CHAPTER V

DISCUSSION

Experiment 1: The effects of genistein on NMU-induced tumorigenesis

Here, it was found that supplement of genistein stimulated NMU-induced tumorigenesis in adult female rats by increasing tumor multiplicity and tumor cross-sectional area. In contrast, Hilakivi-Clarke *et al.* (1999) demonstrated that genistein given at the same dosage (1mg/kg BW in rats) protected the mammary glands from 7,12 dimethylbenzanthracene (DMBA)-induced malignant transformation. One explanation for these contrasting results is the reproductive stage of the rats used in both studies. While we used adult female rats, Hilakivi-Clarke *et al.* (1999) used rats of prepubertal stage. Indeed, it has been reported that neonatal and prepubertal treatment of genistein suppressed the development of DMBA-induced mammary adenocarcinomas in rats (Lamartiniere *et al.*, 1995, Murrill *et al.*, 1996) and provides a protective effect against DMBA- induced mammary cancer. Exposure to phytoestrogen before puberty could alter the ontogeny of the mammary glands and cause precocious maturation of breast terminal end buds to more differentiated lobules and subsequent breast cancer protection (Russo *et al.*, 1979, Lamartiniere *et al.*, 2002). In contrast, combining with the present results, increase of post-pubertal (> 21 days old) or adult (45 days old) exposure to phytoestrogen or estrogen (Clarke *et al.*, 1992) may potentially induce breast cancer risks and render adult animals to be more susceptible to chemically-induced mammary cancer. It has been demonstrated that genistein stimulates MCF-7 cell growth *in vitro*, in the presence of estradiol (Wang and Kurzer, 1998, Shao *et al.*, 2000). Day *et al.* (2001) found that genistein (1g/kg diet) fed during adulthood increased the number of DMBA- induced malignant tumors in the mice. Furthermore, consumption of dietary soy, containing genistein, increased cell proliferation in human breast tissue, and also increased progesterone receptor and pS2 expressions, indicative of an estrogenic stimulus in premenopausal women (McMichael-Phillips *et al.*, 1998, Hargreaves *et al.*, 1999).

We found that serum E_2 levels in both genistein and vehicle groups decreased at 1 month when compared to 0 month or the first day of study period. The reduction of E_2 levels assumed that it could not cause by the genistein treatment because the reduction was found in both vehicle and genistein groups. However, it may be due to stress effect during the tumor development. We found that most rats developed tumors during the first 2 months of NMU injection. The mean latency periods were 59.90 $\pm$ 2.70 and 57.30 $\pm$ 3.30 days in vehicle and genistein groups, respectively. A relation between stress and reproductive dysfunction is well established (River and Vale, 1990, Battaglia *et al.*, 1997). Stress increases the hypothalamic-pituitary-adrenal (HPA) axis activity and concomitantly reduces the hypothalamic-pituitary-gonadal (HPG) axis activity. Briski (1996) showed that physical stress induced an increase in plasma corticosterone but decreased plasma luteinizing hormone in rats. In rhesus monkeys, an induction of a 5-day inflammatory stress episode can activate the adrenal axis and increase cortisol secretion but decrease progesterone secretion (Xiao *et al.*, 1999). The recovery of serum E_2 levels after 1 month in both genistein and vehicle groups may be due to adaptation. The HPA axis adapts its activation in the face of a repeated stressor. For example, repeated exposure to restraint cause a reduction in the elevation of adrenocorticotrophic hormone and corticosterone levels in female and male rats (Kant *et al.*, 1985). Thus, the suppression on the hypothalamic-pituitary axis was attenuated (Briski, 1996)

The serum E_2 levels of genistein group were significantly higher than those of the vehicle groups at 1 and 2 month of treatment period. In the present study, we do not measure the cortisol level, the indicator of stress. However, some studies have shown that genistein decreased cortisol production of H295 cells, human adrenal cells, *in vitro* (Mesiano *et al.*, 1999, Ohno *et al.*, 2002). Therefore, it is possible that the reduction of cortisol production by genistein may result in the higher serum E_2 levels in the genistein group than those of the vehicle group.

It should be noted that the diet fed to the rats in this study was an ordinary diet which contained soy, and hence genistein. By this reason, the same lot of rat chow diet (lot no. 070) was used to feed the experimental rats throughout the study period, which aimed to control the consistency of the phytoestrogen contamination. Hence, the analysis of genistein levels in serum samples of vehicle treated rats showed the presence of genistein, but in low amounts. However,

the total serum genistein levels in genistein treated rats were significantly higher than those of the vehicle treated rats. It can conclude that the enhancement of tumorigenesis occurred in genistein treated rats was an outcome of genistein treatment not from the rat diet.

Surprisingly, there was no difference in free genistein levels between the vehicle and genistein treatment groups. The possible explanations for the lack of the difference in free genistein levels were (i) the low dose of genistein (1 mg/kg daily for 20 weeks) used in the present study, (ii) the elapsed time of blood collection (24 hours) after the last dose of genistein treatment and (iii) the mechanism of equilibrium (or homeostasis) of genistein levels in the blood. In rats, after absorption from the intestine, genistein was then metabolized to predominantly glucoronide and to a lesser extent as sulfate metabolites (conjugated forms) by Phase II conjugated enzymes in the intestine and liver. The conjugated forms were the predominant forms of genistein in peripheral blood (Hendrich *et al.*, 1999). The change of free form of genistein was found in case of high dose genistein used, reflection of saturation of Phase II conjugation (Sfakianos *et al.*, 1997). As quoted above, the serum used for analysis was collected 24 hours after the last genistein injection. The disappearance half-life of plasma genistein was approximately 6 hours for purified genistein feeding and 18 hours for genistein from soy extract (King *et al.*, 1996). It can assume that most of free genistein was cleared up from the rats body within 24 hours before the blood sample collection. On the other hand, because the total genistein levels were higher in genistein treated rats, it can conclude that rats maintained the homeostatic levels of free genistein in the blood by keeping it in the conjugated forms.

Recently, it was reported that in female rats, given the 100 and 500 ppm genistein, the free form of genistein concentrations in mammary gland was approximately 50 % of total genistein in serum which higher than that observed in serum (approximately 1-5% of total genistein in the blood) (Chang *et al*, 2000). Presumably because of its higher lipid content, the concentration of free form of genistein in the mammary gland is higher than the blood circulation. Therefore, in this study, genistein treatment may produce sufficient genistein concentration in the mammary tissue and can stimulate tumorigenesis.

Both groups of rats showed a comparable gain in body weight and food consumption that reflects no effect of genistein on rat growth. This result is consistent with the study of Hilakivi-

Clarke *et al.* (1999), which demonstrated that at low dosage genistein did not affect the body weight gain.

Since there was no significant difference in the length of estrous cycle, uterine and ovarian weights between genistein and vehicle group, this suggests that genistein at the dose of 1 mg/kg BW did not high enough to have an effect on female reproductive organs and function. These results are supported by the study of Santell *et al.* (1997), that is, feeding of the dietary genistein at 0.375 mg or 0.75 mg/kg did not affect the uterine weight of intact rats. In ovariectomized rats in which uterus tissues are more sensitive to exogenous estrogenic chemicals than that of intact animal, genistein as high as 2.5 mg/kg BW/day s.c. did not induce uterotrophic and vaginal epithelium proliferation (Makela *et al.*, 1999, Malaivijitnond *et al.*, 2006). Therefore, in this study, not surprising, the treatment with 1 mg/kg BW of genistein daily did not increase the uterine and ovarian weights and no disruption on vaginal cyclicity. These results support the possibility that genistein may be a "tissue-selective" estrogen with minimal effects on the reproductive organs, but high potential for breast tissue. Indeed, the length of the estrous cycle increased significantly in both groups (vehicle: 6.08 $\pm$ 0.43 days; genistein: 6.88 $\pm$ 0.79 days, mean $\pm$ SE) during the last three months of the study. These changes in the estrous cycles may be due to the transition period to the middle-age of rats, which eventually leads to an acyclic reproductive state (Lu *et al.*, 1979).

In this study, genistein at a dose of 1 mg/kg BW was chosen because it is comparable to the approximately daily consumption in Asian people on mg/kg BW basis (Nagata *et al.*, 1998, Wakai *et al.*, 1999). It can be concluded that long-term exposure to genistein at the dosage comparable to the ordinary human consumption did not affect reproductive system, but it can cause the enhancement of NMU-induced tumorigenesis in intact adult female rats.

Experiment 2: The effects and mechanism of genistein and tamoxifen on NMU-induced mammary tumor growth

Consideration of the effects of genistein on the mammary growth in Experiment 2, genistein showed a trend to stimulate mammary tumor growth. At the end of treatment, the average cross - sectional area tended to be larger in genistein group when compared with the vehicle group (1,081 $\pm$ 116 versus 776 $\pm$ 227 mm^2, respectively). The ability of genistein to stimulate mammary tumor growth in ovariectomized rat and mice was previously evaluated (Hsieh *et al.*, 1998, Ju *et al.*, 2001, Allred *et al.*, 2001, 2004). Most of the previous studies, including the result of Experiment 1, showed that genistein was able to contribute substantial estrogenic action, stimulating mammary tumor growth, increasing cancer cell proliferation and inducing the expression of estrogen responsive genes. Therefore, it can conclude that the supplement of phytoestrogen as genistein to the intact female rats could stimulate the mammary tumor growth. On the other hand, treatment with tamoxifen could decrease the tumor cross-sectional area, tumor weights and stabilized the tumor multiplicity. These results are consistent with previous studies that tamoxifen suppressed the tumor progression in rats (Jordan, 1976, Gottardis and Jordan, 1987, Osborn *et al.*, 1992).

To explore possible mechanisms of genistein and tamoxifen on mammary tumor growth, the expressions of cancer related genes, including ER related genes (ERα, ERβ and pS2), growth factor related genes (IGF-1 and *neu*) and metastasis suppressor gene (GPR54) were determined by using semi-quantitative RT-PCR technique. At the molecular level, tamoxifen was found to significantly down-regulate the expression of ERα mRNA which is consistent with the previous report (Lacobelle, 1986). The reduction of ERα expression led to decrease the capacity of the cell to respond to endogenous estrogen, as shown by the reduction in the estrogen responsive pS2 gene mRNA expression. These results showed that antitumor mechanisms of tamoxifen are mediated through ER pathway, and consequentially supports the use of tamoxifen in the treatment of estrogen dependent breast cancer in premenopausal women.

It has been shown in breast tumor tissue that the ratio between ERβ and ERα is decreased as compared to normal breast epithelium from the same patient (Leygue *et al.*, 1998). Speirs *et al.* (1999) has compared expression of ERα and ERβ in normal and malignant breast. While biopsy from normal breast predominantly express ERβ (22% of sample), this was not

observed in any of the breast tumors investigated. Most breast tumor expressed ERα, either alone or in combination with ERβ. The specific functions of ERβ in the breast are not known, but there is some evidence that this receptor may inhibit cellular proliferation by antagonizing the actions of ERα (Petterson *et al.*, 2000, Lazennec *et al.*, 2001). ERβ is high in resting breast epithelium as compared to proliferating breast tissues (Roger *et al.*, 2001). In agreement with the previous reports, the expression of ERβ in mammary tumor is low in the present study; it could be detected only after nested RT-PCR. By then changes of ERβ expression after genistein and tamoxifen treatment could not be detected. To assess whether ERβ has a role to modulate and antagonize the effects of ERα in breast cancer tissues, a more sensitive method which can discriminate the small amount of changes of ERβ expression, such as a quantitative-RT-PCR method, should be used. Thus, it may facilitate us a better understanding on mechanisms of genistein and tamoxifen on ERβ expression.

Genistein treatment resulted in a reduced ERα expression which is consistent with the study of the other researchers (Maggiolini *et al.*, 2001). This reduction was significantly smaller than that induced by tamoxifen treatment and did not result in reduced expression of the estrogen responsive pS2 gene mRNA levels. However, despite the inhibition of ERα by both genistein and tamoxifen, within the single doses tested in this study, genistein does not show either an additive or a synergistic effect with tamoxifen in inhibition of ER receptor or responsive gene mRNA expression levels. Further studies using a range of dose responses of both tamoxifen and genistein are required to evaluate if any additive or synergistic effects are present and thus potential biochemical interactions.

Interestingly, the present result showed that, at least at the dose tested here, genistein treatment resulted in increased expression of IGF-1 mRNA level, and therefore genistein could be involved in the IGF-1 signaling system. This finding is supported by the recent study of Chen and Wong (2004) who demonstrated that 1 μM genistein caused the induction of IGF-1R and IRS-1 expression in MCF-7 cells. IGF-1 is a known mitogen in breast tissues, exerting its action via paracrine and/or autocrine mechanisms, and activation of the IGF system plays a critical role in the development and progression of human breast cancer (Surmacz, 2000). IGF-1 increased DNA, RNA and protein synthesis, mainly via activation of mitogen-activated protein kinase (MAPK) and phosphoinositide 3'-kinase (PI3-K) pathways (Sathyamoorthy *et al.*, 2002, Qiang *et al.*, 2002). IGF-1 also promotes cell cycle progression in mammary epithelium and other tissues via the induction of cyclin D and cyclin E, a progression factor required to overcome the G_1-S

checkpoint in the cell cycle (Stull *et al.*, 2002). Anti-apoptotic mechanisms of IGF-1 are believed to involve the activation of the MAP-K and PI3-K pathways (Qiang *et al.*, 2002) as well as induction of antiapoptotic proteins such as Bcl-2 (Beierrie *et al.*, 2002). Activation of the IGF-IR also regulated several cellular functions that could impact on the metastatic potential of the cells, including cellular proliferation, anchorage-independent growth, cell migration and invasion (Brodt *et al.*, 2000). Furthermore, there is accumulating evidence that cross-talk exists between ER and IGF-R–mediated pathway in ER-positive breast cancer cells (Yee and Lee, 2000). There is also a proposal that IGF-1 can activate the ER, while ER transcriptionally regulates gene required for IGF-1 action (Martin and Stoica, 2002). The importance of IGF components in ER action and breast cancer growth is provided by Lee *et al.* (2005) who demonstrated that estrogen enhances IGF-1 signaling by inducing expression of the key IGF regulatory molecules, the type 1 IGF receptor (IGFR1) and its downstream signaling molecules, insulin receptor substrate (IRS)-1 and IRS-2 of ER-positive breast cancer cell lines *in vitro* and MCF-7 xenografts tumor studies. It does not know whether the induction of IGF-1 expression by genistein is modulated via ER pathway or other pathways. However, as mention above, the upregulation of IGF-1 signaling pathway could in turn increase the transcriptional activity of ER, indicated by the reduction of ERα expression and no changes of the estrogen responsive pS2 gene expression after the genistein treatment. Thus, the present results clearly show that the supplementation of genistein has the potential to promote mammary tumor growth through enhanced activation of the IGF-1 signaling pathway.

Genistein treatment increased the percentage of metastases to liver and uterus (50%, 10%, 10% and 0%, in genistein, vehicle, tamoxifen+genistein and tamoxifen groups, respectively). It is note worthy that the reduction of GPR54 expression, metastasis suppressor related gene, in tumor obtained from genistein treated group, and the partial inhibition of this by cotreatment with tamoxifen, may be associated with the potentially increased level of metastases of breast cancers to the other organs in these rats. GPR54 is an orphan G protein-coupled receptor. Metastin, a product of Kiss-1 gene, is a candidate endogenous ligand for GPR54 (Stafford *et al.*, 2002), and has been identified as a metastasis-suppressor gene, inhibiting both cell migration and cell growth. Binding of metastin to GPR54 leads to the activation of phospholipase C and increase in intracellular inositol-(1,4,5)-triphosphate and Ca^{2+}, and activation of ERK and MAP-kinase pathways (Kotani *et al.*, 2001, Stafford *et al.*, 2002). Transfection with *Kiss-1* gene to MDA-MB-435, human breast cancer cell line, and then injection into the mammary fat pads of athymic nude mice reduced metastatic potential by 95% compared to non-

transfected cells (Lee and Welch, 1997). Although a physiological role of GPR54 has yet been ascertained, the presence of this mRNA in NMU-induced mammary tumors may facilitate our understanding of the role of GPR54 in these tumors, and in particular whether it is directly or indirectly associated with the anti-metastatic process. Upon the latest information, the specific activity of genistein on GPR54 expression has not been evaluated, and further *in vitro* studies therefore need to be designed in order to address this issue.

The stimulation of tumor growth was, however, obscured when genistein was treated to the rats in combination with tamoxifen. The combination of tamoxifen and genistein treatment also decreased the tumor cross-sectional area comparable to tamoxifen treatment alone. It seems that there was no difference effect between tamoxifen and tamoxifen plus genistein treatment. However, at least, it was found that treatment of tamoxifen along with genistein showed the molecular difference from the treatment of tamoxifen only. The combination of tamoxifen and genistein treatment resulted in a significantly higher IGF-1 mRNA expression level compared to tamoxifen alone. Thus, it appears that genistein may antagonize the antitumor action of tamoxifen by increasing IGF-1 expression thus potentially stimulate mammary tumor growth through the IGF-1 signaling pathway. The antagonistic action of genistein to tamoxifen is supported by recent studies. It was shown that genistein can reverse the inhibitory effects of tamoxifen on E_2-stimulated ER-mediated reporter gene activity *in vitro* (Schwartz *et al.*, 1998) and negate the beneficial effects of tamoxifen on E_2-dependent breast tumor growth *in vivo* (Ju *et al.*, 2002). However, in this study, at this dose any antagonistic action of genistein to tamoxifen on mammary tumor growth could not be observed, although as already stated dose response trials are now required.

The expression of *neu*, another growth factor related gene, was detected in tumor obtained from four treated groups. There were no statistically significant differences in the expression of *neu* in these tumors. It is consistent with the study of Gallo *et al.* (2002) which showed that dietary soy did not modulate the expression of *neu* in DMBA-induced rat mammary tumors. These results suggested that tamoxifen and genistein had a minor effect on the expression of *neu* gene.

In conclusions, in this study, the obtained data indicated that (i) tamoxifen inhibited mammary tumor growth in a manner consistent with the downregulation of ERα expression, thus, reducing the capacity of the cancer cell to respond to endogenous estrogen. (ii) Genistein induced

elevated IGF-1 mRNA expression and this was potentially associated with a stimulation of the mammary tumor growth. (iii) The decrease in expression of GPR54, the orphan receptor of metastasis suppressor gene, in genistein treated group may play a role in the metastatic potential of cancer cells to other organs. Thus, (iv) taken together this study raises concern about the supplementation of genistein in premenopausal women who have the estrogen-dependent breast cancer. The stimulation of tumor growth was, however, obscured when genistein was treated together with tamoxifen. Further investigation on the interaction of genistein and tamoxifen on mammary tumor growth is needed

Genistein treatment did not modulate the serum E_2 level when the E_2 levels were determined at the end of study or after 10 weeks of genistein treatment. In agreement with the Experiment 1, treatment with 1 mg/kg of genistein for 20 weeks did not change the estradiol levels throughout the study period, except for the first month, when the hormonal levels were determined monthly. In contrary, both of tamoxifen treatment, alone or in the combination with genistein, increased serum E_2 level. This results are consistent with the study of Baptista *et al.* (1997) which reported that tamoxifen at the same dosage used in this study (100 μg) significantly increase estradiol levels in female rats. In humans, modulation of E_2 circulating level by tamoxifen has been reported (Jordan *et al.*, 1987) but the mechanism responsible for these effects is unclear. It could be related to a negative feed-back loop activated by the antagonistic effects of tamoxifen on estrogen binding sites in the brain, or to stimulation of gonadotrophin levels (Jordan *et al.*, 1987). Ravin *et al.* (1988) reported that long-term tamoxifen treatment increased levels of estradiol and follicular-stimulating hormone. Since tamoxifen is considered to be a competitive antagonist of estrogen in the breast tissues, therefore, increase in serum estrogen might be inappropriate for long-term tamoxifen therapy in premenopausal patients.

In the present study, SD rats given either tamoxifen alone or tamoxifen plus genistein were significantly ($P<0.05$) decreased in body weights. The decrement of body weight after tamoxifen treatment was also observed in many experiments (Gray and Wade, 1981, Gray *et al.*, 1993, Baptista *et al.*, 1997, Cho *et al.*, 2003). The mechanism is still unclear. One explanation is that tamoxifen treatment mimicked the effects of estradiol and caused significant decrease in body weight (Wade and Heller, 1993). Estradiol plays a critical role in the regulation of food intake and body weight in female rat, because it acts both on the brain and peripheral tissues (such as the liver and adipose tissue) to affect regulation behaviour, as well as the partitioning and metabolism of metabolic fuels. In the adipose tissues, estradiol decreases lipoprotein lipase

activity and in situ lipogenesis, and increases lipolysis and fatty acid release (Wade and Schneider, 1992). Ultimately, all these effects decrease body weight. Wade and Heller (1993) reported that treatment with estradiol benzoate significant reduced body weight gain in ovarectomized rats. In adult female rats, ovariectomy resulted in an increase in food intake and body weight (Gray *et al.*, 1993, Wade and Heller, 1993).

Because the increase of estradiol levels was observed after tamoxifen treatment, changes at weights and cellular levels (under histological examination) of uterus and ovary were examined. The uterine and ovarian weights of rat treated with tamoxifen and tamoxifen plus genistein were significantly decreased compared with those of vehicle and genistein groups, and then, histological changes in reproductive tissues were clearly observed. Tamoxifen treatment had an antiestroginic effect on uterus by decreased uterine sizes and weights in intact rats (Alan *et al.*, 1999, Nephew *et al.*, 2000, Cho *et al.*, 2003). Nephew *et al.* (2000) reported that the oral tamoxifen administration (1 and 10 mg/kg) to intact female rats for 28 days produced a decrease ($p< 0.05$) in overall uterine size and myometrial thickness, however, uterine luminal and glandular epithelial cell hight increase when compared with control rats. Alan *et al.* (1999) reported that decreased ovarian weights and histopathological changes, e.g., decreased copora lutea of ovary were detected in female rats treated orally with tamoxifen (250 g/kg diet per day) for 13-weeks. It has been reported that tamoxifen treatment increased the incidence of ovarian cysts and the significantly higher E_2 serum levels in the premenopausal women (Cohen *et al.*, 1999). Together with the observation in this study, tamoxifen may act directly on the ovary, inducing follicular cystic changes and massive ovarian steriodogenesis, thus causing supraphysiological E_2 levels.

No changes were observed in the liver tissues after tamoxifen treatments, even though the weight was significantly reduced. It has been reported that tamoxifen is a genotoxic mutagenic liver carcinogen. It is transformed to a reactive metabolite (alpha hydroxyl-tamoxifen) which give rise to high levels of DNA adducts in the liver (Martin *et al.*, 1998). However, the genotoxic dosage is 300 times higher than the dosage used in this study (Greaves *et al.*, 1993).