein is able to inhibit tyrosine kinase activity in vitro and proposed that it could be the mechanism that genistein inhibits tumor cell growth. Our results however fail to reveal a significant difference in protein tyrosine phosphorylation among control, EGF (100 nM), genistein (300 μM) and dihydrogenistein (300 μM) treated MCF-7 cells (Fig. 13). Since genistein (300 μM) has been reported to inhibit protein tyrosine kinase activities in several cancer cell lines (Koroma et al., 1994 and Clark et al., 1996). Several explanations could account for these results. First, in MCF-7 cells (the transformed tumor cells), the receptor tyrosine kinases or cellular tyrosine kinases might be already overexpressed. Therefore, any effect of weak stimulators (EGF) or inhibitors (genistein) may not be detected. Second,

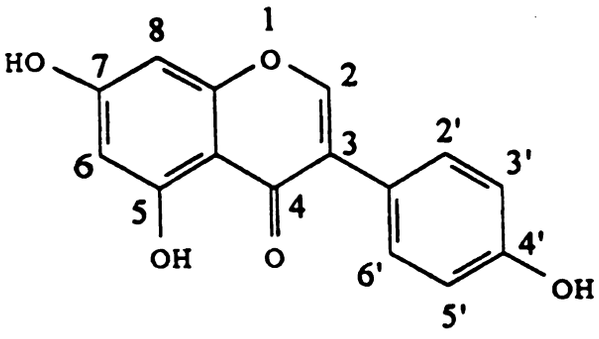
genistein may specifically inhibit tyrosine phosphorylation of certain proteins whose expression may be low and not detectable by assessing the overall levels of tyrosine phosphorylation. Third, cellular metabolism or drug exclusion mechanisms may reduce the intracellular genistein concentrations to levels below that needed to inhibit tyrosine phosphorylation in cells. In contrast to the study of overall tyrosine phosphorylation, the specific study of EGF-R tyrosine phosphorylation revealed that both genistein and dihydrogenistein were capable of inhibiting the tyrosine phosphorylation. Therefore, the differential response of MCF-7 cells to higher concentrations of genistein and dihydrogenistein in cell growth could not be attributed to the differential ability of the two compounds to inhibit protein phosphorylation. Therefore other mechanisms differentially exerted by higher concentrations of genistein and dihydrogenistein must be considered.

The ability of genistein to induce the $p21^{CIP1/WAF1}$ protein expression could be a mechanism that genistein inhibits cell proliferation of MCF-7 cells at cytostatic concentrations from 25 to 50 μM. Genistein appears not to affect the expression of other cell cycle regulatory proteins in MCF-7 cells, such as p16 (another family of cyclin kinase inhibitors), cyclin D1, cdc2 (a G2/M cyclin dependent kinase) and pRb (retinoblastoma protein). The functions of $p21^{CIP1/WAF1}$ could contribute to the following three different mechanisms. First, $p21^{CIP1/WAF1}$ could form a complex with cyclin D ,and CDK 4 or 6 (in G1/S transition) and inhibit CDK-dependent phosphoryaltion and inactivation of pRb (Harper et al., 1995) resulting in G1 arrest. Second, $p21^{CIP1/WAF1}$ could bind to PCNA and inhibit DNA synthesis in S phase (Baylin,

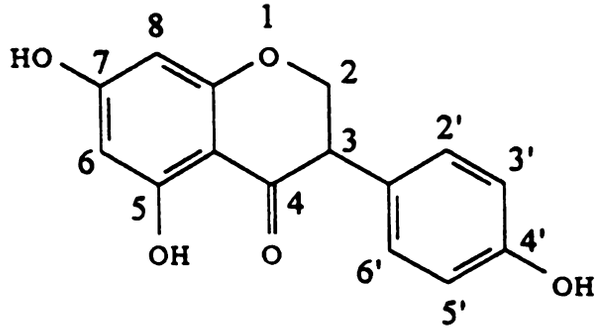
1997). In this manner, p21 $^{CIP1/WAF1}$ would work as a tumor suppressor gene inhibiting tumor cell growth. Third, p21 $^{CIP1/WAF1}$ could bind to cyclin A or B and cdc2 kinase complex (in G2/M transition) to inhibit cell cycle progression from G2 to mitosis (M) phase resulting in tumor cell arrest at G2 phase. The mechanism by which genistein induces the p21 $^{CIP1/WAF1}$ protein expression in MCF-7 cells, however, is not clear. In this regard, dihydrogenistein differs from its parent compound, genistein, by the loss its ability to induce the p21 $^{CIP1/WAF1}$ protein expression at cytostatic concentrations (25 to 50 μM). This mechanism provides potential explanation for the difference in cell growth inhibition at higher concentrations between dihydrogenistein and genistein.

In summary, our results indicate that a chemical modification of genistein results in the loss of growth inhibitory effect at high (>25 μM) concentrations of the isoflavone and no significant change in estrogenic activity. At the biological level, dihydrogenistein, similar to genistein, is expected to promote breast cancer growth at physiological concentrations.

Figure 6. The chemical structures of genistein and dihydrogenistein.



Genistein



Dihydrogenistein

Figure 7. The effect of dihydrogenistein on the proliferation of estrogen receptor-positive MCF-7 human breast cancer cells in culture. MCF-7 cells were cultured in the presence of various concentrations of dihydrogenistein (DHG) (1 nM-80 μM) or estrogen (E2) at 1 nM for 96 hr, in IMEM medium containing 5% fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere of 5% CO_2 in air. Proliferation was assessed by DNA content as measured using Hoechst reagent and fluorometric analysis (expressed as percent of control). Fluorescence was measured under excitation at 350 nm and emission at 455 nm and was used to determine the DNA content. The results (mean of 8 duplicates) are expressed relative to cells grown without dihydrogenistein. Bars represent standard error of mean (Mean±SEM). C represents vehicle control containing 1 to 1000 dilution of DMSO in growth medium.

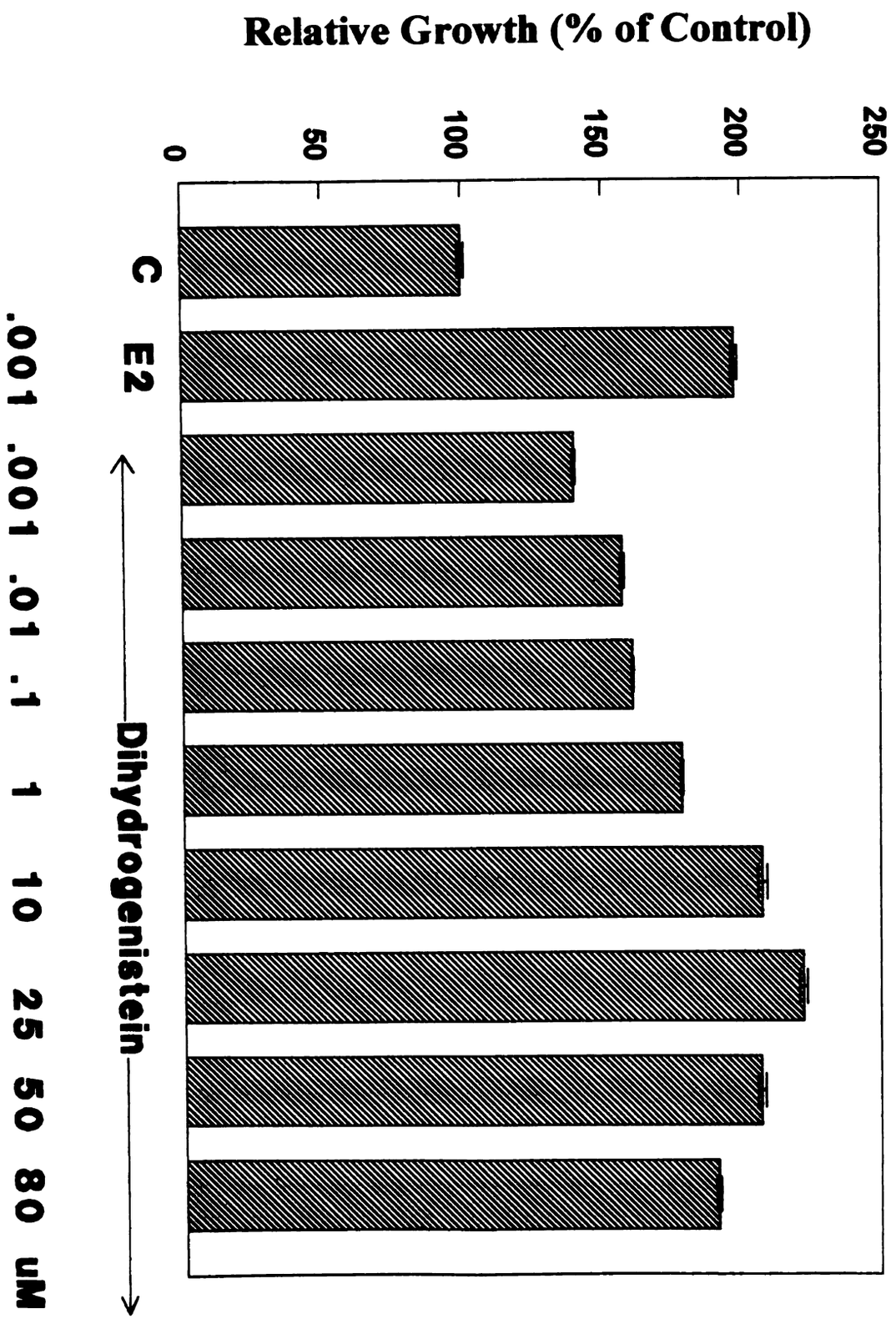

Relative Growth (% of Control)
0
50
100
150
200
250
C
E2
Dihydrogenistein
.001 .001 .001 .01 .1 1 10 25 50 80 uM

Figure 8. The effect of dihydrogenistein on the proliferation of estrogen receptor - negative MDA-mB-231 human breast cancer cells in culture. MDA-mB-231 cells were cultured in the presence of various concentrations of dihydrogenistein (25, 50 and 80 μM) or estrogen (E2) at 1 nM for 96 hr, in IMEM medium containing 5% fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere of 5% CO_2 in air. Proliferation was assessed by DNA content as measured using Hoechst reagent and fluorometric analysis (expressed as percent of control). Fluorescence was measured under excitation at 350 nm and emission at 455 nm and was used to determine the DNA content. The results (mean of 8 duplicates) are expressed relative to cells grown without dihydrogenistein. Bars represent standard error of mean (Mean±SEM). C represents the vehicle control containing 1 to 1000 dilution of DMSO in growth medium.

Relative Growth (% of Control)
0
30
60
90
120
150
C
E2
DIHYDROGENISTEIN
1nM
25uM
50uM
80uM

Figure 9. The effect of dihydrogenistein (DHG) on the expression of the estrogen responsive pS2 gene in estrogen receptor positive MCF-7 human breast cancer cells in vitro. MCF-7 cells were cultured in IMEM medium in the presence of various concentrations of dihydrogenistein (DHG) (10 nM-10 μM) or estrogen (E2) (1 nM). RNA was isolated after 72 hr treatment. Northern blot analysis and detection of pS2 was performed as described in methods. The control represents the vehicle control containing a 1 : 1000 dilution of DMSO in growth media. Northern blots were also probed with human glucose-3-phosphopate-dehydrogenase (G3PDH) cDNA (Clontech Laboratories, Inc., Palo Alto, CA) as an internal control, to confirm equal loading of RNA among treatment and control groups.

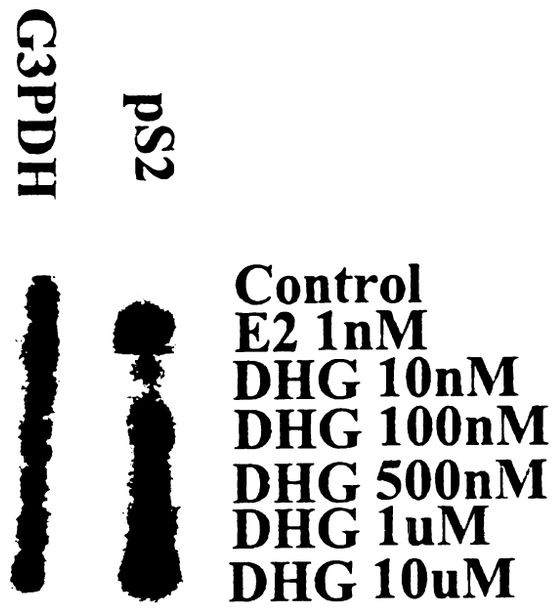

G3PDH
pS2
Control
E2 1nM
DHG 10nM
DHG 100nM
DHG 500nM
DHG 1uM
DHG 10uM

Figure 10. The effect of estrogen receptor antagonist (ICI 164,384) (ICI) (100 nM) on dihydrogenistein (DHG) stimulated cell growth in estrogen receptor positive MCF-7 human breast cancer cells cultured in vitro. MCF-7 cells were cultured in IMEM medium as described above in the presence of DHG (1 μM), genistein (GEN) (1 μM), estrogen (E2) (1 nM) with or without ICI (100 nM). Proliferation was assessed by DNA content as measured using Hoechst reagent and fluorometric analysis (expressed as percent of control). Fluorescence was measured under excitation at 350 nm and emission at 455 nm and was used to determine the DNA content. The results (mean of 8 duplicates) are expressed relative to cells grown without dihydrogenistein. Bars represent standard error of mean (Mean±SEM). C represents the vehicle control containing a 1 : 1000 dilution of DMSO in growth medium.

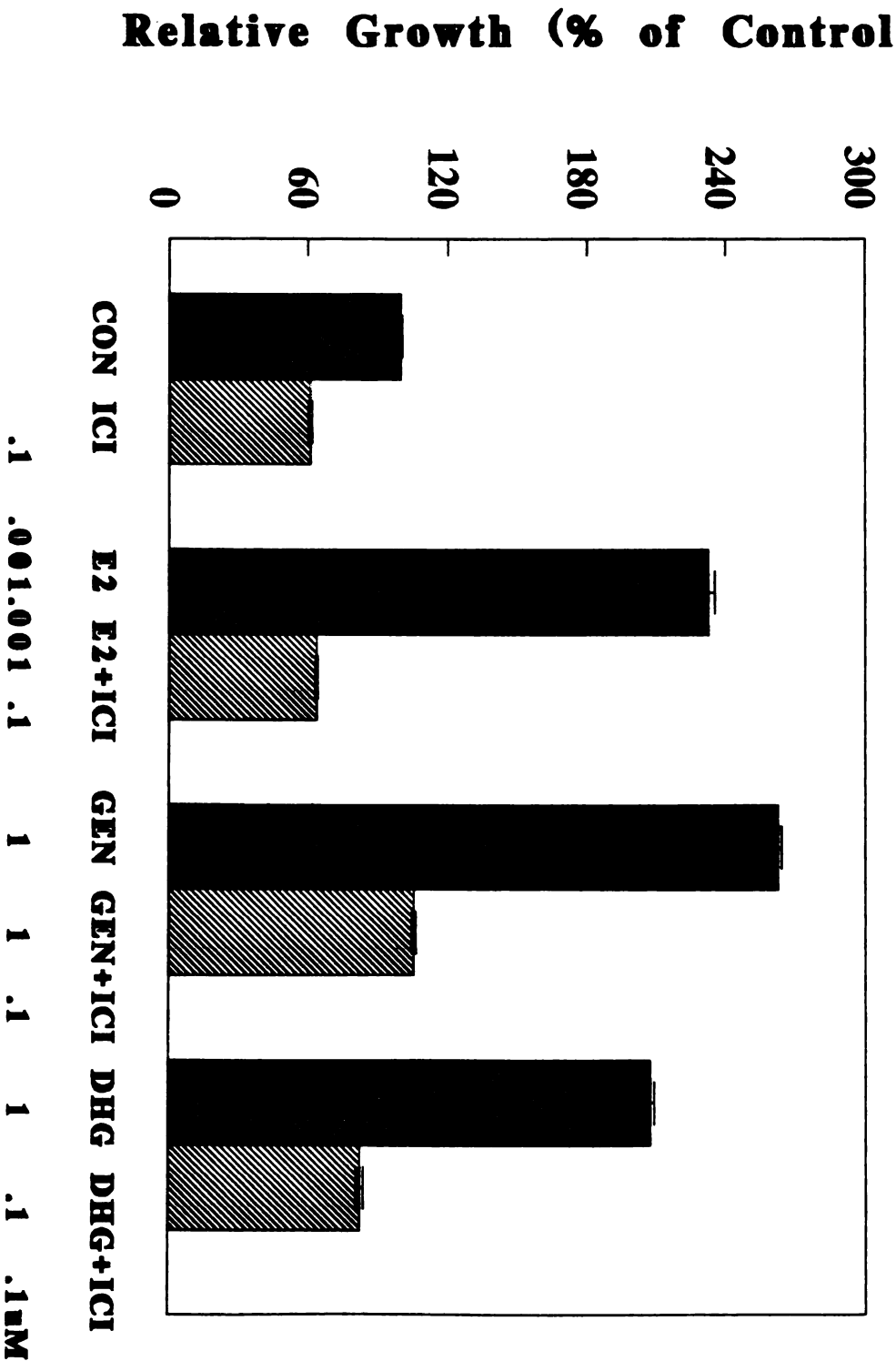

Relative Growth (% of Control)
0
60
120
180
240
300
CON
ICI .1
E2 .001
E2+ICI .001 .1
GEN 1
GEN+ICI 1 .1
DHG 1
DHG+ICI 1 .1
uM

Figure 11. The effect of the estrogen antagonist (ICI 164,384) (ICI) (100 nM) on dihydrogenistein (DHG) stimulated pS2 expression in estrogen receptor positive MCF-7 human breast cancer cells cultured in vitro. MCF-7 cells were cultured in IMEM medium in the presence of DHG (1 μM), genistein (GEN) (0.1 μM) or estrogen (E2) (1 nM) with or without ICI (100 nM). RNA was isolated after 72 hr of treatment. Northern blot analysis and detection of pS2 was performed as described in methods. C represents the vehicle control containing a 1 : 1000 dilution of DMSO in growth medium. Northern blots were also probed with human glucose-3-phosphopate-dehydrogenase (G3PDH) cDNA (Clontech Laboratories, Inc., Palo Alto, CA) as an internal control, to confirm equal loading of RNA among treatment and control groups.

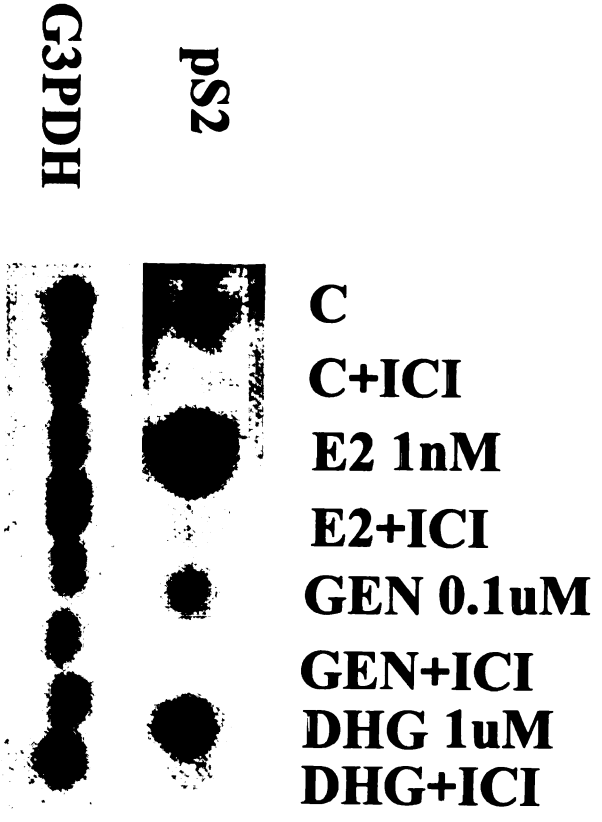

G3PDH
pS2
C
C+ICI
E2 1nM
E2+ICI
GEN 0.1uM
GEN+ICI
DHG 1uM
DHG+ICI

Figure 12. Effects of dihydrogenistein (DHG) and genistein (GEN) on the binding of [^{3}H]estradiol to the ER. ER binding assays were performed as described in Materials and Methods. Competitors [estradiol (E2), GEN, DHG] were added at the indicated concentrations with 1 nM [^{3}H]estradiol. The results (mean±SE, n = 3) are expressed as a percentage of control.

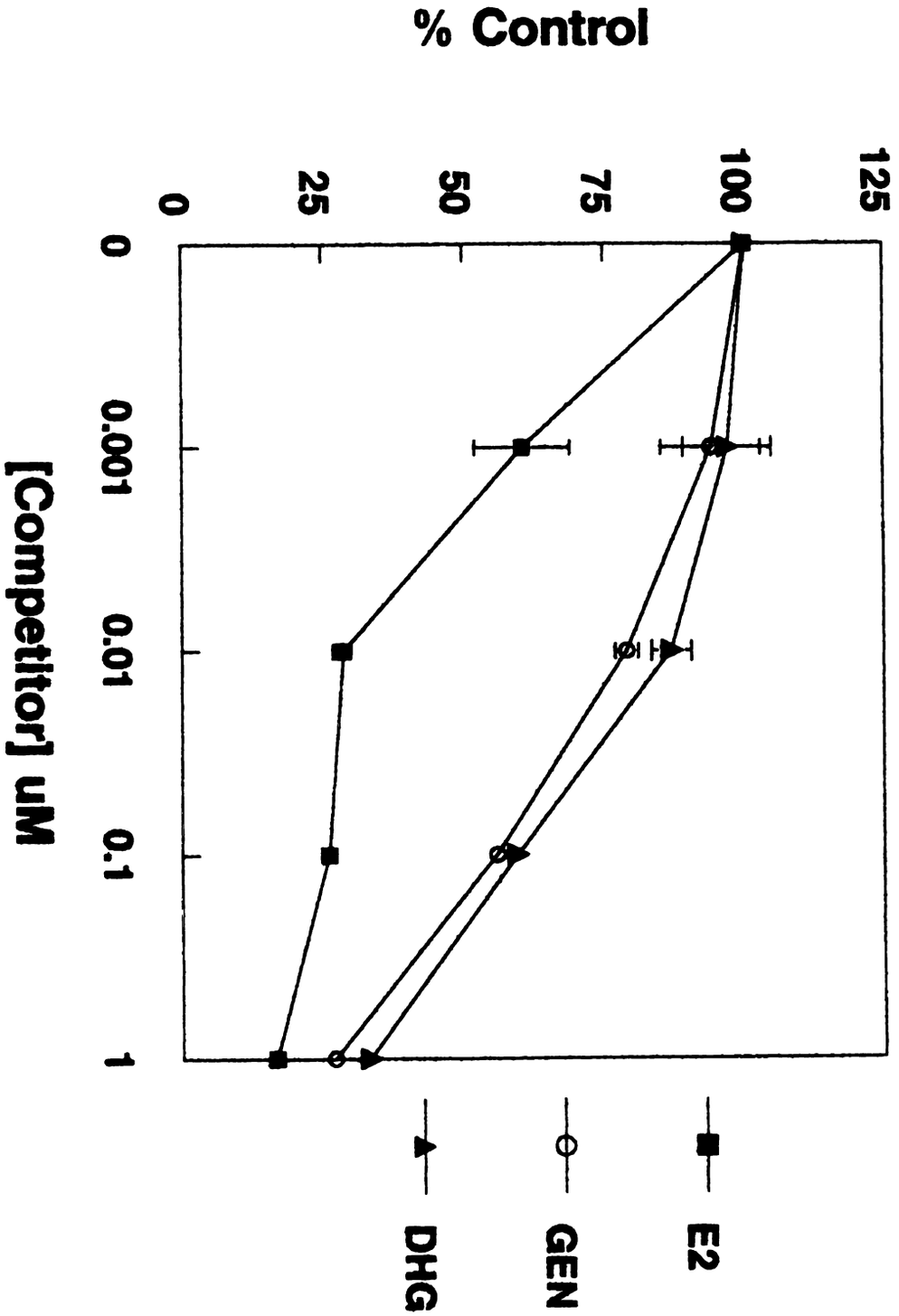

% Control
0
25
50
75
100
125
0
0.001
0.01
0.1
1
[Competitor] uM
DHG
GEN
E2

Figure 13. Effects of dihydrogenistein (DHG) and genistein (GEN) on overall protein tyrosine phosphorylation in EGF-preteated MCF-7 cells. MCF-7 cells were cultured in the presence of dihydrogenistein (DHG) (300 μM) or genistein (GEN) (300 μM) with or without EGF (100 nM) which was added 15 min before the start of treatment. Total protein was collected after 24 hr. Western blot and detection for protein tyrosine phosphorylation was performed using procedures described in Materials and Methods. C represented the vehicle control which contained a 1 : 1000 dilution of DMSO in growth media.

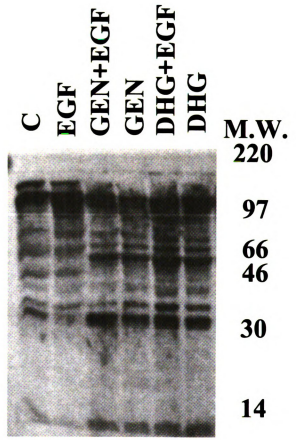

C
EGF
GEN+EGF
GEN
DHG+EGF
DHG
M.W.
220
97
66
46
30
14
P-tyr

Figure 14. Effects of dihydrogenistein (DHG) and genistein (GEN) on EGF receptor protein expression (A) and overall protein tyrosine phosphorylation (B) in EGF-preteated MDA-mB-231 cells. MDA-mB-231 cells were cultured in the presence of dihydrogenistein (DHG) (300 μM) or genistein (GEN) (300 μM) with or without EGF (100 nM) which was added 15 min before the start of treatments. Total protein was collected after 24 hr. Western blot analysis and detection of protein tyrosine phosphorylation (B) or EGF receptor (A) was performed using procedures described in Materials and Methods. A431 cell lysate was used as a positive control for EGF receptor (A). C represents the vehicle control containing a 1 : 1000 dilution of DMSO in growth media.

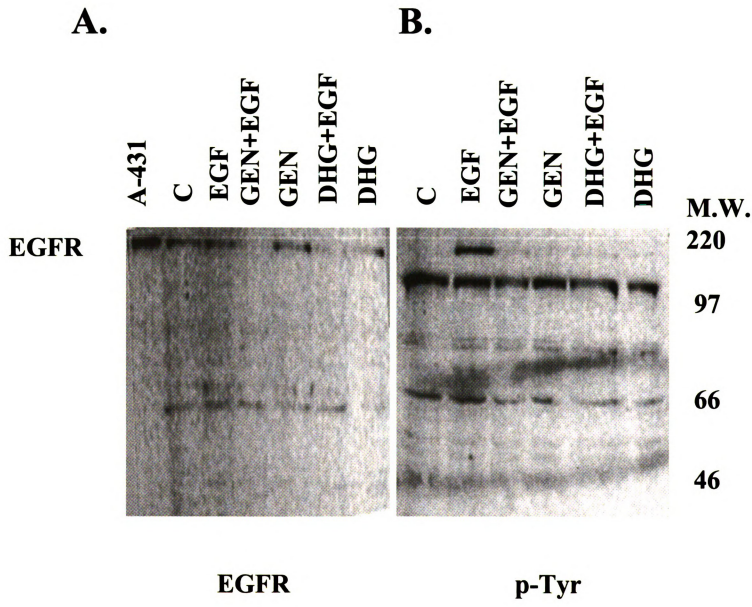
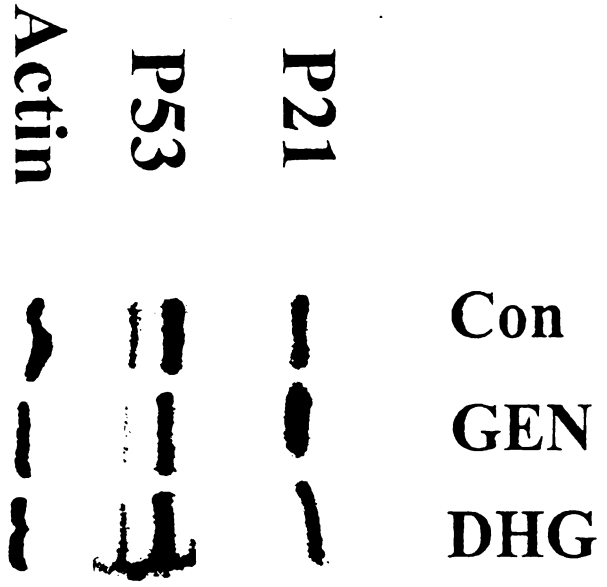

A.
B.
A-431
C
EGF
GEN+EGF
GEN
DHG+EGF
DHG
C
EGF
GEN+EGF
GEN
DHG+EGF
DHG
M.W.
EGFR
220
97
66
46
EGFR
p-Tyr

Figure 15. Effects of dihydrogenistein (DHG) and genistein (GEN) on p21 $^{CIP1/WAF1}$ and p53 protein expression in MCF-7 cells. MCF-7 cells were cultured in the presence of dihydrogenistein (DHG) (50 μM) or genistein (GEN) (50 μM). Total protein was collected after 48 hr of treatment. Western blot analysis and detection of p21 $^{CIP1/WAF1}$, p53 and actin was performed using procedures described in Materials and Methods. C represents the vehicle control which containing a 1 : 1000 dilution of DMSO in growth medium. Western blot was also probed for anti-human actin as an internal control to confirm equal loading of total protein among treatment and control groups.


P21
P53
Actin
Con
GEN
DHG

CHAPTER 5

Genistein Suppresses the Proliferation and Induces the Differentiation of a Normal Human Breast Epithelial Cell Type With Stem Cell Characteristics in Vitro: a Possible Chemopreventive Mechanism of Genistein for Human Breast Cancer

ABSTRACT

Genistein, a natural isoflavonoid phyto-estrogen found in soy and other plant foods, has been shown to inhibit the growth of some breast cancer cell lines in vitro at higher concentrations. The low incidence of breast cancer in countries with a flavonoid-rich soy-based diet and the suppression of experimental mammary tumors by prepubertal genistein treatment in rats suggest that genistein may exert a chemopreventive effect on human breast cancer. Two types of morphologically distinguishable normal human breast epithelial cells (HBEC) have been derived from reduction mammoplasty. Type I HBEC have luminal epithelial and stem cell characteristics; i.e. the ability to differentiate into Type II cells with basal epithelial cell phenotypes and the ability to form budding/ductal structures on Matrigel. These cell cultures were to assess the potential effect of genistein on chemoprevention of human breast cancer. We have analyzed the effects of genistein on cell proliferation, cell differentiation and cell cycle progression (flow cytometric analysis with propidium iodide-stained cells) in both Type I and Type II HBEC. Genistein, at concentrations lower than 1 μM, significantly increased the differentiation of Type I HBEC to Type II cells in one of two primary cultures derived

from different human subjects. Genistein completely arrested cell growth of Type I HBEC at concentrations higher than 5 μM and Type II HBEC at concentrations higher than 50 μM after 72 h treatment in all the 6 independent primary cultures examined. Flow cytometric analysis revealed that genistein was able to arrest cell cycle progression of both Type I and Type II HBEC at both G1/S and G2/M checkpoints. Western blot analysis showed that the level of $p21^{WAF1/CIP1}$, which negatively regulates the G1/S transition, and cdc2 protein, which positively regulates the G2/M transition, are significant enhanced and decreased respectively after 72 h genistein treatment (50 μM) in both Type I and Type II HBEC. Type I HBEC have been shown in previous studies to be more susceptible to neoplastic transformation than Type II cells and Type I cells have also been shown in this study to be more sensitive to growth inhibition. Therefore, the reduction in target stem cells for neoplastic transformation might be a chemopreventive mechanism for genistein.

INTRODUCTION

Epidemiological data suggest that one explanation for the low incidence of breast cancer in oriental women is the consumption of a soy-rich diet (Messina et al., 1991). Genistein, which is one of the principle isoflavonoids in human soy-rich diet (tofu, soy-milk, soy-flour etc.), could be the chemical responsible for the chemopreventive activity of soy products. Indeed, genistein has been shown to suppress mammary tumors induced by dimethylbenz[a]anthracene (DMBA) and to enhance mammary gland differentiation in female Sprague-Dawley SD rats which were exposed to genistein at the

prepubertal stage (Murrill et al. 1996 and Lamartiniere et al. 1995). These data imply that genistein may play a role in human breast cancer prevention.

Although the studies of Murrill et al., 1996 and Lamartiniere et al., 1995 demonstrate a chemopreventive effect of genistein for rat mammary tumors and imply that the induction of mammary gland differentiation is a possible chemopreventive mechanism of genistein, the biological effects and mechanisms of function of genistein in human mammary gland and normal human breast epithilial cells are largely unknown.

Recently, a culture method has been developed to grow two morphologically distinguishable cell types of normal HBEC (Type I and Type II), derived from reduction mammoplasty tissues (Kao et al., 1995). Type II cells express basal epithelial cell markers (i.e. cytokeratin 14 and α 6 integrin) whereas Type I HBEC express luminal epithelial cell phenotypes (i.e. cytokeratin 18 and epithelial membrane antigen, EMA) (Kao et al., 1995). Furthermore, Type I cells are deficient in gap junctional intercellular communication and possess stem cell characteristics (i.e. the ability to differentiate into Type II cells by cyclic AMP enhancing agents and the ability to form budding/ ductal structures on Matrigel) (Kao et al., 1995; Chang et al., 1996). Significantly, Type I HBEC express estrogen receptors (Kang et al., 1997) and have been found to be more susceptible to neoplastic transformation. After SV40 large T-antigen transformation, the Type I cells acquire anchorage independent growth and become immortal spontaneously at high frequency (Kao et al., 1995).

This HBEC system appears to be a relevant system to examine the effect of genistein on normal HBEC in relation to carcinogenesis. It provides an opportunity to test 1) if genistein is capable of inducing differentiation of Type I HBEC into Type cells, 2) if Type I and Type II HBEC proliferation is differentially affected by genistein, and 3) if the expression of cell cycle regulating genes is affected by genistein. These are the objectives of this study.

MATERIALS AND METHODS

Culture Media

The culture medium used in these studies to culture Type I and Type II HBEC is the MSU-1 medium which is a 1:1 mixture (vol/vol) of a modified Eagle's MEM (GIBCO BRL Life Technologies, Grand Island, NY) (D medium) (Chang et al., 1981) and a modified MCDB 153 (M-7403, Sigma) (Pittelkow et al., 1986) supplemented with EGF (0.5 ng/ml) (E-1264, Sigma Chemical Co., St. Louis, MO), insulin (5 μg/ml) (I-1882, Sigma), hydrocortisone (5 μg/ml) (H-0888, Sigma), human transferin (5 μg/ml) (T-7786, Sigma), and 17 β-estradiol (E_2) (1 x 10^{-8} M) (E-2257, Sigma). The modified Eagle's MEM (D medium) contains Earle's balanced salt solution with 1 mg/ml sodium bicarbonate and 7.64 mg/ml sodium chloride, a 50% increase in all vitamins and essential amino acids (except glutamine), a 100% increase in all nonessential amino acids, and 1 mM sodium pyruvate (pH adjusted to 6.5 before the addition of sodium bicarbonate).

The Modified Eagle's MEM (D medium) with 5% fetal bovine serum (FBS) (GIBCO) and penicillin (100 units/ml) and streptomycin (100 μg/ml) was used to grow MCF-7 and MDA-mB-231 cells (American Type Culture Collection).

Acquisition, Processing, and Culturing of Human Breast Epithelial Cells (HBEC)

Reduction mammoplasty tissues were obtained from six female patients of 18, 56, 23, 37, 38 and 18 year of age. The HBEC obtained from these reduction mammoplasty tissue specimens were designated as HME-21, HME-22, HME-23, HME-24, HME-25 and HME-27 in order. The tissue specimens were minced into small pieces with scalpels, then digested in collagenase-Type IA (C-2674, Sigma) solution (1 g tissue per 5,000 units of collagenase in 10 ml medium) at 37°C in a waterbath overnight (16-18 hours). The next morning, the solution containing the digested tissues was centrifuged to remove the collagenase solution. The cellular pellet was washed once with MSU-1 medium before being suspended in the MSU-1 medium supplemented with 5% fetal bovine serum (FBS) (GIBCO). Subsequently, the cells were plated in two flasks (150 cm^2). After a 2 hour incubation, the cells (or cell aggregates) that remained in suspension were transferred to four to six flasks (75 cm^2) for the purpose of reducing the number of attached fibroblasts. After an overnight incubation, the medium was changed to the FBS-free MSU-1 medium. The MSU-1 medium was changed once every 2 days for 1 week. Subsequently, the cells were removed with a solution of trypsin (0.01%) (Sigma) and ethylenediaminetetraacetic acid (EDTA) (0.02%) (Sigma) and stored in solution [phosphate buffered saline (PBS) containing 10% dimethylsulfoxide (DMSO)]

in liquid nitrogen. During this 1 week period, almost all of the fibroblasts can be removed by treatment (one to two times) with diluted trypsin (0.002%) solution.

To start a culture from stored frozen cells, the frozen cells in liquid nitrogen were thawed and placed in MSU-1 medium supplemented with 5% FBS for 4 hours for the attachment of residual fibroblasts. The epithelial cells in suspension were transferred to new plates and cultured in the FBS-free MSU-1 medium. All cultures were incubated at 37°C in incubators supplied with humidified air and 5% CO_2.

Separation of Type I and Type II HBEC

The first passage of HBEC, recovered from liquid nitrogen storage, was plated in the MSU-1 medium supplemented with 5% FBS as described above. After overnight culture, the cells that remained in suspension were transferred to new plates. Continued culture of these suspended cells, which later attached, in the FBS-containing medium gave rise to Type I cells. The attached cells, in the overnight culture, incubated in the FBS-free MSU-1 medium supplemented with 0.4% BPE (Pel-Freez, Rogers, AR) gave rise to Type II cells.

Assessment of HBEC Differentiation in vitro

To determine the effect of genistein on Type I HBEC differentiation, Type I HBEC were grown in several different treatment groups containing MSU-1 medium and various concentrations of genistein (0.01, 0.1 and 1 μM) or DMSO at a 1:1000 dilution (solvent

control). Cholera toxin (CT) (1 ng/ml) (Sigma) was used as a positive control (Kao et al., 1995). Starting from single cell plating of pure Type I cells, the differentiation of Type I HEBC was measured by counting the number of Type II HEBC colonies and colonies of Type I surrounded by Type II cells. The percentage of these colonies among total colonies (Type I, Type II and Type I surrounded by Type II colonies) indicates the differentiation potential of Type I cells under different treatments. Briefly, Type I cells(1×10^4) were plated in 60 mm plates in triplicate in the 5% FBS-containing MSU-1 medium (which promotes the growth of Type I and inhibits Type II cell growth). The next day, the medium was replaced by the FBS-free MSU-1 medium (which supports the growth of both Type I and Type II cells) for one day. Then chemicals for various treatments as described above were added to the FBS-free MSU-1 medium. All cells were incubated for 7 days at 37°C (media changed twice). At day 7 after chemical treatments, the cells were washed twice with PBS and fixed with 2 ml of 70% ethanol containing 0.1% acetic acid. 2 ml crystal violet solution per plate was added to stain the colonies. Then, Type I, Type I surrounded by Type II and Type II colonies were visually identified under a microscope and quantitated (Fig.16). The identity of treatment for each plate was unknown (blind) during counting to ensure objectivity.

Assessment of HBEC proliferation <u>in vitro</u>

To determine the effect of genistein on growth of both Type I and Type II HBEC, HBEC were grown in MSU-1 medium containing various concentrations of genistein (0.1 to 100 uM). The growth of HEBC was measured by quantitation of total nucleic acid

extracted from the culture (Li et al., 1990). Briefly, Type I (1 x 10^4 cells) and Type II (1 x 10^4 cells) HBEC (passage 2) were plated in 35 mm plates in triplicate in the 5% FBS-containing MSU-1 medium for Type I HBEC and in the 0.4% BPE-containing MSU-1 medium for Type II HBEC. The next day, the medium was replaced by the FBS- and BPE-free MSU-1 medium for one day, then various concentrations (0.1 to 100 uM) of genistein were added to the FBS and BPE-free MSU-1 medium. Cells were incubated for 7 days at 37°C (media changed twice) for measuring the dose-dependent growth at day 7. For measuring the growth curves with various concentrations of genistein treatments, the cells were harvested at the day the treatments started (day 0), and at day 1, 3, 5, 8 after the treatments. To harvest cells, the cells were washed twice with PBS and lysed with 2 ml of 0.1 N sodium hydroxide. The lysate was transferred into a 2.2 ml Eppendorf tube and centrifuged at 14,000 rpm for 2-3 min. The absorbance of the clear lysate at 260 nm was measured using a Beckman DU-7400 spectrophotometer (Schaumburg, IL). Each treatment was done in triplicate plates and the Lorentzian wave form curve fitting formula was used to determine the IC_{50} and the IC_{100} of genistein treatments in both Type I and Type II HBEC cells.

Flow Cytometric Measurement and Cell Cycle Analysis

A quantitative measure of cell cycle distribution was obtained by flow cytometric analysis of DNA content - cell number frequency histograms, as described in Fraker et al. (1995). Type I and Type II HBEC were incubated for 72 hours then the medium was replaced with fresh maintenance medium (MSU-1) containing genistein at 0, 5, 10, 25,

50, and 100 μM and collected after 3 days of treatment. Type II HBEC were also collected after incubation for 6 and 13 hours in MSU-1 medium following the 72 hours treatment with genistein at 50 μM for cell cycle recovery analysis. Briefly, there were two steps in this procedure. 1) **Fixation of cells**: HBEC (second passage) were collected from T-25 flasks at 80% confluence by washing two times with PBS followed by trypsinization. Cell number was determined using a hemocytometer and the cell suspension diluted to approximately 1 x 10^6 cells/ml in MSU-1 medium. For analysis of cell viability, a 50 μl aliquot from each sample was collected into a 0.5 ml microfuge tube. After the addition of 25 μl of 10% trypan blue, the sample was incubated at room temperature for 5 min., and cell viability was determined by the exclusion of trypan blue. For flow-cytometry analysis, the remaining samples were centrifuged at 350 x g for 5 min. and the supernatant removed. Then, the cells were resuspended in 5 ml of PBS and transferred to a Falcon 2056 tube. The cells were pelleted by low speed centrifugation (350 x g) for 5 min. The supernatant was aspirated and the pellets were washed with PBS. The cell pellet was resuspended, at a density of 1 x 10^6 cells/ml, in ice cold 70% ethanol with rapid but gentle mixing. After the cells were fixed in ethanol for 1 to 3 hours at 4°C, the sample could be stored at -20 °C until analysis. 2) **Cell staining for the flow activated cell sorter (FACS)**: the cells were centrifuged at 400 x g for 5 minutes to remove the ethanol, the cellular pellet was washed with PBS, and then flow cytometric DNA staining reagents: 0.1 mM EDTA (pH 7.4), 0.1% of Triton X-100, 0.05 mg/ml RNase A (50 units/mg), and 50 μg/ml propidium iodide (PI) in PBS (pH 7.4). The tubes were gently vortexed and placed in the dark at 4°C overnight until reading on the flow activated cell sorter (FACS). Fluorescence was assessed on a FACS Vantage

(Beckton Dickinson) by excitation with an Argon laser at 488 nm and the emission measured at 620 to 700 nm. Data were collected with Lysis II software and the percent of cells in each phase of the cell cycle calculated with MPLUS software (Phoenix Flow).

SDS-PAGE and Western blot analysis

Proteins were extracted from normal HBEC and from MCF-7 cells grown in 60 mm dishes by treatment with 20% SDS lysis solution containing several protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 1 μM antipain, 0.1 μM aprotinin, 0.1 μM sodium orthovanadate, 5 mM sodium fluoride). After sonication at three 10-s pulses from a probe sonicator, the cell lysates were stored at -20°C until use (Kang et al., 1996). The protein amounts were determined by the DC protein assay kit (Bio-Rad Co., Richmond, CA). Proteins were separated on 12.5% SDS polyacrylamide gels and transferred to Immobilon PVDF membranes (Millipore Co., Bedford, MA) at 90 V for 2 hours. The blots were blocked with 5 % dried skim milk in PBS containing 0.1 % Tween 20. $P21^{CIP1/WAF1}$ was detected by the anti-$p21^{CIP1/WAF1}$ polyclonal antibody [(C-19)-G], (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) which recognizes amino acids 146-164 mapping at the carboxyl terminus of human $p21^{CIP1/WAF1}$. P53 was detected by the anti-p53 monoclonal antibody (Ab-2, Oncogene Science, Cambridge, MA) which recognizes amino acids 46-55 of human p53 and reacts with human wild-type and mutant p53. Cdc2 kinase was detected by the anti-cdc2 polyclonal antibody (Ab-1, Oncogene Science, Cambridge, MA) which recognizes the carboxyl terminal 8 amino acids of human cdc2. The anti-actin antibody (Oncogene Science,

Cambridge, MA) was utilized to detect actin, an internal indicator of total protein level in samples. Western blots were incubated with Horseradish peroxidase-conjugated secondary antibody and detected with ECL chemiluminescent detection reagent (Amersham Co., Arlington Heights, IL). The membranes were exposed to X-ray film for 15 sec to 1 min.

RESULTS

The effect of genistein on the differentiation of Type I HBEC into Type II HBEC. Two types of normal HBEC can be developed in pure culture from reduction mammoplasty tissues. Only Type I HBEC at the second passage were used for induction of differentiation into Type II cells by genistein. As shown in Table 1, genistein at concentrations from 0.01 μM to 1 μM significantly increased the frequency of colonies containing Type II cells in HME 21. The effect of genistein was not as potent as cholera toxin, a positive control known to induce the differentiation (Kao et al., 1995). The results from the experiment using a different HBEC culture derived from a different woman (HME 23), however, failure to show the differentiation ability of genistein (Table 1). The induction of differentiation of Type I to Type II HBEC by low concentrations of genistein is usually accompanied by enhanced total cell proliferation in the population. This was seen with HME 21 and HME 23 responded differently to low concentrations of genistein.

The effects of different concentrations (5-100 μM) of genistein on growth of

Type I and Type II HBEC in a 8-day time-course. Pure cultures of Type I and Type II HBEC at passage 2 were used to study the growth response to different concentrations of genistein. The results of these studies are presented in Figure 17, 18 and Table 2.

In Figure 17, growth response curves in a 8-day time course were obtained for Type I and Type II HBEC of HME 21 exposed to different concentrations of genistein. The results show that 5 μM or higher concentrations of genistein completely inhibited the growth of Type I cells (Figure 17a). For Type II cells, 5-25 μM of genistein showed partial inhibition of cell growth in a dose-dependent manner. A complete inhibition of cell growth was observed when the cells were exposed to 50 μM or higher concentrations of genistein. It is clear that Type I cells were much more sensitive to growth inhibition by genistein than Type II cells.

The effect of increasing concentrations of genistein on the growth of Type I and Type II HBEC: dose-dependent inhibition. Results in Figure 18 showed that Type I cells of HME 21 (Fig. 18a) and HME 23 (Fig. 18b) responded differently to low doses of genistein. Genistein at 0.01- 1 μM stimulated cell growth in HME 21 Type I cells, but not HME 23 Type I cells. The stimulation of cell growth by low doses of genistein in HME 21 cells is believed to be due to the induction of Type I to Type II cell differentiation by genistein. The newly converted Type II cells are known to be highly proliferative compared to Type I cells. Genistein at 1 to 5 μM could completely arrest cell growth in HME 21 Type I cells and could completely arrest cell growth at concentration around 50 μM in Type II cells (Fig. 18a). Genistein at concentration

around 10 μM could completely arrest cell growth in HME 23 Type I cells and at concentration around 50 μM could completely arrest cell growth in Type II cells (Fig. 18b). Other different HBEC cultures have also been examined. Table 2 list the concentrations of genistein for complete (IC_{100}) and 50% (IC_{50}) arrest of cell growth among cancer cells, Type I and Type II HBEC of different women after 7 day treatment. Consistently, the results indicate that Type I human breast epithelial cells derived from different women were more sensitive to growth inhibition by genistein than Type II cells (Fig. 3 and Table 2). Furthermore, normal human breast epithelial cells were found to be much more sensitive to growth inhibition by genistein than the breast carcinoma, MCF-7, cell line(Table 2).

The effect of different concentrations of genistein on cell cycle progression of both Type I and Type II HBEC. The flow cytometric analysis revealed that genistein at 5-25 μM did not significantly change the distribution of cells at different phases of cell cycle in HME 21 Type I cells. From Fig. 17a, it is known that 5-25 μM genistein completely arrested the cell growth. It seems that these concentrations of genistein stop the cell cycle progression resulting in no accumulation of cells in a particular phase.

For Type II cells, genistein at 50-100 μM significantly arrested the cells at G2. This effect seems to be reversible when genistein was removed from the medium (Fig. 19 and Table 3).

The effect of genistein on p21 $^{WAF1/CIP1}$ and cdc2 kinase protein expression in

both Type I and Type II HBEC. Western blot analysis showed that the expression of $p21^{WAF1/CIP1}$, which negatively regulates the G1/S transition, and cdc2 kinase, which regulates the G2/M transition, were significantly enhanced or decreased respectively after 72 h of 50 μM genistein (or 25 μM, data not shown) treatment in both Type I and Type II HBEC (Fig. 20) of HME 24 (or HME 21, data not shown). There were no change in the level of p53 and p16 protein expression (data not shown). Therefore, the cell cycle progression arrest could be mediated through a p53, p16-independent mechanism. Thus, our results showed that genistein at 50 μM, which is a cytostatic concentration, can increase the $p21^{WAF1/CIP1}$ protein expression and decrease the cdc2 kinase protein expression to cause cell cycle arrest.

DISCUSSION

Genistein, a component of soy, is suspected to be a chemopreventive agent for breast cancer (Barnes et al., 1990). This is supported by animal experiments showing that genistein treatment during the prepubertal period can suppress the development of chemically-induced mammary tumors in rats (Murrill at al., 1996). Although the effect of genistein has been studied in vitro in breast cancer cell lines as described previously, its effect on normal human breast epithelial cells has not been reported. We believe the study of genistein on normal HBEC is more relevant to evidence concerning its chemopreventive potential. Furthermore, since we have developed two types of normal HBEC from reduction mammoplasty tissues and showed that one of the cell types possessed stem cell characteristics and was more susceptible to neoplastic transformation,

we have a very relevant cell culture system to study the effect of genistein.

The major result from this study is the finding that Type I HBEC were more sensitive to growth inhibition by genistein than Type II cells in all six different HBEC cultures examined. The doses of genistein that inhibited Type I cell growth are at low concentrations (0.1 to 1 μM) which can be easily attained by some vegetarian women (Adlercreutz et al., 1993). In contrast, Type II HBEC and breast cancer cells (MCF-7) were inhibited by higher concentrations of genistein (> 50 μM) which are beyond the physiological dose. The implication of this differential response is that the growth of basal or myoepithelial cells and cancer cells may not be inhibited by physiological doses of genistein in the body. On the other hand, the inhibition of Type I cell growth by physiological doses of genistein could reduce the number of Type I cells in the mammary gland. Since Type I HBEC have stem cell characteristics and have been found to be more susceptible to neoplastic transformation, genistein could effectively reduce the number of target cells for neoplastic transformation and thereby function as a chemopreventive agent.

Our study also revealed a possible mechanism by which genistein inhibited the growth of normal HBEC, i.e. by affecting the expression of cell-cycle regulating genes that arrested the cell cycle progression. Specifically, the expression of $p21^{WAF1/CIP1}$ was enhanced while the expresson of cdc2 was decreased by genistein. The modulation of the expression of these genes could effectively arrest cells at G1 and G2 respectively. Cell cycle analysis confirmed that in Type I HBEC the distribution of cells at G1 and G2

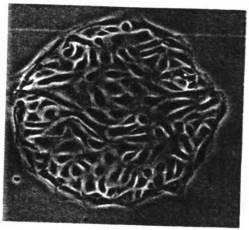
was not affected by concentrations of genistein that completely stopped cell growth. For Type II cells, there was a clear accumulation of cells at G2 by genistein that inhibit cell growth. The difference between the response of Type I and Type II cells to genistein could reflect the fact that there were more cells at S phase in the initial population of Type II cells than Type I cells.

The study of the ability of genistein to induce differentiation of Type I to Type II cells resulted in the finding of positive response in one of two independent HBEC cultures studied. The positive response in HME 21 appears to be real, since it coincided with the increase in cell proliferation by the low doses of genistein. The latter effect is interpreted as due to the induction of differentiation of Type I cells to Type II cells. These newly differentiated cells are highly proliferative. The variable response of different cell cultures derived from different individuals to genistein-induced differentiation is not clear. It could be due to developmental or genetic factors and should be investigated in the future.

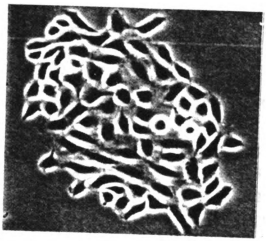
Since Type I cells in conjunction with Type II HBEC are able to form mammary organoid (budding/ductal structures) on Matrigel, it is possible to study the effect of genistein on the development of human mammary gland in the in vitro system. The effect and mechanism of function of genistein revealed by this study should be confirmed by using this system and other studies in the future.

Figure 16. Normal human breast epithelial cells (HBEC) cultured in MSU-1 medium contained three types o f epithelial cell colonies: Type I cells (A), Type II cells (B), and Type I cells surrounded by Type II cells (C) (Kao et al., 1995). In pure Type I cell culture, the appearance of the latter two colonies indicates the differentiation of Type I to Type II cells.

A



B



C

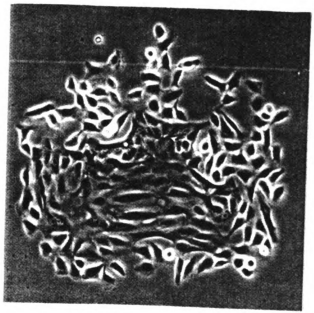


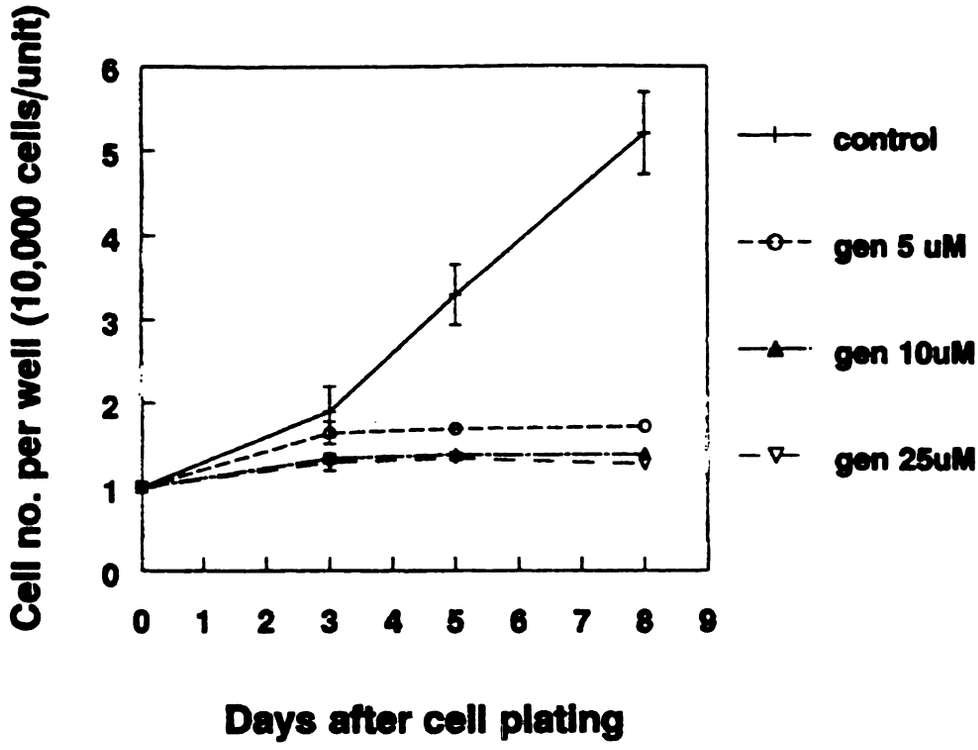
Table 1. The effect of genistein on differentiation of Type I HBEC into Type II HBEC.

Treatments	No. of Type II and Type I surrounded by Type II colonies/ total colony no. (% of Type II and Type I/II colonies)
HME 21	
Control*	57/159 (36.0%)
Cholera toxin (1 ng/ml)	214/245 (87.4%)**
Genistein (0.01 μM)	65/118 (55.1%)**
Genistein (0.1 μM)	68/108 (63.0%)**
Genistein (1 μM)	49/73 (67.0%)**
HME 23	
Control*	22/42 (52.8%)
Cholera toxin (1 ng/ml)	43/54 (78.5%)**
Genistein (0.01 μM)	20/36 (56.1%)
Genistein (0.1 μM)	20/34 (58.4%)
Genistein (1 μM)	12/25 (48.0%)

*Vehicle control contains 1 to 1000 dilution of DMSO in MSU-1 medium. ** highly significantly different from the control ($P<0.01$).

Figure 17. The effects of different concentrations (5-100 μM) of genistein on HME 21 Type I (a) and Type II (b) HBEC growth in a 8-day time-course experiment. Type I and II HBEC were cultured in the presence of various concentrations of genistein(5 μM-100 μM) or DMSO as control for 1,3, 5 and 8 days, in MSU-1 medium at 37°C in a humidified atmosphere of 5% CO_2 in air. Proliferation was assessed by DNA content as measured by lysis of cells with 0.1 N NaOH and reading the absorbance of the clear lysate at 260 nm. The units on the Y axis are expressed as equivalent cell number per well (12 well dishes) determined by DNA content. Results are expressed as average of triplicate plates ± S.E. (standard error).

(a)


Cell no. per well (10,000 cells/unit)
0
1
2
3
4
5
6
0 1 2 3 4 5 6 7 8 9
Days after cell plating
control
gen 5 uM
gen 10uM
gen 25uM

(b)

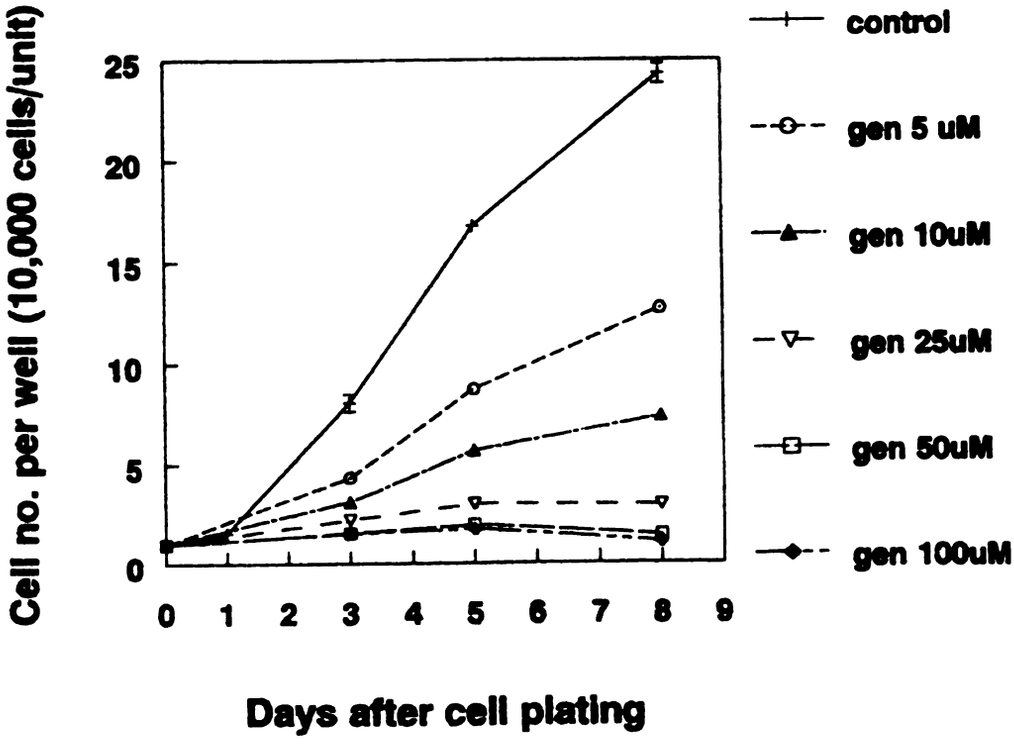
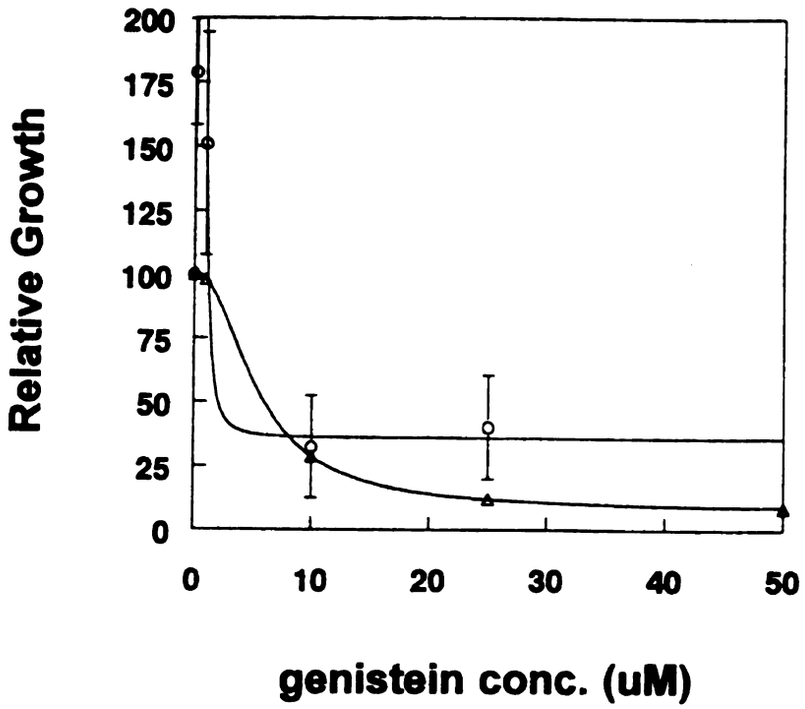

Cell no. per well (10,000 cells/unit)
0
5
10
15
20
25
0 1 2 3 4 5 6 7 8 9
Days after cell plating
control
gen 5 uM
gen 10uM
gen 25uM
gen 50uM
gen 100uM

Figure 18. Dose-dependent inhibition of Type I and Type II HBEC growth by genistein treatment for 7 days. Two different cell cultures were used: (a) results of HME 21 and (b) results of HME 23 (open circles, Type I cells; open triangles, Type II cells). Type I and II HBEC were cultured in the presence of various concentrations of genistein (0.1, 1, 10, 25 and 50 μM) or DMSO (solvent control) for 7 days, in MSU-1 medium at 37°C in a humidified atmosphere of 5% CO_2 in air. Proliferation was assessed by DNA content as measured by lysis of cells with 0.1 N NaOH and reading the absorbance of the clear lysate at 260 nm. Results shown are mean ± S.E. (standard error) from triplicate experiments relative to cell growth without genistein treatment.

(a)


Relative Growth
200
175
150
125
100
75
50
25
0
0
10
20
30
40
50
genistein conc. (uM)

(b)

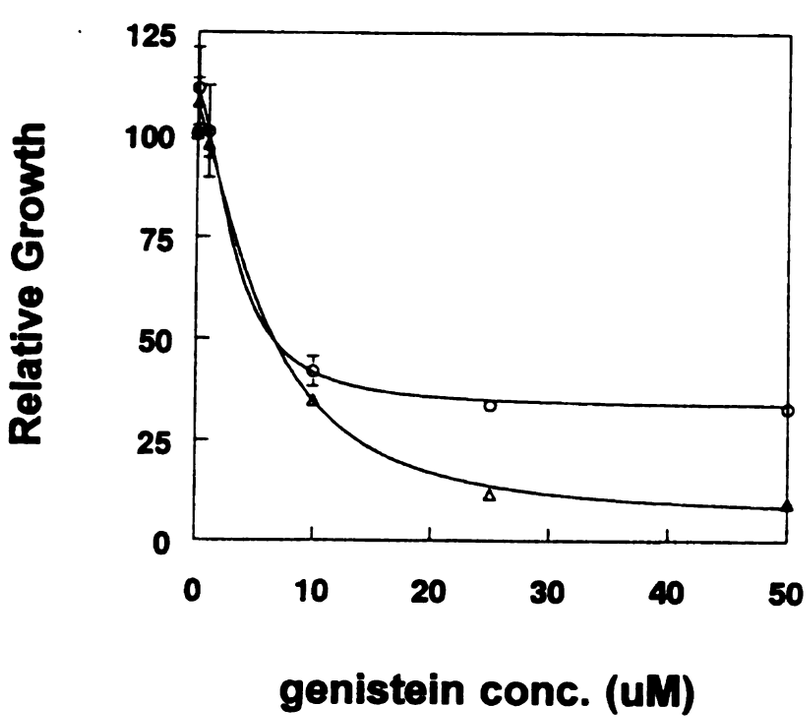

Relative Growth
125
100
75
50
25
0
0
10
20
30
40
50
genistein conc. (uM)

Table 2. The concentrations of genistein for complete (IC_{100}) and 50% (IC_{50}) arrest of cell growth among cancer cells, Type I and Type II HBEC after 7 day treatment.

Cell lines	The concentrations of genistein (IC_{50}) for 50% arrest of cell growth	The concentrations of genistein (IC_{100}) for complete arrest of cell growth
Cancer cell lines		
MCF-7	25 μM	> 100 μM
MDA-mB-231	30 μM	> 100 μM
HME 21		
Type I HBEC	1 μM	1 μM
Type II HBEC	40 μM	50 μM
HME 22		
Type I HBEC	1 μM	10 μM
Type II HBEC	7 μM	25 μM
HME 23		
Type I HBEC	5 μM	10 μM
Type II HBEC	7 μM	50 μM
HME 24		
Type I HBEC	5 μM	10 μM
Type II HBEC	7 μM	50 μM
HME 25		
Type I HBEC	5 μM	10 μM
Type II HBEC	16 μM	50 μM
HME 27		
Type I HBEC	2 μM	10 μM
Type II HBEC	19 μM	50 μM

* HBEC designates normal human breast epithelial cells.

Figure 19. Flow-cytometric analysis of cell cycle distribution of HME 21 Type I (a) and Type II (b) HBEC exposed to different concentrations of genistein. Type I and II HBEC were cultured in the presence of various concentrations of genistein(5 μM-100 μM) or DMSO (solvent control) for 3 days, in MSU-1 medium at 37°C in a humidified atmosphere of 5% CO_2 in air. The DNA content-cell number frequency histograms display the dose-dependent effect of a 72-h treatment with increasing genistein doses in a representative experiment (S-phase reduction, G1/S and G2/M arrest).

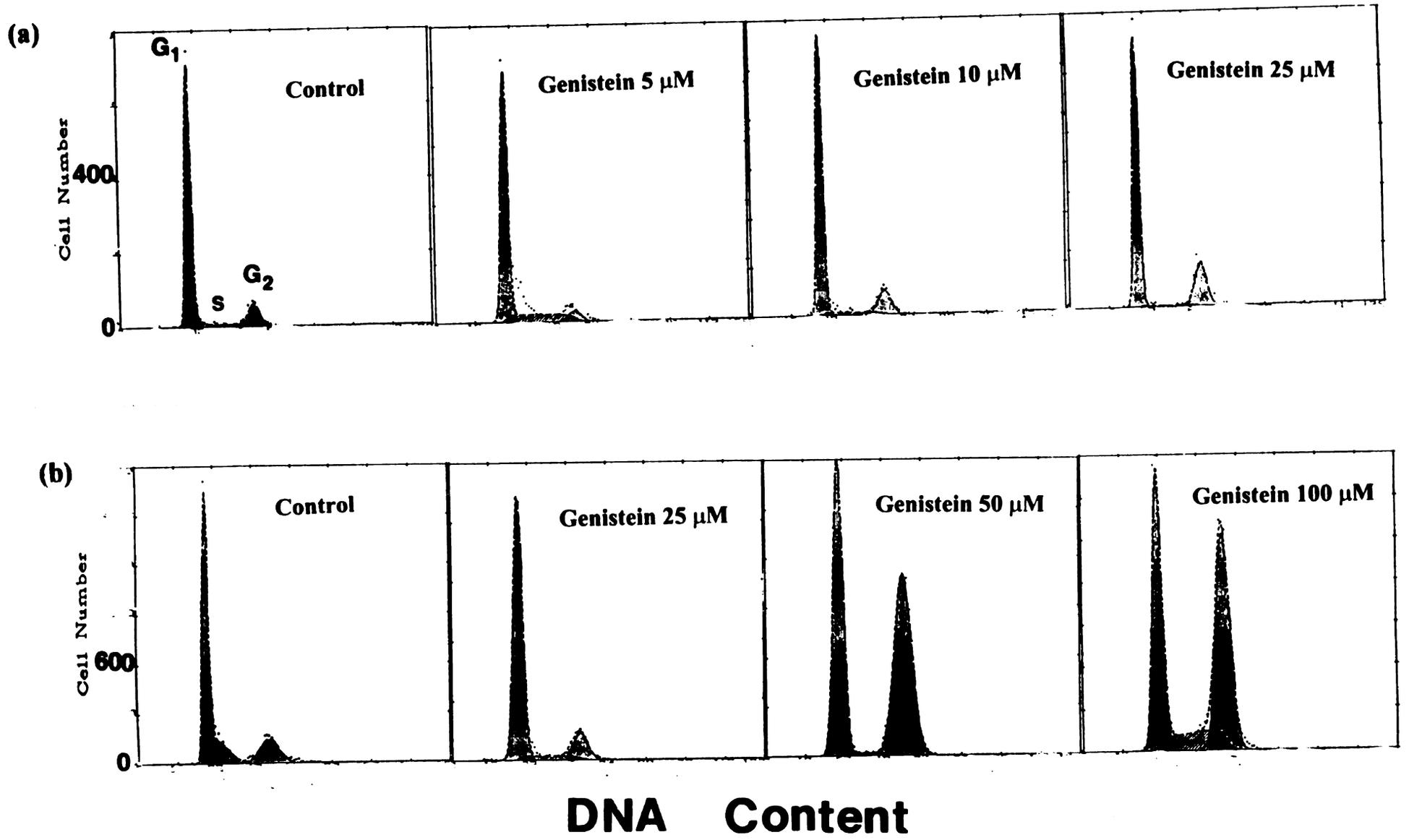
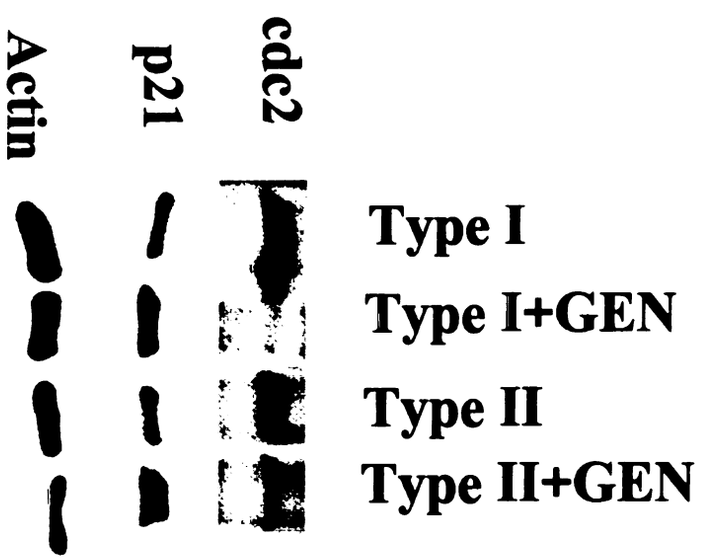

(a)
G1
S
G2
Control
Genistein 5 μM
Genistein 10 μM
Genistein 25 μM
Cell Number
400
0
(b)
Control
Genistein 25 μM
Genistein 50 μM
Genistein 100 μM
Cell Number
600
0
DNA Content

Table 3. The effect of different genistein concentrations on cell-cycle distribution of Type I and Type II HBEC after 72-h treatment.

Treatments	G1 phase (%)	S phase (%)	G2 phase (%)
HME21 type II:			
Control	61.6	19.4	19.0
Genistein (10 μM)	64.0	19.8	16.2
Genistein (25 μM)	65.2	16.5	18.3
Genistein (50 μM)	47.5	2.4	50.1
6 h recovery*	51.9	5.3	42.8
13 h recovery*	66.8	8.1	25.1
Genistein (100 μM)	39.6	14.5	45.9
HME21 type I:			
Control	80.0	8.3	11.7
Genistein (5 μM)	76.4	7.5	16.1
Genistein (10 μM)	79.3	6.1	14.5
Genistein (25 μM)	73.7	2.9	23.4

Data are expressed as percent of the cells in each phase of the cell cycle. Viabilities assessed by trypan blue exclusion were greater than 93% for all concentrations and time points. Vehicle control contained 1 to 1000 dilution of DMSO in MSU-1 medium. *Cells were allowed to recover for 6 hr or 13 hr in fresh growth medium after the 72 hr treatment.

Figure 20. The effect of genistein on the expression of p21 $^{WAF1/CIP1}$ and cdc2 kinase protein in Type I and Type II HBEC. HME 24 Type I and Type II HBEC were cultured as described above in the presence genistein (GEN) (50 μM) or DMSO (solvent control). Total proteins were collected after 48 hours of treatment. Western blot and detection of p21 $^{WAF1/CIP1}$, cdc2 kinase and actin were performed using procedures described in Materials and Methods. Vehicle control contained a 1 : 1000 dilution of DMSO in growth medium. Western blot was also probed for actin using an anti-human actin as an internal control, to confirm equal loading of total proteins among treatment and control groups.


cdc2
p21
Actin
Type I
Type I+GEN
Type II
Type II+GEN

CHAPTER 6

CONCLUSIONS AND IMPLICATIONS OF THE STUDY

This study attempted to elucidate the biological effect and biochemical functions of genistein and dihydrogenistein related to breast carcinogenesis. The following conclusions and implications may be drawn from the results obtained:

1. Genistein at concentrations lower than 10 μM, which is within the physiological range, is able to promote the growth of tumors formed by ER-positive breast cancer cells in vivo through its estrogenic action. Therefore genistein could be detrimental to postmenopausal women who have low levels of circulating estrogen and develop mostly ER-positive breast cancer.

2. The IC_{50} of genistein to inhibit cancer cell growth is always higher than 15-30 μM in several cancer cell lines studied in vitro (Barnes, 1995 and this study). The maximum physiological concentration of genistein has been estimated to be no more than 15 μM by dietary administration (Barnes, 1995). Therefore, it is unlikely that cancer can be cured by eating soy or soy products. On the other hand, if genistein is administered as a pill to reach a high level (> 50 μM) in the body, it could kill normal breast cells as well as cancer cells according to this study. Therefore, it may not be feasible to use genistein as a chemotherapeutic drug. However, genistein has been conjugated to an antibody to target CD 19 cell surface receptor present in B-cell precursor leukemia to selectively inhibit CD 19-associated transmembrane receptor tyrosine kinases and triggered rapid apoptotic cell death (Uckum et al., 1995). The

potential use of this strategy in solid tumors is not clear.

3. Similar to genistein, dihydrogenistein promotes breast cancer cell growth in a dose-dependent manner from 1 nM to 80 μM in ER-positive but not in ER-negative breast cancer cells. The growth promoting effect can be blocked by the estrogen antagonist and coincides with its induction of the expression of the ER-responsive, pS2 protein. Dihydrogenistein differs from genistein in failure to inhibit cell growth at higher concentrations (about 25 μM) in either ER-positive or ER-negative breast cancer cells and in the failure to induce $p21^{WAF1/CIP1}$ expression at 50 μM. This indicates that dihydrogenistein retains the estrogenic activity of the parental compound, genistein, but loses its ability to block cell-cycle progression and to inhibit cancer cell growth. Therefore, dihydrogenistein may be as harmful as genistein and probably no better than genistein as a chemopreventic agent.

4. Genistein preferentially inhibits the growth of a normal HBEC type with stem cell characteristics (Type I HBEC) at physiological concentrations (0.1 to 1 μM). Since Type I HBEC have been shown to be more susceptible to neoplastic transformation in vitro, genistein could reduce the number of target cells for breast carcinogenesis, thereby functions as a chemopreventive agent. The mechanism appears to be mediated by the ability of genistein to induce the $p21^{WAF1/CIP1}$ and to inhibit the cdc2 cell-cycle regulation protein expression.

The conclusions from these studies using in vitro or animal models have their limitations. The potential mechanism that mediates the chemopreventive function of genistein as suggested by this study is consistent with evidence that genistein enhances

rat mammary gland differentiation (Murrill et al., 1996). The hypothesis needs to be substantiated by other epidemiological, clinical trial or in vitro human mammary organoid studies.

Genistein was found to induce the differentiation of Type I to Type II HBEC in one of two cell cultures derived from different human subjects. The extent, mechanism and significance of this variable response are not known.

The amount of dihydrogenistein in the human body as a result of metabloism, absorption and elimination is not known. These are some of the issues need to be investigated in future studies.

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