Figure 1

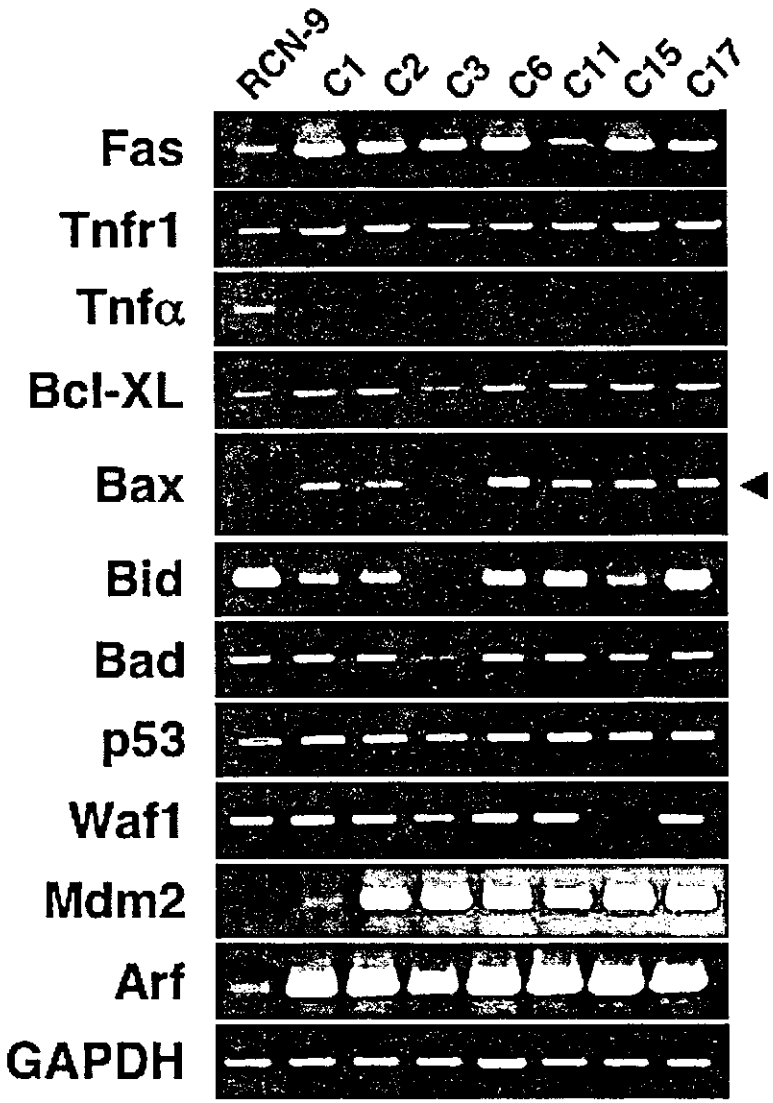

Figure 1 Expression of apoptosis-related genes in the rat mammary and colon carcinoma cell lines.
Various levels of the pro- and anti-apoptotic genes were expressed in the mammary (C1-C17) and colon (RCN-9) carcinoma cell lines. Arrow head indicates the PCR products corresponding to *bax*.

Figure 2

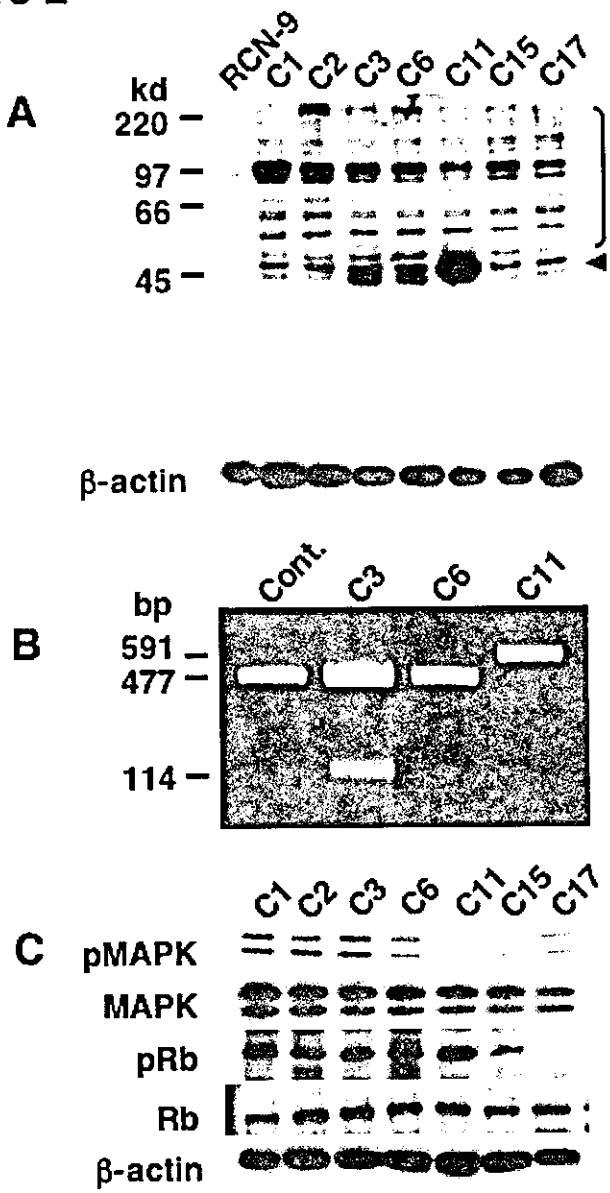

Figure 2 Analyses of protein levels and mutations of p53 tumor suppressor and activities of growth-regulating molecules in the rat mammary carcinoma cell lines.
(A) Western blot analysis of p53 protein. Proteins were separated by 10% SDS-polyacrylamide gel electrophoresis. Un-ubiquitinated (arrow head) and ubiquitinated (parenthesis) forms of the protein were observed in the mammary carcinoma cell lysates. A trace amount of the un-ubiquitinated protein was detected in RCN-9 cells after a longer exposure. (B) Representative RFLP analysis data of codon 246 of the p53 genes in rat mammary carcinoma cells. The PCR fragments derived from the wild-type allele give 477 bp and 114 bp bands after Aci I digestion whereas the ones from the mutant allele give only a 591 bp band. Cont., retinal DNA from a wild-type rat. (C) Phosphorylation levels of p42/44-MAPK and Rb. Note lack of detectable phosphorylation of p42/44-MAPK in C11 cells, and faint phosphorylation of Rb in C17 cells.

Figure 3

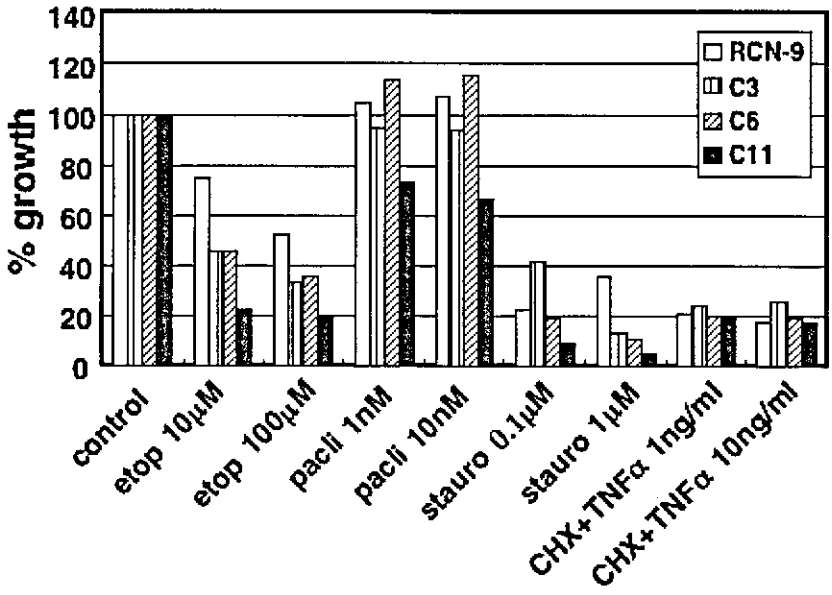

Figure 3 Effects of apoptosis-inducing reagents on cell growth of the mammary carcinoma cells.
Cells were exposed to the indicated concentration of etoposide (etop), paclitaxel (pacli), staurosporine (stauro), or 1 μg/ml cycloheximide (CHX) and TNFα for 24 hr. Data are expressed as the mean of 2 independent experiments in triplicate.

Figure 4

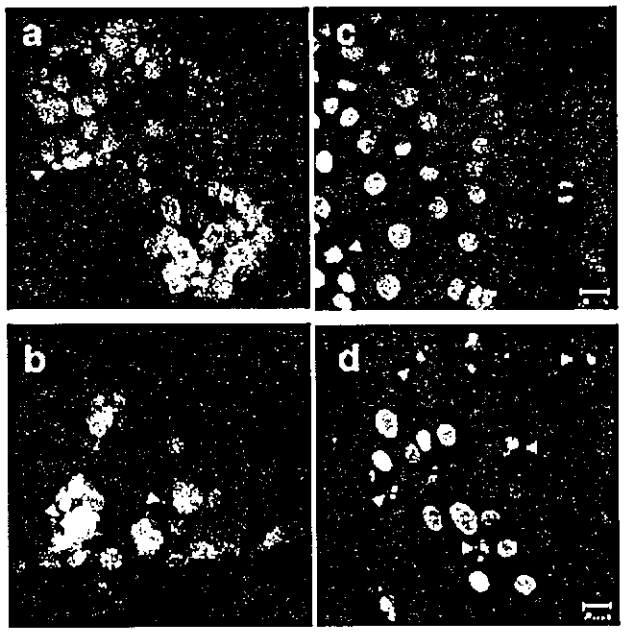

Figure 4 Etoposide-induced apoptosis is prominent in C11 mammary carcinoma cells.
C3 (a and b) and C11 (c and d) cells were incubated in the absence (a and c) or presence (b and d) of 10 μM etoposide for 48 hr. Then, the cells were stained with DAPI, and the nuclear morphology was observed under a confocal microscope. Arrow heads indicate the apoptotic nuclei. Scale bar: 20 μm.

Figure 5

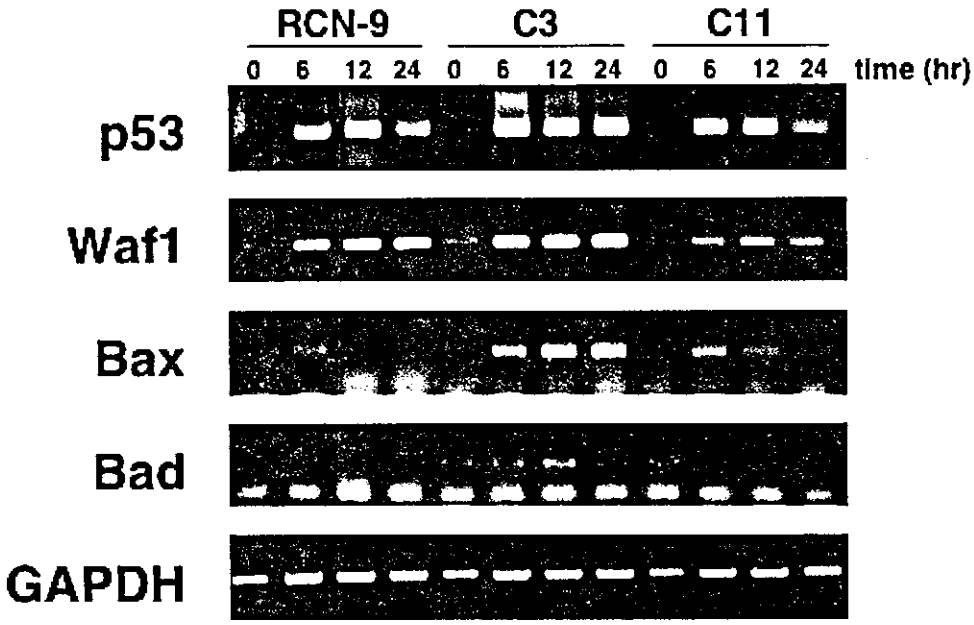

Figure 5 Lower expression of p53-targeted genes after cisplatin treatment in C11 cells containing high levels of mutant p53.
RT-PCR analyses of gene expression in RCN-9, C3 and C11 cells after treatment with cisplatin (0.5 mg/ml) for the indicated time.

Dietary factors modifying breast cancer risk and relation to time of intake

Airo Tsubura, Norihisa Uehara, Yasuhiko Kiyozuka and Nobuaki Shikata

Department of Pathology II, Kansai Medical University, Moriguchi, Osaka 570-8506, Japan.

Running head: Dietary factors and breast cancer risk

Correspondence to: Airo Tsubura, Department of Pathology II, Kansai Medical University, Moriguchi, Osaka 570-8506, Japan. Tel: +81-6-6993-9431; Fax: +81-6-6992-5023; e-mail: tsubura@takii.kmu.ac.jp

ABSTRACT

Multiple factors contribute to the development of human breast cancer. However, environmental factors, especially dietary factors, appear to have the greatest effects. Evidence obtained in epidemiological studies has been corroborated by laboratory findings. Dietary components strongly associated with breast cancer include fat and phytochemicals. A diet high in n-3 polyunsaturated fatty acid (PUFA) or monounsaturated fatty acid (MUFA) and low in n-6 PUFA is protective against breast cancer. Some phytochemicals present in fruits and vegetables are also protective. Time of intake appears to be important: lifetime protection may be achieved if one is exposed to a dietary factor that lowers breast cancer risk early in life. Synergistic and antisynergistic interactions between dietary factors can modify breast cancer risk. The available evidence suggests that breast cancer risk can be reduced by early dietary intervention.

KEY WORDS: breast cancer; mammary cancer; diet; nutrition; fat; phytochemical; phytoestrogen.

Abbreviations used: CDHA, conjugated docosahexaenoic acid; CLA, conjugated linoleic acid; DADS, diallyl disulfide; DHA, docosahexaenoic acid; DMBA, 7,12-dimethylbenz(α)anthracene; EGFR, epidermal growth factor receptor; EPA, eicosapentaenoic acid; ER, estrogen receptor; LA, linoleic acid; MNU, *N*-methyl-*N*-nitrosourea; MUFA, monounsaturated fatty acid; OA, oleic acid; PCNA, proliferating cell nuclear antigen; PgR, progesterone receptor; PhIP, 2-amino-1-methyl-6-phenylimidazo [4-5-b] pyridine; POH, perillyl alcohol; PUFA, polyunsaturated fatty acid; SDG, secoisolariciresinol diglycoside; SFA, saturated fatty acid; TEB, terminal end bud; TGF, transforming growth factor.

INTRODUCTION

The etiology of human breast cancer is largely unknown. Genetic susceptibility, hormonal effects and environmental factors appear to be major determinants. However, known genetic risk factors are present in only 10 to 15% of breast cancer cases (1). Epidemiological studies indicate that incidence and mortality of breast cancer is up to 5-fold higher in Western countries than in some Asian countries (2). Also, Asian migrants to the United States eventually acquire the breast cancer incidence of their host country, suggesting that environmental factors are more important than genetic factors (2). It is generally thought that about one third of cancers are related to dietary factors (3). Although many dietary components have been evaluated for their influence on breast cancer in epidemiological or experimental studies, the roles of specific dietary factors that modify breast cancer risk are not completely understood. Studies suggest that dietary fat and dietary phytochemicals affect the etiology of breast cancer.

Human breast cancer occurs with 100-fold greater incidence in females than males, and reproductive factors such as early age of menarche, late age of menopause and late age of full-term pregnancy are associated with increased risk (2). Such findings suggest that estrogens play an important role in the etiology of breast cancer. Some phytochemicals are classified as phytoestrogens (4), and humans are exposed through diet to a variety of such substances. The effects of phytoestrogens or other dietary components that alter estrogen levels on breast cancer risk may depend on the endogenous estrogen levels at the time they are ingested. Estrogen exposure early in life, when endogenous estrogen levels are low, may have profound effects, including effects on breast carcinogenesis. Relationships between dietary factors and breast cancer may result from effects that occur before maturity when the breasts are still developing (1,2,5). Thus, it is important to include the time of ingestion in analysis of modification of breast cancer risk by dietary factors. In addition, there may be interaction between dietary components of different food items that are consumed concurrently. Some dietary components may interact in a synergistic way to modify breast cancer risk, and others may interact in an antisynergistic fashion. This review examines evidence regarding relationships between dietary factors and breast cancer risk, and relation to time of intake. Combination effects among dietary components are also discussed.

DIETARY FACTORS MODIFYING BREAST CANCER IN ADULTS

Fatty acids and conjugated fatty acids

The hypothesis that dietary fat increases breast cancer risk is based on the observation that national per capita fat consumption strongly correlates with breast cancer mortality rate (6). Although evidence from large prospective studies strongly suggests that there is no association between overall dietary fat intake and breast cancer in humans (7), such findings do not necessarily mean that fat has no effect on breast cancer, because other findings indicate that different types of dietary fat may have different effects on breast carcinogenesis (8). Epidemiological data indicate that Eskimos (9,10) and costal- and rural-dwelling Japanese (11), who traditionally consume large amounts of fish or marine products containing high levels of n-3 polyunsaturated fatty acids (PUFAs), have low breast cancer rates (12,13). Mediterranean diets rich in olive oil, which is high in monounsaturated fatty acid (MUFA), appear to be associated with reduced breast cancer risk (14). Typical American high-fat diets containing high levels of n-6 PUFA, particularly linoleic acid (LA), appear to increase risk (15). Associations between fat consumption and mammary carcinogenesis in animals are well established.

Tannenbaum and Silverstone (16) first demonstrated that increased dietary fat could enhance mammary tumorigenesis in rodents. Since then, the hypothesis that amounts and type of dietary fat affect development of mammary tumors has been confirmed in experimental models (8). A high-fat diet rich in n-6 PUFAs, especially LA, accelerates mammary carcinogenesis and tumor progression, whereas n-3 PUFA (17) and MUFA (18) exert inhibitory effects (Fig. 1); saturated fatty acids (SFAs) do not appear to directly promote mammary tumors (19). Eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA, an n-3 PUFA) and oleic acid (OA, a MUFA) exhibit beneficial effects in experimental mammary carcinogenesis. When *N*-methyl-*N*-nitrosourea (MNU)-treated female rats were fed diets containing 10% fatty acid consisting of EPA, DHA or a 1:1 mixture of EPA and DHA, tumor incidence and tumor multiplicity were 73% and 1.67, 23% and 0.23, and 65% and 1.59, respectively (20). DHA appears to be more effective than EPA in reducing mammary carcinogenesis. The growth of human breast cancer cell lines transplanted into female athymic mice is influenced by the type of fat in the diet (21). A diet high in n-6 PUFA (10% LA), but not a diet low in n-6 PUFA (4.1% LA), enhances the tumor growth of transplanted human breast cancer cells, compared with a high-SFA (9.5% palmitic acid) diet; in contrast, a diet

high in n-3 PUFA (9.5% EPA) inhibits tumor growth (22). In athymic mouse models, metastasis can be enhanced by a diet rich in n-6 PUFA, whereas n-3 PUFA can suppress metastasis (22,23). In culture, n-6 PUFA stimulates human breast cancer cell growth, whereas n-3 PUFA and SFA suppress growth (22,24). n-3 PUFA-induced reduction of tumor cell growth is apparently due to disturbance of cell cycle progression (25) and induction of apoptosis (26, 27). Among n-3 PUFAs, DHA suppresses tumor cell growth more effectively than EPA *in vitro* (24). DHA affects cell cycle progression by inducing G1 arrest involving an increase in p53 and $p21^{Cip1/Waf1}$ and a decrease in cyclin D1, and accelerates apoptosis via down-regulation of Bcl-2 (28). Olive oil, which is high in OA and low in LA, suppresses growth of MNU-induced mammary carcinomas (29). Experimental studies indicate that EPA, DHA and OA strongly suppress breast carcinoma. Some types of dietary fat act as promoters (rather than initiators) of mammary carcinogenesis (30). Thus, in order to affect mammary tumor growth when fed to adult animals, some fatty acids must be present in the diet for a long duration.

Conjugated fatty acids are positional and geometrical isomers with conjugated double bonds. The most widely studied conjugated fatty acid is conjugated linoleic acid (CLA), a LA derivative (Fig. 1). LA increases the risk of breast cancer, whereas CLA has been shown to have anticarcinogenic effects (31). This finding that CLA and LA have opposite effects is of particular interest. CLA reduces the risk of MNU-induced mammary cancer in adult rats when fed continuously during the entire experimental period (32), and inhibits growth of human breast cancer cells in culture (33). n-3 PUFAs have been found to inhibit mammary carcinogenesis, and the opposite effects of CLA and LA suggest that conjugated fatty acids derived from n-3PUFAs have greater tumor-suppressing activity than their parent n-3 PUFAs. In culture, conjugated DHA (CDHA) inhibits human breast cancer cell growth more effectively than DHA (IC_{50} for 72 hrs: 97 μM and 270 μM, respectively), and a dietary dose of 1.0% CDHA suppresses growth of human breast cancer cells transplanted into female athymic mice (28). Conjugated fatty acids (CLA, CDHA and conjugated linolenic acid) are considered powerful anticancer agents, because a dietary dose of 1.0% (and sometimes less) is sufficient to achieve anticancer effects (28, 34, 35), whereas n-3 PUFAs such as EPA or DHA require a dietary dose of 5 to 10% to obtain comparable effects (20,22, 36). The growth inhibitory mechanisms of CDHA, which are similar to those of DHA, include induction of apoptosis (down-regulation of Bcl-2) and disturbance of cell cycle progression causing G1 arrest (up-regulation of p53 and $p21^{Cip1/Waf1}$, and down-regulation of cyclin D1) (28).

Phytochemicals

Genistein found in soy

Anecdotal evidence suggests that soy foods and their constituents (especially genistein) can inhibit growth of breast cancer. However, epidemiologic studies have given conflicting results (37). Genistein is present at high levels in soybeans and soy products such as miso and tofu, and is structurally similar to mammalian estrogens (Fig. 2). Genistein has been found to enhance the growth of estrogen receptor (ER)-positive human breast cancer cells transplanted into ovariectomized female athymic mice (a low-endogenous-estrogen environment) (38), whereas it has been found to reduce the growth of ER-positive and ER-negative human breast cancer cells transplanted into non-ovariectomized athymic mice (39). In cell culture studies, genistein stimulates growth of ER-positive human breast cancer cells at low concentrations (4 nM - 4 μM), but inhibits growth of breast cancer cell lines and breast epithelial cell lines of human and animal origin at high concentrations, regardless of receptor status, with an IC_{50} of 7 to 272 μM after incubation for 72 hours (40). Thus, genistein can have widely varying effects depending on dose and cellular environment. Cells exposed to high concentrations of genistein exhibit G2/M arrest followed by the appearance of a sub-G1 fraction and a characteristic nuclear shape, consisting of multiple small chromatin spheres,

which is indicative of apoptosis (40). The apoptosis cascade is initiated by up-regulation of Bax protein, down-regulation of Bcl-X_L protein, and activation of caspase-3 (40). Animal experiments show that feeding soy protein to adult rats tends to inhibit MNU-induced mammary carcinogenesis, but the effect is not statistically significant (41). In one study, genistein was very effective in reducing tumor multiplicity but was only marginally effective in reducing MNU-induced mammary tumor incidence in adult rats (42). However, significant protective effects have been seen when genistein was fed to rats before puberty (see below).

Enterolactone and enterodiol derived from flax

Lignans, a group of dimeric phenylpropanoids, are mainly found in oil seeds. Flaxseed (linseed) is the most abundant source of plant lignans, but they are also present in whole grains and berries (43). Mammalian lignans, enterolactone and enterodiol (Fig. 2), which are structurally similar to endogenous sex steroid hormones, are produced from the precursor secoisolariciresinol diglycoside (SDG) by the action of bacteria in the colon of mammals (43). Plant lignans do not have inherent estrogenic activity, but are converted to weakly estrogenic compounds in the colon (43). Mammalian lignans enter the enterohepatic circulation and are excreted in the urine. Some epidemiological studies show an inverse association between the urinary or serum enterolactone content and the risk of breast cancer (44, 45), but at least one case control study shows no such association (46). In adult rats, continuous consumption of a diet containing 5% flaxseed or a similar amount of SDG decreases the incidence, number and size of 7,12-dimethylhenz(α)anthracene (DMBA)-induced mammary tumors (47). Studies have demonstrated that a 10% flaxseed diet inhibits the growth and metastasis of human breast cancer in athymic mice; these effects are partly due to down-regulation of insulin-like growth factor I, epidermal growth factor receptor (EGFR), and/or extracellular levels of vascular endothelial growth factor. (48,49). Daily *per os* administration of 10 mg/kg body weight enterolactone for 7 weeks at the promotion stage significantly inhibits growth of DMBA-induced mammary tumors in rats, but a dose of 1 mg/kg did not have this effect (50). In culture, enterolactone stimulates growth of ER-positive human breast cancer cells at low concentrations but inhibits growth of ER-positive and ER-negative human breast cancer cells at high concentrations; these cancer cell lines are more sensitive than normal cells (51,52). Enterolactone is more effective than enterodiol in suppressing cancer cell growth *in vitro* (53,54).

Diallyl disulfide in allium vegetables

Numerous reports imply that high consumption of certain vegetables may reduce the risk of breast cancer. Allium genus vegetables, including garlic, onions, leek, scallions, chives and shallots, have been shown to have beneficial effects against cancers. Garlic (Allium sativum L.), fresh garlic extracts, garlic compounds and synthetically prepared substitutes appear to affect breast cancer risk significantly. A review of epidemiologic studies shows that in two case-control studies, high consumption of garlic and onions was found to be associated with a decreased risk of breast cancer (55,56), whereas a cohort study showed no effect of allium vegetables (57). Laboratory investigations have provided evidence that garlic powder inhibits the incidence of DMBA-induced mammary tumors (58). Garlic contains a complex mixture of oil- and water-soluble organosulfur compounds (59). Animal studies provide evidence that some components of garlic have anticarcinogenic effects. Several lines of evidence suggest that allyl sulfur compounds are potentially important antitumorigenic agents. Oil-soluble diallyl disulfide (DADS) (Fig. 2) has been found to reduce the incidence and delay the onset of MNU-induced mammary tumors (60), and reduce the multiplicity of 2-amino-1-methyl-6-phenylimidazo [4-5-b] pyridine (PhIP)-induced mammary tumors (61), whereas water-soluble S-allyl cysteine has been found to be less effective or ineffective (60,62). In cell culture studies, oil-soluble compounds such as DADS, diallyl sulfide and diallyl trisulfide have exhibited antiproliferative effects against canine mammary tumor cells,

whereas the water-soluble compounds S-allyl cysteine, S-ethyl cysteine and S-propyl cysteine have exhibited little or no inhibitory effect (63). DADS has been found to inhibit growth of ER-positive and ER-negative human breast cancer cell lines, with an IC_{50} of 2 to 18 μM after 72 hours of incubation (64). In a study using female athymic mice, intraperitoneal injection of 1 or 2 mg DADS 3 times a week from the day of human breast cancer cell inoculation until the end of the experiment (35 days later) caused growth retardation and 43% reduction of primary tumor weight, respectively, compared with DADS-untreated mice (64). Formation of DNA adducts is believed to be an initial step in induction of carcinogenesis by some chemicals; levels of adducts in mammary epithelial cells correlate positively with mammary tumor incidence (58). Garlic powder protects against DNA adduct formation in mammary epithelial cells (65). A decrease in MNU-induced DNA methylation has been observed in mammary tissue of rats fed a diet supplemented with garlic powder (60). Furthermore, shifts in the cell cycle have been found to correlate with the suppression of tumor growth by DADS (66).

The inhibitive effects of DADS on growth of human breast cancer cells appear to be related to apoptosis resulting from up-regulation of Bax protein, down-regulation of Bcl-X_L protein, and activation of caspase-3 (64). The protective effect of garlic and its component DADS may be mediated by several mechanisms, including blockage of DNA adduct formation, reduction of DNA methylation, and reduced cell proliferation and/or induction of apoptosis.

Perillyl alcohol found in citrus fruits

Monoterpenes are nonnutritive compounds found in the essential oils of citrus fruits, cherries, mints and herbs. They may function as chemoattractants or chemorepellents, as they are largely responsible for the pleasant fragrance of these plants (67). Two monoterpenes, limonene and perillyl alcohol (POH) (Fig. 2), exhibit chemopreventive and chemotherapeutic effects against experimental mammary cancer. Limonene, the simplest monocyclic monoterpene, when administered in the diet at mass percentages of 1 to 5%, inhibits both DMBA- and MNU-induced mammary carcinogenesis in rats (68,69). Also, dietary limonene can cause regression of both DMBA- and MNU-induced rat mammary carcinomas (70). POH is a hydroxylated product of *d*-limonene, which is formed by the condensation of two isoprene molecules, and is 5 to 10 times more potent than limonene in its ability to cause regression of DMBA-induced mammary carcinomas (71). POH has been shown to inhibit growth of human breast cancer cells transplanted into athymic mice. For example, POH at a dose of 75 mg/kg administered intraperitoneally 3 times a week throughout the experiment (6 weeks) suppressed growth of orthotopically transplanted human breast cancer cells and regional lymph node metastasis (72). This growth suppression effect was the result of inhibition of tumor cell proliferation. In culture, POH inhibits cell proliferation in a variety of cell lines at a dose within a pharmacologically achievable range (73). *In vitro* studies indicate that growth of human breast cancer cell lines is inhibited by both POH and limonene (72, 74), and that POH is a more potent inhibitor of cell proliferation than limonene (74). POH at a dose of 500 μM has been found to exert a cytostatic effect on both ER-positive and ER-negative human breast cancer cell lines (72). Most anticancer drugs are cytotoxic, whereas POH is a cytostatic agent that blocks cell proliferation. Proposed mechanisms of action of POH include the transforming growth factor (TGF) β pathway (75) and inhibition of $p21^{ras}$ signaling (76). POH has also been found to affect several cell cycle regulatory molecules. It causes cell arrest in G1 phase by decreasing levels of cyclin D1 and cyclin E, which is followed by increased levels of $p21^{Cip1/Waf1}$, and has been found to decrease expression of proliferating cell nuclear antigen (PCNA) (72).

Resveratrol present in grapes and grape products

Resveratrol is a naturally occurring phytochemical that is found in approximately

72 plant species, including grapes, peanuts and various herbs (77). It is present at particularly high concentrations in grape skin, and is also abundant in red wine (78). Resveratrol is structurally related to estrogens (Fig. 2), and may play roles in protecting plants against fungal infections and in conferring disease resistance (acting as a phytoalexin). It has been suggested that resveratrol shows potential as an agent in cancer prevention and therapy. Resveratrol exerts anticarcinogenic activity in animal models of mammary carcinogenesis. In one rat study, intragastric administration of 10 or 100 mg/kg resveratrol 5 days per week, starting 7 days before administration of MNU and continuing for the entire period of the study suppressed induction of mammary carcinomas by MNU (79). At the end of the study (120 days after MNU administration), 100 mg/kg resveratrol was found to have reduced the multiplicity of tumors (6.0 vs. 3.9), decreased the total number of tumors, and increased the latency (median time for the appearance of the first tumor was 52 days in the control and 80 days in 100 mg/kg group), compared with untreated controls; a dose of 10 mg/kg resveratrol had significantly weaker effects (79). In another rat study, dietary administration of 10 ppm resveratrol beginning 1 week before DMBA administration (age of rats, 45 days) until termination of the experiment (age of rats, 210 days) suppressed mammary tumors with no effect on body weight gain (80). Compared with the controls, the resveratrol diet produced striking reductions in mammary tumor incidence (75% vs. 41%), multiplicity (2.41 vs. 1.08), and extended latency (77 days vs. 98 days after DMBA), but there was no effect on tumor volume (80). The suppression of DMBA-induced mammary carcinogenesis by resveratrol correlated with down-regulation of expression of NF-κB, cyclooxigenase-2 and matrix metalloprotease-9 (80). In cell culture studies, low concentrations of resveratrol have been found to cause proliferation of ER-positive human breast cancer cells, whereas high concentrations of resveratrol have been found to inhibit the growth of both ER-positive and ER-negative human breast cancer cell lines (78,81). Growth suppression by resveratrol is the result of S/G2M arrest (80,82) and modulation of the apoptosis cascade (up-regulation of Bax and Bak protein, down-regulation of Bcl-X_L protein, and activation of caspase-3) (81).

Certain types of fat and several plant-derived chemicals have exhibited protective effects against mammary carcinogenesis in experimental models. In culture, inhibition of growth of breast cancer cell lines, regardless of ER status, has been observed after incubation with certain food chemicals (Table I). However, in chemically induced animal models of mammary carcinogenesis and experiments in which tumors are transplanted into athymic mice, some dietary components must be fed to the adult animals for a long duration to obtain a significant effect.

MATERNAL NUTRITION DURING PREGNANCY AND LACTATION AND DIET BEFORE MATURITY

Epidemiological observations indicate that women who have experienced pregnancy early in life have lower risk of breast cancer than nulliparous women (83). This parity protection against breast carcinogenesis is apparently universal, having been observed in women of many ethnic backgrounds. Early, but not late, pregnancy has been shown to protect against breast cancer, suggesting that events early in a woman's life are particularly important factors in subsequent development of breast cancer. This phenomenon has also been observed in rats (84), and protection can also be achieved by short-term estrogen and progesterone treatment (85). In contrast, epidemiologic studies show that high *in utero* estrogen levels increase risk of breast cancer (86). Short-term exposure to hormones during critical periods of development may offer lifetime protection against breast cancer or may promote breast cancer later in life. Events at certain stages of development, including ingestion of certain substances (such as estrogenic chemicals or chemicals that modify estrogen levels), may alter estrogen target tissues (87) in ways that affect breast cancer risk later in life. Relationships between breast cancer and diet during childhood and adolescence

are of particular interest (5). Animal experiments have confirmed the importance of diet early in life.

Dietary fat and CLA

The timing of fat intake appears to be crucial, as studies indicate that maternal intake of high amounts of dietary fat during pregnancy affects breast cancer risk in female rat offspring. In a rat study, mammary tumor incidence was higher and latency for tumor appearance was shorter among the rats that were exposed *in utero* via maternal feeding to a diet high in n-6 PUFA, compared with the offspring of mothers fed an isocaloric diet low in n-6 PUFA (88). In another rat study, *in utero* exposure to a diet high in n-3 PUFA significantly reduced the risk of mammary cancer in offspring, compared with offspring exposed to a diet high in n-6 PUFA (89). In the same study, circulating levels of total estradiol during pregnancy were significantly higher in pregnant females fed a diet high in n-3 or n-6 PUFA than in animals fed a low-fat diet (89). Thus, high fetal estrogenicity is not sufficient to account for subsequent mammary tumor development in female offspring. A maternal diet high in OA during pregnancy and lactation also appears to have a protective effect against future development of mammary cancer in female rat offspring (90). Mammary carcinogenesis has been found to be associated with the development and differentiation of the mammary glands during early life (91). Studies have shown correlation between tumor yield and the number of terminal end buds (TEBs) at the time of carcinogen exposure (91). TEBs are relatively undifferentiated, and are particularly susceptible to chemical carcinogens. In contrast to the effects of exposure to high levels of n-6 PUFA *in utero*, incidence of mammary tumors in virgin adult rats was not altered by exposure to high levels of n-6 PUFA for 3 weeks (the length of the gestation period of rats) (92). Feeding rats a 1% CLA diet during early postweaning and adolescence (for 3 weeks from 21 to 42 days of age) has been found to reduce the incidence of MNU-induced mammary tumorigenesis; with adult rats, long-term continuous intake of CLA is necessary to achieve optimal inhibition of tumorigenesis (32). Intake of CLA during early postweaning and adolescence is associated with reduced proliferation of the epithelial cells within the TEBs and lobular epithelium, resulting in decreased epithelial density; thus, the carcinogen-sensitive target population is reduced (93). The timing of short-term exposure to dietary fat or CLA during development appears to be a critical factor in its effects on mammary cancer development later in life.

Phytoestrogens and mycoestrogens

Humans are exposed to a variety of estrogen-like chemicals through the food chain. Most natural estrogens are produced by plants. Genistein (an isoflavone), resveratrol (a stilben), and enterolactone and enterodiol (lignans) are classified as phytoestrogens, and zearalenone (a β-resorcyclic acid lactone), which is produced by molds that contaminate cereal crops, is classified as a mycoestrogen (4). Evidence suggests that exposure to phytoestrogens or mycoestrogens early in life influences breast cancer risk and causes endocrine disruption.

Genistein

Epidemiological studies indicate that high soy intake during adolescence reduces the risk of breast cancer later in life (37). The timing (stage of development) of exposure to estrogenic chemicals is an important factor in physiological outcome, especially if endogeneous estrogen activity is low. Several studies using rodents have shown that a genistein-rich diet can significantly reduce risk of DMBA-induced mammary cancer if exposure occurs before puberty or during adolescence (94). In rats, genistein exposure before puberty or during adolescence up-regulates TGFα and EGFR, stimulates cell proliferation in the mammary gland, enhances mammary gland differentiation, and significantly decreases the number of TEBs at the time of carcinogen administration, resulting in reduction of mammary cancer risk (94). It is possible that genistein exerts its beneficial effects via mechanisms other

than acceleration of mammary gland differentiation. The effects of prenatal and prepubertal genistein exposure on mammary tumorigenesis were evaluated in rats that were administered a carcinogen (MNU) before differentiation of the mammary glands (at 28 days of age) and sacrificed when the largest mammary tumor was ≥1 cm in size (95). In that study, both prenatal and prepubertal genistein exposure, at 1.5 mg/kg daily (comparable to average daily intake in Asian countries) or 30 mg/kg daily over a period of 5 days (at 15-19 days of gestation, or at 15-19 days of age), tended to suppress incidence of MNU-induced mammary carcinoma (prenatal low-dose, 54%; prenatal high-dose, 57%; prepubertal low-dose, 42%; prepubertal high-dose, 48%), compared with control groups (70%) at 26 weeks of age; suppression was significant in the prepubertal low-dose group (95). All groups exhibited comparable development of the mammary gland at the time of MNU administration (95). Thus, mammary gland differentiation had not been altered by prenatal or prepubertal genistein treatment at the time the carcinogen was administered. However, at the time of MNU administration, genistein-exposed TEBs had lower levels of ERα- and progesterone receptor (PgR)-positive cells (presumed to be progenitor cells of MNU-induced mammary carcinomas), p63-positive mammary progenitor/stem cells (involved in cell renewal) and PCNA-positive cells (necessary for cell proliferation) (95). Estrogen-like chemicals are thought to cause reproductive abnormalities. In these genistein-exposed rats, disturbance of estrous cyclicity was observed, but all rats were cycling (95).

Resveratrol

In a recent study, prepubertal rats (15-19 days of age) were given daily subcutaneous injections of 10 or 100 mg/kg resveratrol or vehicle for 5 days, were administered MNU at 49 days of age, and were monitored for occurrence of mammary carcinoma ≥1 cm in diameter (96). The dose of 100 mg/kg resveratrol significantly increased the incidence of mammary carcinoma and cancer multiplicity (58.3%, and 1.71, respectively), compared with controls (29.2%, and 0.92, respectively), at 32 weeks after MNU treatment; 10 mg/kg resveratrol had no significant effect (29.2%, and 1.00, respectively) (96). Resveratrol treatment tended to increase the population of ER- and PgR-positive cells, which are presumed to be the progenitors of the majority of MNU-induced mammary carcinomas (84). Resveratrol did not affect body weight gain, but early vaginal opening and elongation of the estrus phase were observed, indicating an estrogenic effect (96). These findings suggest that foods containing high levels of resveratrol should not be consumed by young children. However, daily doses of 10 and 100 mg/kg resveratrol are 500 and 5000 times the amount that is consumed by a person who drinks one glass of red wine a day, respectively (97), and 10 mg/kg resveratrol had no effect on mammary carcinogenesis.

Enterolactone

Maternal exposure to a 10% flaxseed diet or a level of SDG equivalent to a 10% flaxseed diet during pregnancy and lactation alters reproductive indices in female rat offspring (97,98). When rat dams were fed a 10% flaxseed diet during pregnancy and lactation, the female offspring had shortened anogenital distance, greater uterine and ovarian relative weights, earlier onset of puberty, lighter body weight at puberty, lengthened estrous cycle and persistent estrus, compared with controls, suggesting an estrogenic effect (98). These findings suggest that the amount of flaxseed consumed during human pregnancy and lactation should be carefully controlled. In contrast, maternal exposure to a 10% flaxseed diet or an equivalent level of SDG during the lactation period only or during lactation and the post-lactation period did not alter reproductive indices in female rat offspring (100). The only significant effect that has been observed in rats after maternal exposure to a 10% flaxseed diet or an equivalent level of SDG during pregnancy and lactation or during lactation only is promotion of differentiation of mammary glands in female offspring (99,100). Mammary glands may be more sensitive to lignans than other estrogen target tissue at that period of development. Exposure to a 10%

flaxseed or an equivalent level of SDG during lactation has been found to inhibit DMBA-induced mammary tumorigenesis in rats (101); compared with the control group, at 21 weeks after DMBA administration, the flaxseed and SDG groups had significantly lower tumor incidence (31.3% and 42.0% lower, respectively), total tumor load (50.8% and 62.5% lower, respectively), mean tumor size (43.9% and 67.7% lower, respectively) and tumor number (46.9% and 44.8% lower, respectively). Flaxseed consumption by lactating mothers may permanently protect the offspring from developing mammary cancer without noticeable effects on reproductive organs. Enterolactone has approximately 10 times greater estrogenic activity than enterodiol (51), and may help prevent breast cancer if administered at a critical period of development.

Zearalenone

Zearalenone is a mycotoxin synthesized by *Fusarium* molds (Fig. 2). Zearalenone is found in a variety of host plants and debris from soil around these molds, and is present as a natural contaminant in food as a result of infection of grain by *Fusarium* species. Thus, humans may be exposed to zearalenone by ingestion of contaminated grain products. In one study, maternal exposure to 20 μg (0.1 mg/kg) of zearalenone between 15 and 20 days of gestation did not significantly influence incidence of DMBA-induced mammary tumors in female rat offspring, although it tended to reduce the incidence (102). When 20 μg of zearalenone was administered to suckling rats at 7, 10, 14, 17 and 20 days of age (0.7-2.0 mg/kg ×5), it caused mammary gland differentiation, and the incidence and multiplicity of DMBA-induced mammary tumors were reduced (103). In another study, rats received daily doses of 0.1 mg/kg or 10 mg/kg of zearalenone between 15 and 19 days of age, and were administered MNU at 28 days of age, when both zearalenone-treated groups and the untreated controls exhibited similar mammary gland differentiation (104). At 33 weeks after MNU administration, mammary tumor incidence (rats with mammary tumor ≥1 cm in size) was significantly reduced in rats treated with 0.1 mg/kg (46%) or 10 mg/kg (50%) zearalenone, compared with untreated controls (79%). Zearalenone inhibited mammary cancer multiplicity including histologically detected small tumors, in a concentration-dependent manner; 10 mg/kg zearalenone significantly reduced multiplicity (1.4±0.6), compared with untreated controls (3.1±0.3), and 0.1 mg/kg zearalenone also reduced multiplicity (2.1±0.5), but not significantly (104). Zearalenone has strong estrogenicity, as indicated by *in vitro* bioassays (105). These findings suggest that zearalenone exposure could have significant effects on mammals with an estrogen-responsive reproductive system. In rats, prepubertal exposure to zearalenone at 0.1 mg/kg and 10 mg/kg did not affect body weight gain, but vaginal opening was accelerated and disruption of estrous cyclicity (persistent estrus or prolonged diestrus) occurred in a considerable number of animals at 8 to 10 weeks of age (104). Also, at 37 weeks of age, the frequency of rats with no newly formed corpora lutea in the ovaries (indicating anovulation) increased in parallel to the zearalenone dose (104). Thus, prepubertal zearalenone treatment appears to disrupt endocrine function and ovarian structure in adulthood. Androgenization of neonatal female rats has been found to cause anovulation and reduce incidence of DMBA-induced mammary tumors, whereas progesterone treatment accelerates DMBA-induced mammary tumorigenesis (106). Those results suggest that the zearalenone treatment produced an altered estrogen/progesterone ratio, which in turn influenced the development of MNU-induced mammary tumors. It has been estimated that people living in the United States ingest 1 to 5 mg/kg (0.02-0.1 mg/kg) of zearalenone daily (107). Thus, prepubertal zearalenone exposure at a dose comparable to the daily intake of Americans suppressed mammary carcinogenesis, but its profound effect on ovarian function (sterility) indicates that consumption of zearalenone-contaminated food in the prepubertal period can have other clinically significant consequences.

COMBINATION EFFECTS OF DIETARY COMPONENTS

Synergistic action by two or more chemicals is defined as therapeutic effects that are greater than those expected from simple addition of the effects of the individual chemicals. Synergistic action against cancer cells may have beneficial effects in breast cancer control. A combination of EPA and the angiogenesis inhibitor TNP-470 exerts synergistic action against human breast cancer cells by accelerating apoptosis (26). Asian diets contain large amounts of soy products and fat derived from fish. EPA (abundant in fish oil) acts synergistically with genistein (found in soy) against human breast cancer cells: against MCF-7 cells at EPA >210.9 μM and genistein >93.2 μM, and against MDA-MB231 cells at EPA >609.3 μM and genistein >176.1 μM (40). Dietary intake of a combination of EPA and genistein may be beneficial for breast cancer control.

Epidemiological studies have shown an inverse correlation between red wine consumption and the incidence of cardiovascular disease, even in people who consume a diet relatively high in fat (the so-called French paradox) (108). It has been suggested that resveratrol is the main cause of this effect. Resveratrol (at 52-74 μM) has been found to antagonize the effects of LA, a potent breast cancer cell stimulator, and to suppress the growth of both ER-positive and ER-negative human breast cancer cells (81). Thus, resveratrol may mitigate the growth stimulatory effect of n-6 PUFAs in populations consuming a Western-style diet.

Garlic is widely cultivated and consumed worldwide. Components of garlic, including allyl sulfur compounds, can modify the action of several dietary chemicals. In a study of human breast carcinoma cells in culture, DADS antagonized the effect of LA (DADS =1.8 μM and LA $\geq 6.5\times10^{2}$ μM), and synergized the effect of EPA (at DADS $>3\times10^{-3}$ μM and EPA $>6.3\times10^{-1}$ μM) (64). DADS has also been found to suppress PhIP-induced mammary carcinogenesis in animals fed a high corn oil diet, which is rich in LA (61). Thus, DADS appears to exert beneficial effects in combination with compounds present in both Western and Asian diets. Further studies are needed to elucidate the underlying mechanisms of combination effects of dietary components effective in breast cancer control.

CONCLUDING REMARKS

Experimental studies have confirmed some of the beneficial and harmful effects of dietary components observed in epidemiological studies of their effects on breast cancer. Dietary fat, fruits and vegetables appear to affect breast cancer risk. For example, studies suggest that high intake of EPA and DHA (both abundant in fish), high intake of OA (found in olive oil), and low intake of LA (n-6 PUFA) can reduce breast cancer risk. Genistein (found in soy), enterolactone (derived from flax), DADS (found in garlic), POH (found in citrus fruits) and resveratrol (found in grapes) have also been shown to be protective. Experiments show that certain fatty acids and phytochemicals interact synergistically or antisynergistically in their effects on breast cancer. Although there are similarities in effects of dietary factors on breast cancer risk between adults and children, the effects appear to be much less pronounced in adults. Adult animals must often be exposed to test compounds for a long duration to obtain significant effects. In contrast, short-term exposure to certain dietary components at an early stage of animal development can have long-lasting effects on mammary carcinogenesis. Thus, the available evidence indicates that the early period of development is a critical time for determining predisposition to subsequent breast cancer risk. It appears that breast cancer risk can be reduced by early dietary intervention.

ACKNOWLEDGEMENTS

We wish to thank our co-workers, Drs. H. Senzaki, Y. Uemura, Y. Singh, K. Shirai, J. Yang, D. Yamamoto, H. Nakagawa, M. Sato, M. Tsujita-Kyutoku, T. Yuri, Y. Nikaido, and R.-J. Pei. We also wish to thank Ms. T. Akamatsu for her technical assistance, and Ms. Y. Yoshida for her help in preparing the manuscript. This work was supported in part by a Health and Labor

Science Research Grant for Research on Risk of Chemical Substances, from the Ministry of Health, Labor and Welfare, Japan, and by a Grant-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science (16510047, 16790766).

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