Phyto-oestrogens and breast cancer

M S Morton, N Bundred¹, D F McMichael-Phillips¹, C Harding¹, R I Nicholson and K Griffiths

Tovus Cancer Research Centre, University of Wales College of Medicine, Cardiff CF4 4XX, UK and ¹Paterson Institute for Cancer Research, Christie Hospital, Manchester M20 4BX, UK

(Requests for offprints should be addressed to M S Morton)

Introduction

Breast cancer and carcinoma of the prostate belong to a group of hormone-dependent cancers that would also include tumours of the ovary and endometrium. These cancers comprise a major proportion of the so-called ‘Western diseases’. The incidence of and mortality from these cancers are high in the Western world relative to the rates associated with Asian populations such as the Chinese, Japanese, Thai and Filipino, and somewhat higher than those for people in countries surrounding the Mediterranean. Cardiovascular disease and osteoporosis follow a similar pattern and would also be considered ‘Western diseases’.

Carcinoma of the breast remains the principal cause of death from cancer in women in the developed countries of the West and particularly in the United States of America and Northern Europe. A recent report (Boyle & Maisonneuve 1995) confirms that the highest incidence rates are exclusively within the United States with an annual rate of 104.2 cases per 100 000 population observed for white women in the bay area of San Francisco. Outside of the United States the highest rates occur in Porto Allegre in Brazil (78.5/100 000 annually) and in Geneva in Switzerland (73.5/100 000 annually). Very much lower rates are reported in Asia with an annual rate of 17.6/100 000 in Yamagata, Japan and 9.5/100 000 in Qidong, China. In England and Wales breast cancer constitutes 28% of all female cancers with 25 000 women annually presenting and 15 000 dying from the condition each year (Dhom 1991).

Epidemiological studies of migrating populations, particularly those populations migrating from China and Japan to Hawaii or to mainland United States, show that the risk of developing prostate or breast cancer increases from a low rate to one closer to that of the indigenous population, within a few generations (Haenzel & Kurihara 1968, Buell 1973, Shimizu et al. 1991). Data from the migrant studies are amassed sufficiently quickly to suggest that the differences can be attributable to dietary and environmental factors and not to the genetic characteristics of the different peoples.

Diet and cancer

The significant differences in the incidences of specific types of cancer in particular countries or regions of the world have directed attention to the possible influence of dietary components on the biological processes concerned with carcinogenesis. As a result of many epidemiological studies, it has often been implied that certain of these dietary factors may be 'causative' with regard to certain cancers, whereas others are seen to some extent as 'protective' agents. Some estimates suggest that approximately 35% of cancer deaths may be attributable to dietary habits; Doll & Peto (1981) provide some support for this estimate but suggest a range of 10-70% would be more appropriate.
In general terms the ‘Western diet’ has been considered to be one in which the fat consumption is high, whereas the ‘fibre’ intake is low. In contrast, the proportion of the total calorific intake related to fat in the more vegetarian-style ‘Eastern diet’ is comparatively low and the fibre content is much higher. Predictably, therefore, Western diseases such as cancers of the breast and prostate, and also colorectal cancer and cardiovascular disease, have been associated with fat as a causative factor. Only recently has it been recognised that constituents of the Eastern diet could possibly be protective against certain diseases and it is the lack of such constituents, rather than a high fat intake, that may be the important factor. The concept is, therefore, that components in the Asian diet and possibly also in the Mediterranean and vegetarian diets, are protective against the development of these diseases that are so prevalent in Western developed countries.

The traditional Asian diet, and to a lesser extent the Mediterranean and vegetarian diets also, are not only low fat/high fibre, they are also a rich source of weak, dietary plant oestrogens. These plant or phyto-oestrogens are excreted in large amounts in the urine of Chinese, Japanese (Adlercreutz et al. 1991) and vegetarian (Adlercreutz et al. 1986) men and women and have many interesting properties which single them out as possible cancer-protective agents in these populations; a concept first proposed by Adlercreutz (1990). Two groups of compounds, namely the isoflavonoids and mammalian lignans, have attracted particular attention. Many foods of plant origin contain varying amounts of isoflavonoids and lignans. Some of these diphenolic phyto-oestrogens possess weak oestrogenic and anti-oestrogenic activity and therefore the potential for exerting an influence on hormone-dependent cancers such as those of the breast and prostate (Setchell & Adlercreutz 1988).

Oestrogenic substances in plants

The presence in plants of non-steroidal substances with oestrogenic activity has been recognised for some time and many hundreds of plants manifest some degree of oestrogenic activity (Bradbury & White 1954, Price & Fenwick 1985). Soya bean and red clover are members of the leguminosae family and are a major source of isoflavonoids (Verdeal et al. 1980, Axelson et al. 1984, Price & Fenwick 1985). Soya is consumed daily in large amounts in a number of forms in China and Japan and in Asia generally. Soya beans contain the glycoside conjugates of the isoflavonoids, genistein and daidzein, which can be metabolised by gut bacteria to their respective aglycones. Genistein can be further metabolised to the non-oestrogenic p-ethylphenol and daidzein is converted to the oestrogenic isoflavan, equol. The aglycones and their metabolites are then absorbed and appear in blood and urine, primarily as glucuronide conjugates, and also sulphates (Setchell & Adlercreutz 1988). Daidzein and genistein were isolated from soya beans more than 60 years ago (Walz 1931) and 100 g fat-free soya beans may yield up to 300 mg genistein (Coward et al. 1993). Generally the presence of isoflavonoids in plants is limited to legumes, although they have recently been identified in beer and bourbon (Van Thiel et al. 1991, Rosenblum et al. 1992) and may be more widely distributed than was previously thought.

Lignans are another group of polyphenolic plant compounds (Price & Fenwick 1985). The plant precursors, matairesinol and secoisolariciresinol, are metabolised after ingestion by intestinal microflora to give rise to the weakly oestrogenic enterolactone and enterodiol respectively. The lignans are absorbed from the gut to appear in blood and other body fluids (Setchell & Adlercreutz 1988). The lignans are widely distributed in nature and precursors are found in many cereals, grains, fruits and vegetables, but the richest source is linseed (flaxseed) and other oilseeds such as sesame (Thompson et al. 1991).

The flavonoids are closely related in structure to the isoflavonoids, the former having a 2-phenylchroman nucleus and the latter a 3-phenylchroman nucleus (Miksicek 1993). Recently several commonly occurring plant flavonoids have been shown to possess weak oestrogenic activity (Miksicek 1993). Unlike isoflavonoids, the flavonoids are ubiquitous in nature, and are found in high concentration in many fruits, vegetables and crop species. In particular, apigenin and kaempferol, both of which are oestrogenic, are regarded as two of the major flavonoids because of their common occurrence among plants, and their significant concentrations when they are present. Apigenin, for example, is found in the leaves, seeds and fruits of flowering plants, with up to 7% of dry weight in leafy
vegetables. Tea leaves are an excellent source of apigenin (Miksicek 1993).

Isoflavonoids and lignans are normal constituents of body fluids and have been identified in most animal and human body fluids by gas chromatography-mass spectrometry (GC-MS) (Setchell & Adlercreutz 1988). They are present in urine (Adlercreutz et al. 1986, Kelly et al. 1993), plasma (Morton et al. 1994), saliva (Finlay et al. 1991) and in semen (Dehennin et al. 1982). Analysis of expressed prostatic fluid found that enterolactone and equol were constituents (Finlay et al. 1991), suggesting that dietary oestrogens can accumulate in the prostate.

The levels of isoflavonoids are high in the urine and plasma of the Japanese and Chinese (Adlercreutz et al. 1991, 1993b), whose traditional foodstuffs contain large amounts of soya in the form of bean curd (tofu), soya bean milk, miso and tempeh. The concentration of lignans is high in the urine of vegetarians (Adlercreutz et al. 1986) whose diet contains whole grain cereals, vegetables and fruits. In Western subjects fed 40 g soya daily, the urinary excretion of equol was found to increase 1000-fold above control levels (Axelson et al. 1984, Setchell et al. 1984). The concentrations of flavonoids in plasma and urine of different populations have yet to be determined and this is an obvious programme for future research. However as tea, fruit and vegetables are the principal sources of flavonoids, it is probable that Asians, with their high consumption of tea, and vegetarians have significant circulating levels of these compounds.

**Biological properties of isoflavonoids and lignans**

**Oestrogenic activity**

The mammalian lignans, enterolactone and enterodiol, the isoflavonoids, daidzein, genistein, coumestrol and equol, and the flavonoids, apigenin, kaempferol and phloretin, all possess weak oestrogenic activity (Pope & Wright 1954, Bickoff 1961, Miksicek 1993). Some antioestrogenic properties have also been described (Folman & Pope 1966, Waters & Knowler 1982).

Genistein stimulates uterine growth in ovariectomised mice (Bickoff et al. 1957), sheep (Braden et al. 1967) and rats (Noteboom & Gorsky 1963). In mice, genistein is roughly 100 000 times less effective than diethylstilboestrol (DES) in stimulating uterine growth (Bickoff et al. 1962). Based on competitive binding to human tumour cell receptors, however, genistein is only 50 times less potent than oestradiol (Bickoff et al. 1962). Affinity for oestradiol receptors, in vitro, clearly indicates a much higher potency for genistein than in vivo uterine weight gain assays. Transport and metabolic effects may be responsible for these differences.

In addition, studies at the Tenovus Institute have shown that both genistein and enterolactone, at $10^{-6}$ M to $10^{-8}$ M concentrations, are as effective as $10^{-9}$ M oestradiol in promoting the growth of MCF-7 cells and the expression of the oestrogen-regulated progesterone receptor and pS2 proteins. At higher concentrations, a growth inhibition of MCF-7 cells has been reported for genistein (Peterson & Barnes 1991).

In contrast to these oestrogenic effects, Folman & Pope (1966) reported that both genistein and coumestrol, in vivo, can act as antioestrogens by inhibiting the DES-promoted uterine weight gain in immature albino mice. In a more recent report, the citrus flavonoid, naringenin, has demonstrated similar properties (Ruh et al. 1995). In rats co-treated with oestradiol plus naringenin, a significant decrease in oestradiol-induced uterine wet weight, DNA synthesis, progesterone receptor binding and peroxygenase activity was observed.

Cancers of the breast and prostate are initially hormone dependent, in that if the source of hormone is removed, by castration for example, the tumour will regress. A large proportion of the hormones, oestradiol and testosterone, circulating in plasma are bound to transport proteins such as sex hormone binding globulin (SHBG), and only about 2% is unbound and this is considered the biologically active fraction which passively diffuses into the target cells (Vermeulen et al. 1972). As weak oestrogens, phyto-oestrogens stimulate the synthesis of SHBG in the liver (Adlercreutz et al. 1987). Therefore any increase in the concentration of SHBG, by phyto-oestrogens for example, causes a reduction in the free fraction of the growth-promoting steroid hormone. Higher plasma levels of SHBG and decreased concentrations of free testosterone have recently been reported for vegetarian men when compared with an omnivorous group (Belanger et al. 1989), and also for Chinese and Japanese men (Vermeulen 1993).
Inhibition of steroid metabolising enzymes

Inhibition of aromatase

The aromatase enzyme is responsible for the irreversible conversion of C19 androgens into C18 oestrogens. In a recent study, the lignan, enterolactone, was shown to be a moderate inhibitor of aromatase activity in placental microsomes (Adlercreutz et al. 1993a). Enterolactone was found to bind at or near to the active site of the aromatase, thereby competing with the androgen substrate of the enzyme. Unpublished studies from the Tenoous Institute also show that the isoflavonoids, biochanin A, coumestrol, genistein and equol, are moderate inhibitors of aromatase in genital skin fibroblasts.

The aromatase activity of adipose and muscle tissue is the major source of oestrogen in the post-menopausal woman and elevated aromatase activity has been observed in adipose tissue adjacent to breast cancer (O’Neill & Miller 1987). With oestrogen synthesis in adipose tissue considered to be implicated in breast cancer progression in the post-menopausal patient, aromatase inhibitors such as aminoglutethimide have been used as a form of second-line breast cancer therapy (Lonning & Kvinnslan 1988, Miller 1996). Plant oestrogens, as aromatase inhibitors, may act as a natural form of therapy in Asian postmenopausal women. In Western women breast cancer incidence continues to rise after the menopause, whereas in Asian women a slight decrease is observed.

Inhibition of 17β-hydroxysteroid dehydrogenase

The enzyme system 17β-hydroxysteroid dehydrogenase catalyses the interconversion of 17-keto and 17-hydroxy steroids and several isozymes have been identified (Tait et al. 1989, Reed 1991). Thus the metabolism of testosterone to androstenedione and that of oestrone to oestradiol is under the control of 17β-hydroxysteroid dehydrogenases. Coumestrol and genistein inhibit the 17β-hydroxysteroid dehydrogenase type I enzyme and hence the conversion of oestrone to oestradiol (Makela et al. 1995). In the report of Evans et al. (1995), several isoflavonoids and lignans were shown to inhibit the conversion of testosterone to androstenedione, with the lignan, enterolactone, the most potent inhibitor. From this study also, the IC50 of an eight-compound cocktail of isoflavonoids and lignans was only 0.7 µM of each compound. Daidzein, genistein, formononetin and biochanin A are strong inhibitors of β-hydroxy-

steroid dehydrogenases of Pseudomonas testosteronii (Keung 1995). Daidzein was shown to inhibit the oxidation not only of testosterone, but also that of pregnenolone, pregnanolone, oestradiol, epiandrosterone and dehydroepiandrosterone. In addition, many plant oestrogens are also inhibitors of the 5α-reductase enzymes (Evans et al. 1995).

As some early breast cancers are dependent on hormones for growth, the ability of natural dietary compounds to alter either the biological availability or metabolism of these hormones may, over a life-time of exposure to phyto-oestrogens, have a significant effect on the development of breast cancer in Asian women.

In addition to the properties listed above which influence hormone metabolism, dietary isoflavonoids and lignans have many other non-hormonal characteristics which are likely to effect the carcinogenesis process.

Inhibition of tyrosine-specific protein kinases

Tyrosine kinases are necessary for the function of several growth factor receptors, including those for epidermal growth factor, platelet-derived growth factor, insulin and insulin-like growth factors (Hunter & Cooper 1985). In addition, several retroviral oncogenes, such as src, abl, fps, yes, fes and ros, code for tyrosine-specific protein kinases (Bishop 1985). Tyrosine phosphorylation plays an important role in cell proliferation and cell transformation and tyrosine kinase-specific inhibitors may well be used as anticancer agents (Schlessinger et al. 1983, Kenyon & Garcia 1987). The isoflavonoid, genistein, has been shown to be a specific inhibitor of tyrosine kinase activity (Akiyama et al. 1987). In addition, the flavonoids, apigenin and kaempferol, reverse the transformed phenotypes of v-H-ras NIH 3T3 cells, an effect mediated via inhibition of tyrosine kinase (Kuo et al. 1994).

Furthermore, genistein induces apoptosis in human breast tumour cells (Kiguchi et al. 1994), inhibits invasion of murine mammary carcinoma cells (Scholar & Toews 1994) and enhances adhesion of endothelial cells (Tiisala et al. 1994).

Inhibition of DNA topoisomerases

DNA topoisomerases are enzymes which alter the conformation of DNA and are crucial to cell division.
(Cummings & Smyth 1993). By a process involving strand cleavage, strand passage and religation, these enzymes are able to untangle supercoiled DNA. Genistein is a potent inhibitor of these enzymes and is considered to act by stabilisation of a putative ‘cleavable complex’ between DNA and the topoisomerase enzyme (McCabe & Orrenius 1993). The flavonoids, quercetin, fisetin and morin, also inhibit DNA topoisomerases I and II, while kaempferol inhibits only DNA topoisomerase II (Constantinou et al. 1995). Inhibition of the topoisomerases is now the target for the design of new anticancer drugs. In addition, genistein is cytostatic, arresting cell cycle progression in G2-M (Matsukawa et al. 1993, Spinozzi et al. 1994) and induces apoptosis in immature human thymocytes by inhibiting DNA topoisomerase II (McCabe & Orrenius 1993). The flavonoid, apigenin, induces morphological differentiation and G2-M arrest in rat neuronal cells (Sato et al. 1994).

Inhibition of angiogenesis

Angiogenesis, or neovascularisation, involves the generation of new capillaries, a process invoking the proliferation and migration of endothelial cells. Normally the process is restricted to wound healing, but it is also enhanced in association with cancer growth. Folkman et al. (1989), Folkman (1985) and Weidner et al. (1991) report that new capillary blood vessels are necessary for a cancer to expand beyond 2 mm in size. Angiogenesis therefore exercises an important role in cancer progression and is essentially seen as the growth towards a focus of cancer, of capillary sprouts and columns of endothelial cells from pre-existing capillaries. The process is probably promoted by the production of growth factors by the cancer cells and fibroblast growth factor (FGF), or members of the FGF family, are recognised as potent angiogenic agents. Genistein inhibits angiogenesis and endothelial cell proliferation (Fotsis et al. 1993). In wound healing, the process is regulated by a balance between angiogenic factors and restraining factors such as transforming growth factor-β (TGF-β). Restraining the process of angiogenesis could inhibit cancer progression and cortisone and heparin treatment has been reported to suppress the metastatic capacity of experimental tumours by these means (Folkman 1985); significantly, genistein may have a similar influence. These effects may relate to the inhibition of the tyrosine kinase-associated FGF receptor (Akiyama et al. 1987).

Anti-oxidant activity

Flavonoids, isoflavonoids and lignans are all polyphenolic compounds and can function as effective antioxidants and radical scavengers. Compounds such as quercetin and cyanidin have antioxidant potentials four times that of Trolox, the vitamin E analogue. Removing the ortho-dihydroxy substitution, as in kaempferol, for example, or reducing the 2,3 double bond in the C-ring, as in catechin or epicatechin, decreases the antioxidant activity by more than 50%, although these structures are still more effective than α-tocopherol or ascorbate (Rice-Evans et al. 1995). In addition, flavonoids such as catechin inhibit the oxidation of low density lipoprotein, an effect consistent with the ability of some flavonoids of similar structure to inhibit lipoxygenases (Mangiapane et al. 1992).

Inhibition of tumorigenesis

Soya-bean isoflavones and lignans inhibit experimental carcinogenesis in a wide variety of systems. Of 26 animal studies in which diets containing soy or soybean isoflavones were employed, 17 (65%) reported protective effects (Messina et al. 1994). No studies reported that soy intake increased tumour development. Most of the studies reporting no inhibition of tumorigenesis employed soya protein isolate, the preparation of which includes treatment with alkali and is responsible for a four- to sixfold reduction in isoflavone concentration (Coward et al. 1993). Of particular interest are the studies of Lamartiniere et al. (1995a,b). In these, rats were given genistein on days 2, 4 and 6 or 16, 18 and 20 postpartum and on day 50 they were exposed to the carcinogen, dimethylbenz(a)anthracene. The rats receiving genistein had a longer latency to first mammary tumour and lower incidence and multiplicity of tumours than control animals. A positive correlation was observed between chemoprevention and reduced cellular proliferation, and differentiation of terminal ductal structures.

Many in vitro tumour model systems are also growth-inhibited by isoflavonoids and lignans, including those for both breast and prostate cancers (Peterson & Barnes 1991, 1993). Genistein and biochanin A, a precursor of genistein, at
high concentrations inhibit the growth of oestrogen receptor-positive (MCF-7) and oestrogen receptor-negative breast cancer cells (MDA-468) (Peterson & Barnes 1991).

In addition, isoflavonoids and flavonoids inhibit the bioactivation of potent chemical carcinogens. Biochanin A inhibits the metabolic activation of benzo(a)pyrene (Chae et al. 1992) and the flavonoid catechins from green and black tea inhibit the activation of the potent tobacco carcinogen 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK) and subsequent lung tumorigenesis in A/J mice (Shi et al. 1994).

Measurement of isoflavonoids and lignans
Isoflavonoids and lignans are normal and natural constituents of both human and animal body fluids and excreta (Setchell & Adlercreutz 1988). They have usually been identified and measured by GC-MS. We have developed isotope dilution GC-MS assays for the determination of isoflavonoids and lignans in biological fluids based upon selected ion monitoring (Morton et al. 1994, 1996). In a pilot study, we have used GC-MS to measure the concentrations of isoflavonoids and lignans in plasma from British postmenopausal women with breast cancer (n=6), cured breast cancer patients (n=4) and healthy controls (n=6). The mean concentrations of genistein in these three groups was 2.3 ng/ml, 3.7 ng/ml and 16.5 ng/ml respectively. While the mean concentration of genistein was highest in the healthy controls at 16.5 ng/ml, this figure is still well below that observed for Japanese people where plasma levels often exceed 100 ng/ml (Adlercreutz et al. 1993).

Summary
Asian and to a lesser extent vegetarian and Mediterranean populations have lower incidences of hormone-dependent cancers than Western people who are omnivores. The lower-risk populations consume large amounts of weak oestrogens in their diet. Soya is a rich source of isoflavonoid phyto-oestrogens and it has recently been estimated that the traditionally eating Chinese consumes, on average, 35 times more soya than their Western counterparts. Vegetarians consume large amounts of fruits, vegetables and cereals, foods which contain the weakly oestrogenic lignans. These plant oestrogens can affect hormone metabolism and availability, and have several other properties which can influence the carcinogenic process. They have been measured in biological fluids by GC-MS, and Asians and vegetarians have high plasma and urinary concentrations of these compounds. A life-time exposure to isoflavonoids and lignans may, in part, be responsible for the lower levels of hormone-dependent cancers and other degenerative diseases in Asian, vegetarian and Mediterranean populations.

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