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Prepubertal exposure to zearalenone or genistein reduces mammary tumorigenesis

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Summary Prepubertal exposure to a pharmacological dose (500 mg kg^{-1}) of the phyto-oestrogen genistein can reduce the incidence and multiplicity of carcinogen-induced mammary tumours in rats. However, such an exposure also disrupts the function of the hypothalamic–pituitary–gonadal axis, making it unsuitable for breast cancer prevention. We studied whether prepubertal exposure to genistein at a total body dose broadly comparable to the level typical of Oriental countries, approximately 1 mg kg^{-1} body weight, affects mammary tumorigenesis. We also studied whether prepubertal exposure to zearalenone, a major source for phyto-oestrogens in the USA, influences breast cancer risk. Prepubertal rats were treated between postnatal days 7 and 20, with 20 µg (~ 1 mg kg^{-1} body weight) of either genistein or zearalenone. Zearalenone exposure significantly reduced both the incidence and multiplicity of mammary tumours induced by 7,12-dimethylbenz(a)anthracene (DMBA). Genistein exposure significantly reduced tumour multiplicity, but not tumour incidence, when compared with vehicle-treated animals. Furthermore, 60% of the tumours in the genistein group were not malignant, while all the tumours analysed for histopathology in the vehicle and zearalenone groups were adenocarcinomas. A higher number of differentiated alveolar buds, and lower number of terminal ducts, were present in the DMBA-treated mammary glands of the phyto-oestrogen exposed rats. The concentration of oestrogen receptor (ER) binding sites after the DMBA treatment was low in the mammary glands of all groups but a significantly higher proportion of the glands in the zearalenone exposed rats were ER-positive (i.e. ER levels ≥ 5 fmol mg^{-1} protein) than the glands of the vehicle controls. Our data suggest that a prepubertal exposure to a low dose of either zearalenone or genistein may protect the mammary gland from carcinogen-induced malignant transformation, possibly by increasing differentiation of the mammary epithelial tree.

Keywords: genistein; zearalenone; prepuberty; mammary tumorigenesis

Asian populations with a high intake of phyto-oestrogens have a relatively low incidence of breast cancer (Setchell et al, 1984). Therefore, it has been suggested that phyto-oestrogens may reduce breast cancer risk (Messina et al, 1994). Phyto-oestrogens are naturally occurring compounds produced by a variety of plants. They are present in several foods, including soybean-based products that are typical for diets of Asia, but not for Western countries. Approximately 50% of the isoflavones in soybeans consists of genistein (Messina et al, 1994). There is some epidemiological evidence in favour of soy/genistein being anti-tumorigenic, but this is controversial. In three of a total of eight studies, a statistically significant association between high soy intake and low breast cancer risk has been found (Nomura et al, 1978; Hirohata et al, 1985; Lee et al, 1991; Yuan et al, 1995; Wu et al, 1996; Witte et al, 1997; Zheng et al, 1999). While most animal studies are supportive of the hypothesis that genistein inhibits mammary tumour promotion (Hawrylewicz et al, 1991; Messina et al, 1994; Barnes, 1997; Gotoh et al, 1998), some studies show an ability of genistein to increase mammary tumorigenesis (Hsieh et al, 1998). Prepubertal exposure via injections to a pharmacological dose of genistein (500 mg kg^{-1}) on days 16, 18 and 20 is reported to dramatically reduce subsequent risk to develop mammary tumours (Murrill et al, 1996). Thus, genistein may be particularly effective in reducing breast cancer risk, if its exposure occurs prior to puberty.

Zearalenone is another phyto-oestrogen that may be linked to mammary tumorigenesis. Zearalenone is mainly produced by the mould *Fusarium graminearum* found in a variety of host plants and debris from soil around the world (Burgess et al, 1982). It is present as a contaminant in stored cereals, being found in barley, corn, corn flakes, rice and wheat at concentrations from 35 to 115 µg kg^{-1} (Hagler et al, 1984; Schoental, 1985; Luo et al, 1990). Zearalenone also is used as an anabolic agent to enhance growth in cattle and lambs (Ralston, 1978; Wiggins et al, 1979). In contrast to genistein, pharmacological doses (10 mg kg^{-1} body weight) of zearalenone have been associated with an increased breast cancer risk in rats (Schoental, 1974).

Enthusiasm concerning the effects of prepubertal genistein exposure is limited because the dose used in previous studies is 5000 times higher than that of human exposure on a mg kg^{-1} body weight basis (Murrill et al, 1996). This pharmacological exposure causes severe perturbations in hypothalamic-ovarian function (Murrill et al, 1996) that may lead to infertility. Asians consume 2–9 g soy protein daily, while most women in Western countries do not consume any soy (Seow et al, 1998; Wu et al, 1998).

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Genistein content of different soy products varies considerably; for example, soy beans, soy milk and tofu contain approximately 2–18 μg genistein per g product, while miso and natto contain 40–330 μg genistein per g product (Lu et al, 1995; Fukutake et al, 1996). Fukutake et al (1996) have estimated that the daily genistein intake is 1.5–4.1 mg (< 0.1 mg kg^{-1}) among Asians. Zearalenone is the main phyto-oestrogen consumed in the USA (Kuiper-Goodman, 1990). The present study examined whether a prepubertal exposure to a more physiological dose of genistein and zearalenone (~ 1 mg kg^{-1} body weight) alters carcinogen-induced mammary tumorigenesis in rats. In addition, we investigated whether the concentrations of total oestrogen receptor (ER) binding sites are altered by prepubertal phyto-oestrogen administration. Low doses of genistein and zearalenone are known to bind to ER and affect its transcriptional regulatory activities (Wang et al, 1996; Collins et al, 1997; Zava and Duwe, 1997). The effect on mammary gland morphology also was studied, since a previous report suggested that an increased differentiation of the mammary epithelial tree by prepubertal genistein exposure may explain its cancer-reducing effects (Murrill et al, 1996).

METHODS

Animals

Pregnant female Sprague-Dawley rats, purchased from Charles Rivers Breeding Laboratories, were obtained at day 10 of gestation. The animals were housed singly, in standard rat plexiglas cages, at a constant temperature (20–22°C) and humidity (60–65%), under a 12-h light–dark cycle (lights on 06:00 h). Two days after the offspring were born, the males were sacrificed and the females cross-fostered. Ten to twelve female pups were housed with a lactating dam. The female offspring were weaned on postnatal day 22, and thereafter housed in groups of 3–5 animals. All studies were performed in accordance with the appropriate institutional and federal requirements.

Phyto-oestrogen exposure

On postnatal day 7, the litters were divided into three groups. The offspring received 20 μg genistein, 20 μg zearalenone (both from Sigma Chemical Co., St Louis, MO, USA), or vehicle, administered as subcutaneous injections, in a volume of 0.05 ml. Phyto-oestrogens were first dissolved in 2% dimethyl sulphoxide (DMSO), and then mixed with peanut oil (this mixture also served as vehicle). The injections were repeated on days 10, 14, 17 and 20. Body weight of a rat pup on day 7 is approximately 10 g and on day 20 25–30 g. Thus, the animals received genistein at the doses ranging from 2 mg kg^{-1} (day 7) to 0.7 mg kg^{-1} (day 20).

Inducing and monitoring of mammary tumorigenesis

Mammary tumors were induced by administration of 10 mg (~ 50 mg kg^{-1} body weight) 7,12-dimethylbenz(a)anthracene (DMBA) (Sigma, St Louis, MO, USA). This is a suboptimal dose used in our laboratory to enable assessments of both reductions and increases in the end points of tumorigenicity (Hilakivi-Clarke et al, 1997*b*). More than 75% of the tumours induced by 10 mg DMBA are adenocarcinomas (Russo and Russo, 1987). The carcinogen was dissolved in peanut oil and administered by oral gavage in a volume of 1 ml. Animals were 45 days old at the time of DMBA administration. The groups that were given phyto-oestrogens or vehicle after birth each contained 30 animals.

The animals were examined regularly for mammary tumours by palpation once per week. The end points for data analysis were (i) latency to tumour appearance, (ii) the number of animals with tumours (tumour incidence), (iii) the number of tumours per animal (tumour multiplicity), and (iv) tumour proliferation. A tumour was designated as proliferating if it increased regularly in size. Tumour sizes were measured by recording the tumour diameters with a caliper and determining the length of the longest axis and the width perpendicular to the longest axis. The animals were sacrificed when detectable tumour burden approximated 10% of total body weight, as required by our institution. All surviving animals, including those that did not appear to develop mammary tumours, were sacrificed 19 weeks after carcinogen administration.

Histopathology of the DMBA-induced mammary tumours was evaluated from 22 haematoxylin and eosin stained samples. Two pathologists at the Lombardi Cancer Center (Georgetown University) independently assessed the tumour samples. The pathologists were blind to the experimental groups.

Oestrogen receptor

The number of ER binding sites in the 4th mammary glands were determined from female rats exposed to genistein, zearalenone, or vehicle during the prepubertal period (n = 7 per group). The animals from which the mammary glands were taken had been treated with DMBA 18 weeks before, and consequently developed at least one mammary tumour. None of the tumours were in the 4th gland in the animals used for ER assays. ERs were detected using a ligand binding assay as described by Nelson et al (1986). The ligand used was [2,4,6,7-^{3}H] 17β oestradiol (specific activity 99 Ci $mmol^{-1}$; Amersham, Arlington Heights, IL, USA). This assay detects both ERα and ERβ with equal efficacy. Thus, ER binding reflects total ER concentrations (ERα + ERβ).

Mammary gland morphology

Whole mounts of the 9th mammary glands of the same female rats whose 4th glands were used for ER assays, were prepared (n = 4–5 per group). At the time of sacrifice, 18 weeks had passed from the DMBA administration. The removed glands were stained with carmine aluminium as previously described by Haslam (1988). We have previously validated a visual scale to study the development of a mouse and rat mammary epithelial tree (Hilakivi-Clarke et al, 1997*a*, 1997*b*). Using this scale, we determined differentiation of mammary epithelial structures in the whole mounts. The mammary epithelial trees were analysed for the density of ductal structures, terminal ducts and differentiated alveolar buds. This analysis was done double-blind under an Olympus dissecting microscope, using a 5-point scale (from 0 = absent to 5 = numerous). Differentiated alveolar buds do not give rise to adenocarcinomas. While terminal ducts occasionally give rise to tumours, the majority of tumours originate from terminal end buds (Russo and Russo, 1987). However, at the time the whole mounts were obtained from 6-month-old rats, all terminal end buds had differentiated to alveolar buds or regressed to terminal ducts. In addition to this quantitative analysis of the mammary whole mounts, a qualitative evaluation was performed.

Table 1 Effects of early postnatal exposure to 20 μg genistein or 20 μg zearalenone on mammary tumour growth

	Tumour latency (weeks)	Tumour area (mm^2)	Tumour multiplicity	Number of non-proliferating tumours
Vehicle (n = 33/17)[d]	11.0 ± 0.7	64.5 ± 13.5	1.8 ± 0.3	2 (6%)
Genistein (n = 15/13)	11.4 ± 0.3	67.7 ± 14.2	1.1 ± 0.1[b]	6 (40%)[c]
Zearalenone (n = 13/9)	14.4 ± 0.3[b]	58.7 ± 15.7	1.2 ± 0.1[a]	5 (38%)[c]

Significantly different from vehicle group: [a]$P < 0.06$, [b]$P < 0.01$, [c]$P < 0.001$. [d]n = Number of tumours/number of animals with tumours. Note: 60% of the tumours in the genistein group are benign, while all tumours examined in the vehicle and zearalenone groups are malignant adenocarcinomas. Data represent the mean ± s.e.m. of latency to tumour appearance, area of tumours at first detection, tumour multiplicity and percentage of non-proliferating tumours. Number of rats per group = 30.

Statistical analyses

Results for the data obtained on weight gain, ER binding sites, density of epithelial ducts, terminal ducts and alveolar buds in the whole mounts, and tumour latency, multiplicity, size upon first detection and growth data were analysed using one-way analysis of variance (Snedecor, 1988; Hanfelt, 1997). Where appropriate, between-group comparisons were done using Fisher's least significant difference. Results of tumour incidence were analysed using a log-rank survival analysis test. Differences in the number of non-proliferating and proliferating tumours, and the percentage of mammary glands that contained ER levels that were either ≥ 5 fmol mg^{-1} protein or < 5 fmol mg^{-1} protein, were determined using a χ^2 test. All probabilities are two-tailed. Statistical tests were performed using the BMDP software (BMDP Statistical Software, Los Angeles, CA, USA).

RESULTS

Effect on weight gain

Early postnatal exposure to either genistein or zearalenone did not affect body weight gain. Between the first (day 7) and last day of phyto-oestrogen exposure (day 21), weight increased by 2.5-fold in the vehicle-treated rats, by 2.5-fold in the genistein-treated rats and by 2.6-fold in the zearalenone-treated rats. Body weights also were similar at the time the carcinogen was administered or 18 weeks after the administration (data not shown).

Mammary tumorigenesis

Tumour latency

The first tumours appeared on week 7 after the DMBA exposure in all groups. The mean tumour latency time to the first tumour per animal was significantly longer in the female rats exposed to zearalenone during early postnatal period than in the vehicle-treated rats ($F(2,37) = 4.91$, $P < 0.01$) (Table 1). Tumour latency was similar in the rats exposed to genistein or vehicle during prepuberty.

Tumour incidence

The incidence of mammary tumours (number of animals with tumours per group) was determined weekly, beginning on week 7 following DMBA administration. At the end of the study, on week 18 following DMBA administration, the percentage of rats with mammary tumours was 57% (17/30) in the vehicle-treated group, 43% (13/30) in the genistein-treated group, and 30% (9/30) in the zearalenone-treated group ($\chi^2 = 14.92$, df = 2, $P < 0.001$).

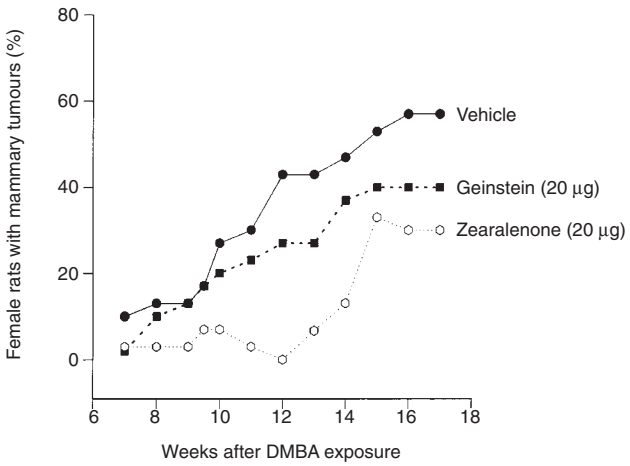

Figure 1 The proportion of female rats exposed to 20 μg genistein, 20 μg zearalenone, or vehicle during postnatal days 7, 10, 14, 17 and 20, that developed DMBA-induced (administered on day 45) mammary tumours. The number of animals per group was 30. Tumour incidence was significantly lower in the zearalenone-treated rats ($P < 0.001$)

Log-rank test analysis indicated that the tumour incidence was significantly lower in the animals exposed to zearalenone during the prepubertal period than in the vehicle-treated controls (z-value = 2.36, $P < 0.018$). The slightly lower tumour incidence in the genistein-exposed rats was not statistically significant, when compared with the controls ($z = 1.03$, $P < 0.30$) (Figure 1).

Tumour multiplicity

The average number of tumours per animal was significantly lower in the rats exposed to genistein ($P < 0.01$) during prepubertal life than in the vehicle-treated rats ($F(2,38) = 4.53$, $P < 0.02$). A reduction that approached statistical significance also was seen in the zearalenone-exposed rats ($P < 0.06$) (Table 1).

Tumour growth rate

The size of the tumours upon first detection was similar among the groups (Table 1). However, the percentage of proliferating tumours in the genistein- (60%) and zearalenone-exposed rats (62%) was significantly lower than in the vehicle-treated group (94%) ($\chi^2 = 9.96$, df = 2, $P < 0.001$).

Tumour histopathology

Histopathological analysis performed for 22 samples indicated that the histotypes of all mammary tumours in the vehicle- and zearalenone-treated groups were adenocarcinomas (100%). In the

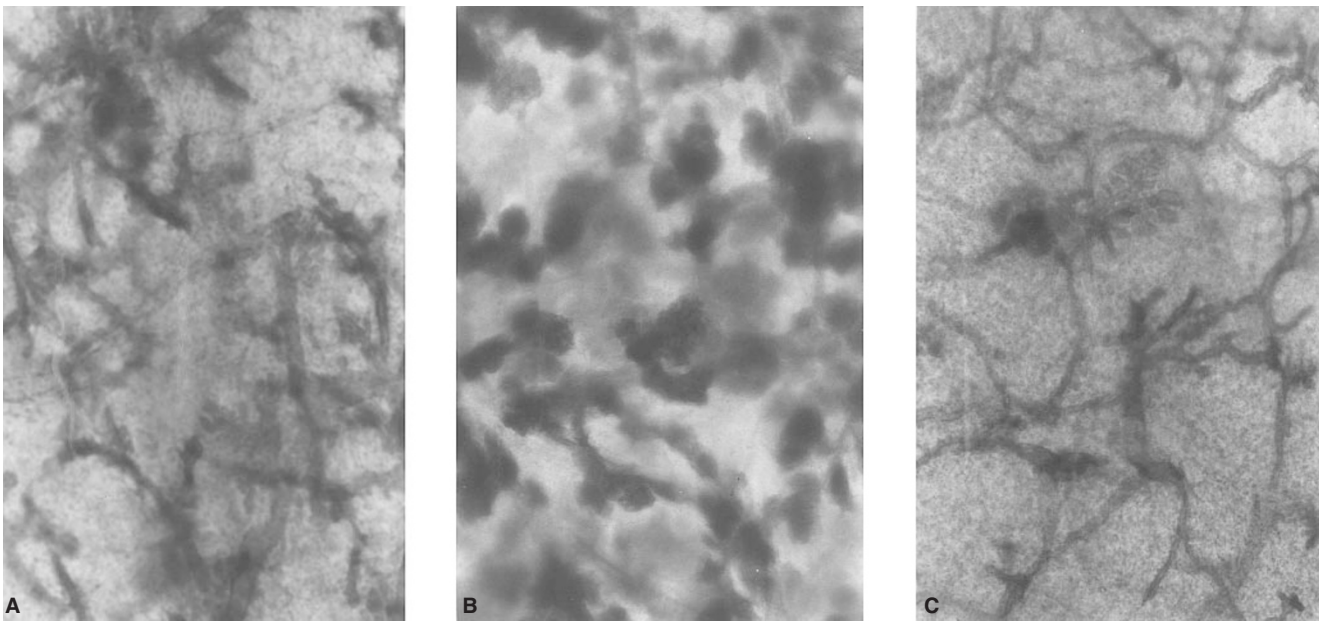

Figure 2 Mammary wholemount preparations (carmine staining) of the 9th abdominal glands obtained from 6-month-old female rats exposed to vehicle (**A**) 20 μg genistein (**B**) or 20 μg zearalenone (**C**) during a prepubertal period, and to DMBA at the age of 45 days. This figure is representative of 4–5 specimens per group. The wholemounts of the genistein group contained high levels of 2–3 type lobules, and the whole mounts of zearalenone group indicated ductal atrophy, combined with higher level of lobular structures than seen in the vehicle group (but clearly less than in the genistein group). Magnification x 6.3

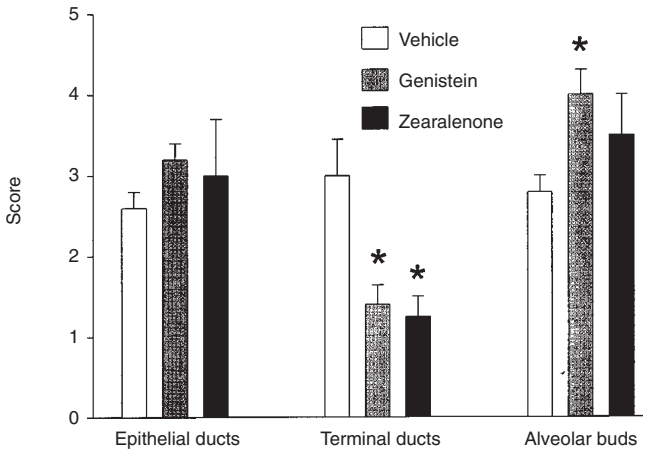

Figure 3 Density of epithelial ducts, terminal ducts (TDs) and differentiated alveolar buds (ABs) of the 9th abdominal gland obtained from 6-month-old female rats exposed to 20 μg genistein, 20 μg zearalenone, or vehicle during a prepubertal period, and to DMBA at the age of 45 days (n = 4–5 female rats per group). These parameters were evaluated using a scoring system with scores ranging from 0 (absent) to 5 (high), as described in Methods. Means ± s.e.m. are shown. Significantly different from the vehicle group: $^*P < 0.05$

genistein-treated group, 40% of the tumours were adenocarcinomas and 60% were non-malignant.

Oestrogen receptor

The concentrations of ER protein were determined in the mammary glands in rats that were exposed to DMBA 18 weeks prior to sacrifice. The ER levels were similar in the animals treated with genistein (3.4 ± 1.4 fmol mg^{-1} protein) or zearalenone (4.3 ± 2.8 fmol mg^{-1} protein) during the prepubertal period, and did not differ from that of the controls (3.9 ± 1.7 fmol mg^{-1} protein). The total ER levels in the present study were lower than we have seen in mice (Hilakivi-Clarke et al, 1998), but comparable to those seen in rats exposed to carcinogens (Thordarson et al, 1995).

We also determined the percentage of mammary glands that had ER levels higher than 5 fmol mg^{-1}. Breast tumours containing ER levels ≥ 5 fmol mg^{-1} protein are often considered ER-positive (Clark and McGuire, 1988; Winstanley et al, 1991). Using this cut-off point the data indicate that the proportion of ER-positive glands was significantly higher in the zearalenone-treated group (43%) than in the genistein- (14%) or vehicle-treated (28%) groups ($\chi^2 = 17.80$, df = 2, $P < 0.01$).

Mammary whole mounts

Analysis of mammary whole mounts obtained from animals that had been exposed to DMBA 18 weeks prior to sacrifice, indicated that prepubertal exposure to genistein and zearalenone induced distinct differences, when compared with the vehicle-controls (Figure 2). The mammary glands of genistein-treated animals showed the most lobular differentiation. These lobules were of type 2 and 3, indicating a high level of differentiation. Zearalenone-treated glands also showed differentiated lobular structures, although less than that seen in the genistein-treated glands.

According to the scale-based quantitative evaluation, total mammary epithelial density did not differ among the groups (Figure 3). However, the density of terminal ducts was significantly lower in the rats exposed to zearalenone ($P < 0.05$) or genistein ($P < 0.05$) during the prepubertal period than in the vehicle controls ($F(2,11) = 8.30$, $P < 0.006$). The density of alveolar buds, in turn, was significantly higher in the genistein-treated group ($P < 0.05$) than in the controls ($F(2,11) = 3.44$, $P < 0.07$).

DISCUSSION

Despite the general perception that consumption of soy-based food products protects from breast cancer, the supporting epidemiological evidence is inconsistent. Three studies suggest that soy intake is associated with lower breast cancer risk. A study in Singaporean women found that high soy intake is associated with a lower breast cancer risk among premenopausal, but not postmenopausal, women (Lee et al, 1991). A study in Asian-American women living in Los Angeles or Hawaii indicated that breast cancer risk decreases with increasing frequency of intake of tofu (bean curd) both in pre- and postmenopausal women (Wu et al, 1996). Finally, a study that measured urinary excretion levels of phyto-oestrogens reported that a high excretion of isoflavones (genistein was not included) was associated with a substantial reduction in breast cancer risk (Ingram et al, 1997). A similar, but more recent, study did not find significant differences in urinary excretion levels of daidzein or genistein between breast cancer cases and their controls in Shanghai; however, total isoflavonoid levels were lower in the cases (Zheng et al, 1999). Soy protein intake was similar in these Shanghai women who were newly diagnosed with breast cancer and randomly selected controls. Four other studies also suggest that the risk of breast cancer is not associated with soy consumption (Hirohata et al, 1985; Yuan et al, 1995; Witte et al, 1997). These significant inconsistencies may reflect differences in the end points used for genistein intake (consumption of tofu, miso, or serum isoflavone concentrations). It also is possible that timing of genistein exposure might be critical.

Treatment during prepuberty, with a pharmacological dose of genistein, has been suggested to reduce the subsequent risk to develop breast cancer. Murrill et al (1996) found that a subcutaneous exposure of prepubertal rats on postnatal days 16, 18 and 20 to 500 mg kg^{-1} genistein significantly reduces mammary tumour incidence and multiplicity. However, this dose is 500–5000 times higher than human genistein intake, and may not be directly relevant to human populations. We have used a genistein dose of ~1 mg kg^{-1} body weight, which should approximate the daily genistein consumption in Asia on a mg kg^{-1} body weight basis. If this dose is further adjusted for interspecies surface area differences (Freireich et al, 1966; Clarke, 1997), a human genistein exposure equivalent to 0.143 mg kg^{-1} body weight is obtained, comparable to the exposure to genistein alone in Oriental populations (~ 0.1 mg kg^{-1} body weight). Our data show that when the rats were exposed to this substantially lower genistein dose between postnatal days 7 and 20, they exhibited reduced mammary tumour multiplicity, but no significant change in tumour incidence. Additionally, a significant proportion of the tumours in the genistein group did not proliferate, and 60% of them were not malignant. Thus, prepubertal exposure to genistein not only reduced the subsequent mammary tumour multiplicity, it also reduced the likelihood that a tumour was malignant.

The lack of significance in the tumour incidence and latency in the genistein-exposed rats in the present study, when compared with the previous study (Murrill et al, 1996), is likely to be caused by the use of a low versus high dose of genistein. Genistein displays a convex dose–response curve for oestrogenic activity. Low genistein doses stimulate ER, while higher doses inhibit this receptor's activity (Wang et al, 1996). Inhibition of ER by high genistein doses may be due to inhibition of the tyrosine kinase activity of the epidermal growth factor receptor (EGFR) (Akiyama et al, 1987), which could lead to a reduced phosphorylation of ER. Another means by which pharmacological doses of genistein may affect tumorigenesis is by influencing the reproductive system. The 500 mg kg^{-1} genistein administration to prepubertal rats caused a permanent impairment of the hypothalamic–gonadal axis (Murrill et al, 1996). It also has been reported that 1 mg genistein (~ 100 mg kg^{-1}) given to rat pups daily between postnatal days 1 and 10, significantly decreases pituitary responsiveness to gonadotrophin releasing hormone (GnRH) (Faber and Hughes, 1991). A ten times lower genistein dose (100 μg ≈ 10 mg kg^{-1}) has the opposite effect, increasing GnRH-induced secretion of luteinizing hormone (Faber and Hughes, 1991). Thus, changes in the magnitude of a genistein exposure may have opposing effects on the reproductive system. We did not perform a detailed analysis of the reproductive system functions, and therefore cannot exclude the possibility that a low genistein dose (1 mg kg^{-1}) disrupts reproductive functions. However, we did not observe any differences in the timing of the onset of sexual maturation with prepubertal exposure to either genistein or zearalenone (not shown), which would be one measure of altered reproductive function.

Zearalenone, when administered during the prepubertal period, effectively reduced DMBA-induced mammary tumorigenesis in female rats. Rats that received 20 μg (~ 1 mg kg^{-1}) zearalenone every 3–4 days between days 7 and 20, subsequently exhibited a longer latency to tumour appearance, and had a lower tumour incidence than the vehicle-treated controls. In addition, one-third of the tumours did not proliferate. However, histopathological evaluation indicated that all the assessed tumours in the zearalenone group were malignant. These findings contrast with earlier data obtained in rats showing that treatment on postnatal days 7 and 14 with 10 mg kg^{-1} zearalenone increases the incidence of spontaneous mammary tumours (Schoental, 1985). The opposite results may be caused by the tenfold difference in the dose of zearalenone used in the two studies. We also treated the rats with a carcinogen and measured tumorigenesis within the following 18 weeks, while in the other study spontaneously arising tumours appeared when the animals were 2 years of age. These differences also may indicate that prepubertal zearalenone exposure inhibits premenopausal breast cancer (that is mimicked by DMBA), and stimulates the growth of postmenopausal breast cancer (that is mimicked by spontaneous tumours in older rats).

Both genistein and zearalenone act as relatively weak oestrogens. They stimulate the growth of human breast cancer cells in vitro (Martin et al, 1978; Wang et al, 1996), and have similar properties to oestradiol in rodent and human breast tissues (Petrakis et al, 1996; Harding et al, 1997; Santell et al, 1997; Hsieh et al, 1998; McMichael-Phillips et al, 1998). Since oestrogens are thought to increase breast cancer risk (Clarke et al, 1992), it is surprising that genistein or zearalenone in the present and previous study (Murrill et al, 1996) reduced the incidence/multiplicity of mammary tumours. Perhaps oestrogens have opposing effects on breast cancer risk depending on the timing of exposure. In utero exposure to oestrogens, or an exposure during the first week after birth, increases the subsequent incidence of mammary tumours in mice and rats (Bern et al, 1985; Lopez et al, 1988; Hilakivi-Clarke et al, 1997*b*), and possibly also in humans (Ekbom et al, 1992; Michels et al, 1996; Sanderson et al, 1996). However, prepubertal or pubertal treatment with oestradiol reduces mammary tumorigenesis in rats (Nagasawa et al, 1974; Grubbs et al, 1985). An exposure to oestrogens during adulthood increases breast cancer risk in animals (Clarke et al, 1992; Russo et al, 1994), and perhaps in humans (Grodstein et al, 1997). These differences may reflect

changes in the responsiveness of the mammary gland to ER oestrogens over time.

In the present study, phyto-oestrogen exposure occurred during a prepubertal period when the mammary epithelial cells are thought not to respond fully to oestrogens, e.g. oestrogens do not cause epithelial cell proliferation or alter ER levels (Haslam, 1989). We did not find any evidence that the prepubertal exposure to genistein or zearalenone permanently affected the levels of ER in the mammary gland. However, the mammary glands of the zearalenone-treated rats were significantly more often ER-positive (had ER levels ≥ 5 fmol mg^{-1} protein) than those of the genistein group. Our findings are consistent with the fact that ligands can alter the number/function of a receptor when they are present, and generally do not cause any persistent changes in receptor expression. One exception is an exposure that occurs during fetal life or immediately after birth, which can produce a permanent change (Verhoeven et al, 1982; Bern et al, 1985; Hilakivi-Clarke et al, 1998). However, since a prepubertal treatment with both a low (interacts with ER) and high (interacts with ER and other targets) dose of genistein or zearalenone reduces subsequent mammary tumorigenesis, prepubertal ERs may be involved as mediators of these effects.

Whether or not the effects we report are a direct consequence of changes in oestrogen-regulated gene function, it seems highly likely that the mechanism for prepubertal oestrogen exposure in affecting breast cancer risk involves changes in the mammary epithelial network. We have proposed that the increased number of terminal end buds and their reduced differentiation to alveolar buds, plays a critical role in increasing breast cancer risk following a perinatal oestrogen exposure (Hilakivi-Clarke et al, 1997*a*). Terminal end buds, and to a lesser degree terminal buds, are the primary sites of carcinogen action in the mammary gland (Russo and Russo, 1987). Consistent with this hypothesis, prepubertal exposure to a pharmacological genistein dose increases differentiation of terminal end buds at the time when DMBA is administered (Murrill et al, 1996). In our study, a more physiological dose of genistein also increased differentiated alveolar buds, when determined 18 weeks after DMBA exposure.

In summary, prepubertal exposure to a low dose of genistein or zearalenone reduces the risk to develop mammary tumours in rats. The mediating mechanisms remain to be established, but are likely to include changes in the differentiation of mammary epithelial tree and may reflect events mediated through activation of the ER.

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