shoulder to the inguinal region, forming the mammary streaks in the fetal rat. Downgrowths of ectodermal epithelium from the mammary streaks form the 12 primary buds from which the 6 pairs of mammary glands develop. There is one pair located in the cervical region, two pairs in the thoracic region, one pair in the abdominal region, and two pairs in the inguinal region [5].

Postnatal stage

The major development of the mammary gland in the rat occurs between birth and puberty. By the end of the first week, each mammary gland consists of a single primary or main lactiferous duct with three to five secondary ducts. During the second week, the secondary ducts branch dichotomously into third, fourth, and fifth generations of ducts.

Juvenile stage

Starting at about the fourth week, growth of the mammary ducts increases significantly. The growth rate of the gland now exceeds the previous isometric rate. Ductal arborization is initiated from the highly proliferative terminal end buds (TEBs) found at the tips of the ductal branches [6] (Figure 1), and ductal morphogenesis and lumen formation is accomplished by a highly regulated process of cell proliferation and cell death [7]. The number of TEBs reaches a maximum at 20 days of age and then decreases as the TEBs differentiate into terminal ductules and alveolar buds (ABs) [5]. TEBs are influenced by systemic steroid hormones and aid the ducts in linear growth as well as the regulation of branching patterns. The ductal pattern is created by the penetration of TEBs through the stromal fat pad. The TEB consists of two histologically distinct cell types. The body cells give rise to mammary epithelial cells while the cap cells are the precursors of the myoepithelial cells [7].

Puberty

Puberty, defined as the onset of estrous cycles, commences in the rat between 35 and 42 days of age. At the onset of puberty, each AB develops into 10 to 12 alveoli to form a lobule, which accumulates progressively over multiple estrous cycles [5, 8]. By 85 days of age, virgin female rats have a relatively constant number and proportion of TEBs, ABs, and lobules, although histological changes in size and secretory development occur within the lobules during the estrous cycle [9-11].

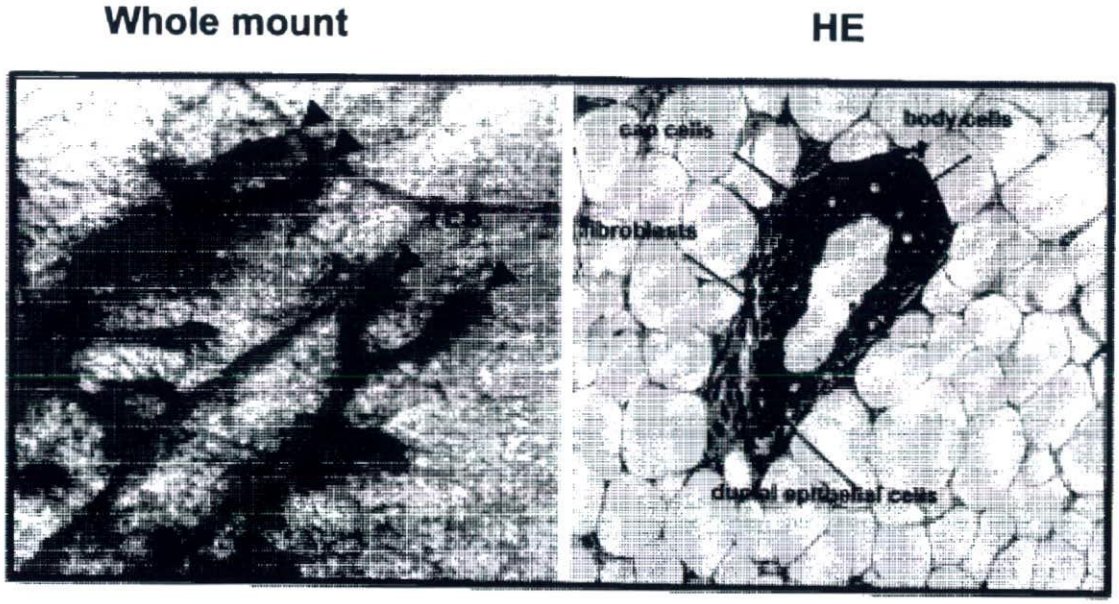

Figure 1. Terminal end buds
Whole mount preparation (left) and hematoxylin-eosin (HE) staining (right) of 49-day-old virgin rat mammary gland showing terminal end buds (TEBs).

Cellular structures of mature mammary gland

The mammary gland can be divided into two compartments: the epithelial (or parenchymal) compartment and the stromal (or mesenchymal) compartment. At the cellular level, the parenchymal compartment of the mammary gland is composed of two different types of epithelial cells with distinct morphologies, functions, and proliferative activities. These cell types are the luminal epithelial cells, which are located along ducts, ductules, terminal ducts, and alveoli, and the myoepithelial cells.

The luminal epithelial cells are cuboidal or columnar in shape [12], and can be defined immunohistochemically by the expression of keratins 8, 18, and 19 [13, 14]. These cells develop the majority of mammary carcinomas [15].

The myoepithelial cells lie between the luminal epithelial cells and the basement membrane [16-19]. The shape, thickness, and continuity of these cells vary during development and between individual epithelial structures in the mammary glands. The myoepithelial cells synthesize and secrete the continuous basement membrane that separates the epithelium from the stromal compartment [12, 20]. The myoepithelial cells express higher levels of cell adhesion receptors and adhesion-associated molecules than the luminal epithelial cells [19]. The former cells can be immunohistochemically distinguished from the latter cells by the expression of intermediate filament proteins (vimentin, keratin 5, and keratin 14) and contractile proteins (myosin and smooth muscle actin) [13, 14, 21].

Pregnant stage

During pregnancy, there is a rapid and continuous increase in the mammary gland epithelium resulting in growth of the lobules and the ducts [12, 22]. The alveoli develop and increase in size and number until the space between ducts is almost completely filled with them. The number of alveoli per unit area shows a large increase from day 5 to day 10 of pregnancy and peaks at day 20 of gestation [12]. During pregnancy, the mammary gland is influenced by estrogen, progesterone, and other placental hormones. The duration of pregnancy is usually 21 days in the rat.

Lactational stage

Lactation is the production and secretion of milk. The initiation of lactation appears to be induced by a decrease in estrogen and progesterone. About 20% of total mammary growth occurs during the first 14 days of lactation. Several hormones, such as prolactin, insulin, and glucocorticoid, are involved in the maintenance of lactation.

Involution

After weaning, there is involution of the glands with a three-fold reduction in the size of lobules; however, the number of lobules remains high, and the gland never returns to the same level of differentiation as seen in virgin female rats of the same age [5]. Involution has been attributed to several factors, including falling levels of circulating prolactin upon the cessation of suckling, mild ischemia as a result of milk engorgement and compression of the vasculature, factors in milk that promote cell death, physical distension of the luminal epithelium, and increased activity of basement membrane-degrading enzymes [12]. Mammary involution comprises two distinct phases. The first phase is apoptosis among the secretory epithelial cells. The second phase is characterized by the degradation of the alveolar structures and the mammary basement membrane and extracellular matrix [23-27]. The apoptosis-induced signals and the loss of survival factors may exert significant control over mammary gland involution.

Induction of mammary lesions in rats

While many strains of rats develop spontaneous tumors later in life, they respond to chemical carcinogens and radiation with faster development of both hormone-dependent and hormone-independent mammary tumors. For the specific induction of mammary tumors in rats via genotoxic mechanisms, the most commonly used agents are 7,12-dimethylbenz[a]anthracene (DMBA) and N-methyl-N-nitrosourea (NMU). A single dose of DMBA (2.5-20 mg) or NMU (25 or 50 mg/kg body weight) by gavage or by intravenous or subcutaneous routes, respectively, induces mammary tumors with latencies of 8 to 21 weeks [28, 29]. Other chemicals such as benzo[a]pyrene, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) and 1,2-dibromo-3-chloropropane can induce mammary carcinomas with lower incidences [5, 30, 31] (Table 1). Sublethal doses of different types of radiation, including x-rays and neutrons,

Table 1. Induction of mammary tumors by chemical or physical agents.

Carcinogen	Lesion Type
Genotoxic Agents	
DMBA	Ductal / Alveolar/ Mesenchymal?
NMU	Ductal / Mesenchymal?
benzo[a]pyrene[30]	Ductal
2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP)	Ductal / Mesenchymal
1,2-Dibromo-3-chloropropane[116]	Ductal
1,2-Dibromoethane[117]	Alveolar/ Mesenchymal?
Ochratoxin A[118]	Alveolar/ Mesenchymal?
Radiation (x-rays, γ-rays, neutrons)	Ductal / Mesenchymal
Non- Genotoxic Agents	
Estrogens[119, 120]	Ductal

induce mammary tumors within a year [32, 33] (Table 1). Induced tumors are histologically benign and malignant, and they generally have features in common with human tumors.

Chemically-induced mammary preneoplastic and neoplastic lesions

With chemical carcinogens, the earliest visible histological changes in the rat mammary glands are focal or multifocal hyperplasias primarily within the terminal ductule or AB or both [34-36]. The TEBs and terminal ducts are the sites of origin of malignancies, whereas benign lesions such as cysts, adenomas, alveolar hyperplasias, and fibroadenomas originate from the ABs in the rat mammary glands [35-39]. These observations indicate that there are two different pathogenetic pathways: one pathway for malignant lesions and another pathway for benign lesions. In addition, benign lesions tend to appear later than the malignant ones, indicating that the former are not precursors of the latter [35, 40].

Ductal hyperplasia pathway

A common type of chemically-induced preneoplastic lesion found in rats is ductal hyperplasia, which is characterized by intraluminal proliferation of epithelial cells (i.e., an increase in the number of epithelial cell layers within a duct). Ductal hyperplasia, which exhibits intraductal epithelial proliferation, progresses through a phenotype very similar to human ductal carcinoma in situ. In histological sections, intraductal proliferation becomes larger (Figure 2) and leads to the formation of micro-adenocarcinomas. When young virgin rats (21 to 50 days old) are inoculated with DMBA or NMU, ductal hyperplasia is detectable within 14 days after inoculation, and intraductal carcinomas are detectable after 20 days. Locally invasive carcinomas develop from these intraductal lesions to form palpable tumors approximately 13 weeks after the injection [28, 36].

In contrast to conventional strains, a rat strain carrying the human c-Ha-ras protooncogene is highly susceptible to mammary chemical carcinogens [41, 42]. When the transgenic rats are intravenously injected with 50 mg/kg body weight of NMU at 50 days of age, atypical ductal hyperplasias develop in 44% of the animals by day 15, and small invasive carcinomas form in almost all animals by day 20. Adenocarcinomas become palpable in all animals by day 56 [43]. This rat model can be used for short-term screening of chemopreventive agents, as well as mid-term screening of promoting agents for mammary carcinogenesis.

Studies of the pathogenesis of rat mammary carcinomas have revealed that carcinogens act on TEBs and terminal ducts mainly when these structures are differentiating into ABs. Transformed TEBs and terminal ducts evolve into ductal hyperplasias, carcinomas in situ, and invasive carcinomas [35, 38, 44]. In humans, ductal hyperplasias with or without atypia and

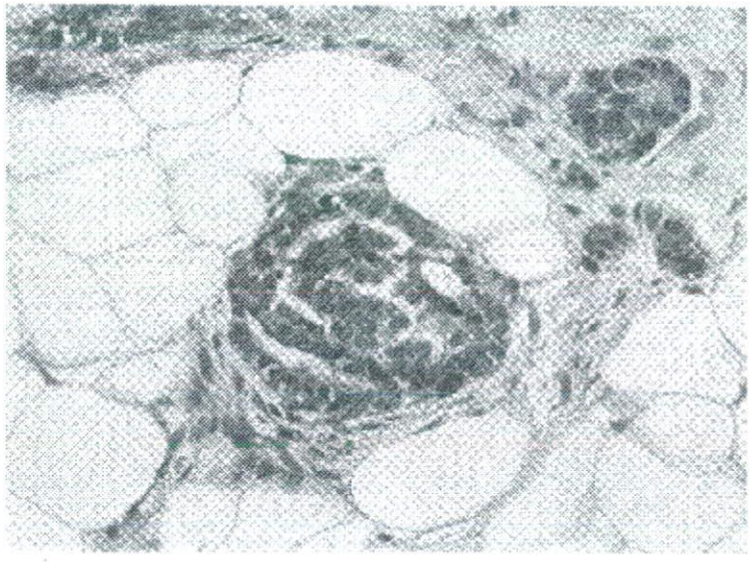

Figure 2. Intraductal epithelial hyperplasia
An enlarged terminal ductal structure exhibiting intraductal proliferations of epithelium.

atypical lobular hyperplasia are considered to be risk factors for subsequent development of invasive breast carcinomas [45-47]. However, the comparison between the pathogenetic pathways of mammary carcinogenesis in rats and humans is only tentative, because the role of the TEBs in humans is unknown. The TEB in the human female is a prepubertal structure, and the biology and differentiation of this structure from prepuberty to puberty needs to be studied. An important difference between the pathogenetic pathways in rats and humans is at the level of the terminal ductal lobular unit. The TEB in the rat would be equivalent to the intralobular terminal duct in the human, the area which is most susceptible to neoplastic growth [28]. Observations of early carcinomas in the human breast are needed to facilitate a clearer understanding of the pathogenetic scheme.

Alveolar hyperplasia pathway

Alveolar hyperplasia is a term commonly applied to enlarged lobules consisting of relatively normal alveoli that resemble the normal prelactating mammary gland [28, 37]. However, minimal or mild degrees of alveolar hyperplasia are difficult to distinguish from the normal state. In rats, the cause and biological behavior of alveolar hyperplasia are unknown, but the lesion is thought to be a precursor of adenoma and/or fibroadenoma (Figure 3). Chemically-induced alveolar hyperplasia does not appear to give rise to adenocarcinomas in rats. By clear contrast, the principal preneoplastic lesion in mouse mammary glands is alveolar hyperplasia or hyperplastic alveolar nodule [48, 49], which can be induced by mouse mammary tumor viruses [50-52], chemical carcinogens [53], X-irradiation [54], and prolonged hormone stimulation [55]. Also, in humans, atypical lobular hyperplasia is thought to progress to invasive lobular carcinoma via lobular carcinoma in situ.

Histopathology of neoplastic lesions

Rat mammary tumors have been classified by several authors [28, 38, 56, 57], and benign tumors, such as intraductal papilloma, papillary cystoadenoma, adenoma, and malignant tumors, such as papillary carcinoma, cribriform carcinoma (Figure 4), comedo carcinoma, and tubular carcinoma, have been recognized. Most of the neoplastic lesions found in the rat mammary glands have their counterpart in human pathology, with the exceptions of human-specific lesions such as lobular carcinoma and Paget's disease. Although lobular carcinoma, in situ or invasive, has not been described in the conventional strains of rats [44], the alveolar epithelia can transform to give rise to carcinomas under a certain genetic

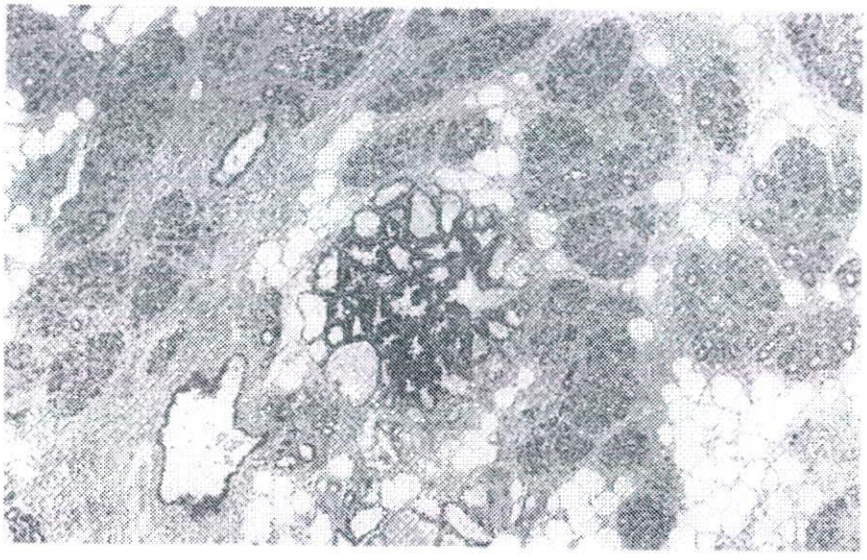

Figure 3. Fibroadenoma with alveolar hyperplasia
Note the proliferation of epithelium resembling alveolar buds and also the papillary growth pattern.

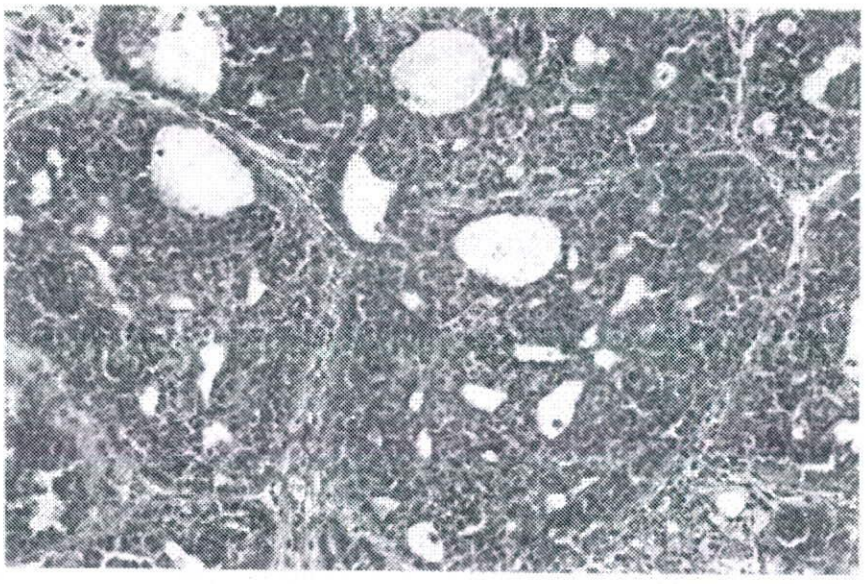

Figure 4. Cribriform carcinoma
Ductal carcinoma with areas of cribriform pattern.

background such as human c-Ha-ras transgenic rats [39] (Figure 5).

Molecular pathology of neoplastic lesions

Mutation analyses of oncogenes and tumor suppressor genes in sporadic human breast cancer and chemically-induced rat mammary carcinomas have revealed both similarities and differences in the mutation spectra of the two types of tumors. Ha-ras mutations are commonly observed with an incidence of 18% to 80% in the rat carcinomas induced by DMBA, NMU, or 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), whereas mutations in Ki-ras, p53, and brca1 are rarely

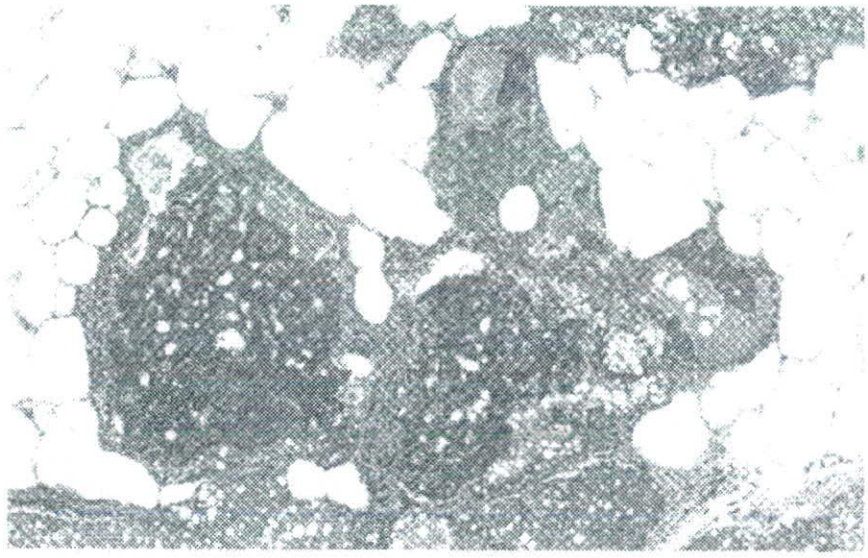

Figure 5. Alveolar hyperplasia with cellular atypia in c-Ha-ras proto-oncogene transgenic rat A marked increase in the number of mammary terminal branches and alveoli in 35-week-old transgenic virgin rats is described in Hamaguchi *et al.* [39]. Occasionally, the epithelial cells of these pre-neoplastic lesions displayed atypia.

Table 2. Genetic alterations in chemically induced rat mammary carcinomas.

	DMBA	NMU	PhIP
Ha-ras	23%[121, 122]	29-80%[41, 121-123]	18-42%[31, 124]
Ki- ras	.....	18%[123]	0%[31]
p53	3% [122]	0%[122]	0-10%[31, 125, 126]
brca 1	.....	.....	0%[127]

detected (Table 2). In contrast, approximately 30% of sporadic human breast cancers have mutations in TP53 (human p53 gene) [58-60], but few human cancers feature Ha-ras or Ki-ras alterations [61, 62]. Amplification and/or overexpression of Ha-, Ki-, and N-ras, cyclin D1, and neu/erbB-2/HER-2 are common in breast malignancies in humans [62, 63]. Two laboratories have demonstrated that overexpression of Ha-ras is sufficient to induce mammary carcinomas in rats [64, Ueda and Tsuda, unpublished data]. Thus, aberrant expression of the ras, cyclin D1, or erbB-2 genes can initiate mammary carcinogenesis in rats as well as in humans.

In both rats and humans there is strong evidence of genetic predisposition. Hereditary breast cancer is characterized by early age at onset (an average of 5 to 15 years earlier than sporadic cases), bilaterality, vertical transmission through both maternal and paternal lines, and familial association with tumors of other organs, particularly the ovary and prostate gland. The search for genes associated with hereditary susceptibility to breast cancer has led to the identification of several susceptibility genes, including BRCA1, BRCA2, TP53, and PTEN/MMAC1. Mutations in the BRCA1 or BRCA2 genes confer a lifetime risk of breast cancer of between 60% and 85% [65, 66]. However, mutations in these genes account for only 2% to 3% of all human breast cancers [67, 68], and susceptibility alleles in TP53 and PTEN/MMAC1 are even less common causes of human breast cancer [69]. The 1100delC mutation

in the cell-cycle-checkpoint kinase gene (CHEK2 or CHK2) has been discovered as an additional gene variant conferring susceptibility to breast cancer [70]. CHEK2 protein is implicated in DNA repair processes involving BRCA1 and p53 [71-73]. While the 1100delC mutation, a truncating variant that abrogates the kinase activity, has a frequency of 1.1% in healthy individuals this variant is present in 5.1% of individuals with breast cancer from 718 families that do not carry mutations in BRCA1 or BRCA2. This mutation doubles the risk of breast cancer among women and increases the risk among men by a factor of 10 [70]. The CHEK2 protein is activated after phosphorylation by the checkpoint gene product ATM and in turn activates BRCA1. The role of ATM mutations in the predisposition to the early onset of breast cancer remains controversial, but some missense mutations appear to increase susceptibility to breast cancer in humans [74] and mice [75]. There is also convincing evidence of the existence of additional high-penetrance genes that increase susceptibility to breast cancer [69].

In human lesions, ductal carcinoma in situ shows multiple losses involving loci on chromosomes 2q, 13q, 16q, 17p, and 17q, which also happen to be sites where important tumor suppressor genes are located [76]. A few studies have been conducted in lobular neoplasia and show losses at 11q, 16q, and 17q in these lesions [47]. Specific genomic alterations have been described in rat mammary carcinomas induced by DMBA, NMU, and PhIP [77, 78]. However, the tumorigenic potential of each of these alterations mostly remains to be tested in rat models.

Risk assessment using the rat carcinogenesis model

Reproductive history is the strongest and most consistent risk factor outside of genetic background and age [79, 80]. Especially, early age of first full-term pregnancy (≦20 years old) is a strong protective factor. On the other hand, a history of induced abortion appears to have little influence on the breast cancer risk [81, 82]. A collaborative re-analysis of data shows that the relative risk of breast cancer decreases by 4.3% for each year that women breastfeed [83]. Protection by parity from mammary carcinogenesis is also observed in rats [84-87], and short-term exposure to pregnancy levels of estrogen is sufficient to this effect and equally protective for even nulliparous rats [88]. Whereas pregnancy alone has been as effective as pregnancy and lactation in most experiments, Yang *et al.* [87] have reported that pregnancy followed by lactation has an additive effect in protection when rats are exposed to NMU prior to pregnancy. Interrupted pregnancy appears to be protective with lower efficiency compared to full-term pregnancy, although the interruption experiments have yielded contradictory results [86, 89, 90].

The age-adjusted death rates from breast cancer are 2 to 8-fold less in Asian countries than in the United States and Western Europe. The smaller death rate appears to be related to the 20- to 50-times greater consumption of soybean products [91]. A case-control study in Shanghai suggested that regular soy consumption reduced the risk of hormone-receptor-positive tumors [92]. Studies using the rat model as well as carcinoma cell lines have indicated that genistein, one of the isoflavones in soybean, may be responsible for tumor suppressive effects [43, 93-96]. A diet rich in folate and carotenoids might also be protective [97-99]. The possibility of a protective role for folate is somewhat controversial in the rat models since moderate folate deficiency inhibits, whereas dietary folate supplementation does not significantly promote, the progression of NMU-induced mammary neoplastic foci [100, 101]. There are relatively few studies of the effects of carotenoids in the rat models. The majority, but not all, of these studies indicate a protective effect of lycopene-rich tomato carotenoid oleoresin, whereas β-carotene shows no protection against the development of mammary cancer [102-104]. A Canadian case-control study found an association with dioxin-like polychlorinated biphenyls, suggesting that exposure to these substances might increase the risk [105]. In rats, however, inconsistent results have been obtained: 3,3',4,4'-tetrachlorobiphenyl significantly inhibits the tumor growth [106] whereas 2,3,7,8-tetrachlorodibenzo-p-dioxin slightly increases the tumor incidence when neonatal rats are initiated with NMU [107].

Greater consumption of dietary fat (especially n-6 polyunsaturated fatty acids) enhances breast cancer

risk [108, 109]. In the rat model, high levels of dietary fat increase the incidence of chemically-induced mammary carcinomas, and affect the promotion phase but not the initiation phase of the carcinogenesis [5]. Both the quantity and the constituents of fat should be considered as the risk factors. An n-6 polyunsaturated fatty acid, linoleic acid, may be responsible for the promoting activity while n-3 polyunsaturated fatty acids, eicosapentaenoic acid and docosahexaenoic acid, may act inversely [110].

An increase in the multiplicity of breast cancer is seen in long-term neuroleptics users [111]. A variety of drugs, such as reserpine and perphenazine, that decrease hypothalamic dopaminergic activity enhance the development, multiplicity, and growth of chemically-induced mammary carcinomas in rats [5, 112]. This effect is probably mediated by prolactin, since dopaminergic activity is primarily responsible for inhibition of prolactin release from the pituitary.

PROSPECTS

Since rat mammary tumors closely resemble the human counterparts in many aspects, genetically engineered rats may serve as a favorable model for human breast cancer research. A major disadvantage of the rat system is that the gene knockout technique has been unavailable. In contrast, almost 100 transgenes, targeted mutations, combinations of transgenes, and combinations of transgenes and targeted mutations have been used to study mammary cancer in mice. Genetically engineered mice tumors have: (1) phenotypes similar to those of non-genetically engineered mice tumors; (2) signature phenotypes specific to the transgene; and (3) some morphological similarities to human breast cancer [113, 114]. However, some investigators did not appreciate the relevance of the murine systems because mouse mammary tumors do not resemble most human breast cancers either morphologically or biologically. Nevertheless, the emergence of knockout and transgenic biologies has provided remarkable evidence that mouse tumors can be produced by the same genes implicated in human breast cancer [115]. The development of the gene knockout technique in rats will be a powerful tool in breast cancer research.

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Purple corn color suppresses Ras protein level and inhibits 7,12-dimethylbenz[*a*]anthracene-induced mammary carcinogenesis in the rat

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Anthocyanins belong to the class of phenolic compounds collectively named flavonoids. Many anthocyanins are reported to have inhibitory effects on carcinogenesis. Purple corn color (PCC), an anthocyanin containing extract of purple corn seeds, is used as a food colorant. The major anthocyanin in PCC is cyanidin 3-*O*-β-D-glucoside (C3-G). The present study was conducted to assess the influence of dietary PCC on 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced mammary carcinogenesis in rats. PCC significantly inhibited DMBA-induced mammary carcinogenesis in human c-Ha-*ras* proto-oncogene transgenic (Hras128) rats and in their non-transgenic counterparts. PCC and C3-G also inhibited cell viability and induced apoptosis in mammary tumor cells derived from Hras128 rat mammary carcinomas. At the molecular level, PCC and C3-G treatment resulted in a preferential activation of caspase-3 and reduction of Ras protein levels in tumor cells. It is proposed that C3-G could act as a chemopreventive and possibly chemotherapeutic agent for cancers with mutations in *ras*. Secondly, the *in vitro–in vivo* system used in this study can be utilized for screening for cancer preventive compounds that act via Ras down-regulation. (*Cancer Sci* 2008; 99: 1841–1846)

Anthocyanins are found throughout the plant kingdom and impart purple, blue and red color to fruits and vegetables. They belong to the class of phenolic compounds collectively named flavonoids. Anthocyanins are naturally present as glycosides having glucose, galactose, rhamnose, xylose or arabinose attached to the aglycon nucleus; the sugar-free aglycon nucleus is known as anthocyanidin. Several hundred anthocyanin species exist depending on the glycoside structure. It is generally accepted that anthocyanin food colors do not exert obvious toxicity, teratogenicity or mutagenicity and, indeed, anthocyanins may inhibit mutagenesis in the Ames test.[1–3] In studies testing the effects of anthocyanins on carcinogenesis, inhibitory effects of anthocyanins have been reported.[4–9]

PCC (Maize morado color) is extracted from the seeds of purple corn, *Zea mays* L., and is used as a beverage colorant (as Chica Morada) in Latin America, especially in Peru. PCC has been shown to inhibit azoxymethane-induced colon tumors in rats.[4] PCC contains six anthocyanins; the major anthocyanin found in PCC is cyanidin 3-*O*-β-D-glucoside (C3-G). *In vitro*, C3-G reacts with peroxyradicals and is converted into the oxidation products 4,6-dihydroxy-2-*O*-β-D-glucosyl-3-oxo-2,3-dihydrobenzofuran and protocatechuic acid (PC); PC is also a radical scavanger.[10] Thus, after C3-G reacts with biological radicals, a second radical scavenger is produced. C3-G also gives rise to PC *in vivo*: after oral administration of C3-G, both C3-G itself and PC are found in the plasma.[11] The plasma C3-G concentration reaches a maximum at 30 min after single oral administration. The half life of plasma C3-G is about 2 h.[11] Therefore, C3-G in the diet is expected to improve the body's antioxidant capability and inhibit carcinogenesis *in vivo*.

We have established a rat line carrying copies of the human c-Ha-*ras* proto-oncogene under the regulation of its own promoter region (Hras128). This line is highly susceptible to *N*-methyl-*N*-nitrosourea (MNU)- and DMBA-induced mammary carcinogenesis.[12–14] Tumors develop in almost all females within as short a period as 8–12 weeks after a single MNU or DMBA treatment. The animals have also been found to be susceptible to *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine-induced urinary bladder,[15] DMBA-induced skin,[16] and 4-nitroquinoline 1-oxide-induced tongue[17] carcinogenesis. This model can be used for the short-term assay of test compounds including chemopreventive compounds,[18] genotoxic compounds[19] and non-genotoxic promoting agents.[20] In the present study, this short-term tumor model was employed to investigate the effects of PCC on DMBA mammary carcinogenesis. Non-transgenic rats were then used to confirm the effects that PCC had on transgenic rats. PCC inhibited mammary carcinogenesis in both transgenic and non-transgenic rats.

Recently, we established cell lines from mammary carcinomas induced by DMBA in Hras128 rats.[21] These cells can be utilized for mechanistic analysis of compounds showing a modifying influence on mammary carcinogenesis in Hras128 rats. Accordingly, we used the cell lines for the analysis of the mechanism by which PCC inhibited carcinogenesis.

Materials and Methods

Animals. Female c-Ha-*ras* transgenic (Hras128, Tg) and non-transgenic (non-Tg) rats were bred by CLEA Japan, Tokyo, Japan. They were maintained in plastic cages in an air-conditioned room with a 12-h light/12-h dark cycle. In total, 62 Tg and non-Tg rats received a single dose of DMBA (Tokyo Chemical Industry, Tokyo, Japan) (25 mg/kg body weight) by gavage at 7 weeks of age. One day thereafter, they were placed on powdered basal diet MF (Oriental Yeast, Tokyo, Japan) containing either purple corn color (PCC) (San-Ei Gen F.F.I., Osaka, Japan) or no supplement. PCC was prepared as described previously.[4] The specifications of PCC used in this study were as follows: trade name San RED No.5, lot No. 040421, purity 33.7% as anthocyanin concentration. Gross observation and palpation of the mammary gland were regularly performed to monitor the development of mammary tumors after DMBA

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Abbreviations: DMBA, 7,12-dimethylbenz[*a*]anthracene; PCC, purple corn color; C3-G, cyanidin 3-*O*-β-D-glucoside; PC, protocatechuic acid.

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treatment. The surviving animals were killed by exsanguination under deep ether anesthesia at the end of week 8 for Tg and week 22 for non-Tg rats. The numbers of visible tumors were recorded before they were measured and sampled for histological examination. Values are expressed as average tumor weight of the total tumors for each rat. Body and liver and kidney weights were also recorded. The experiments were conducted according to the 'Guidelines for Animal Experiments of the Nagoya City University Graduate School of Medical Sciences'.

Cell culture. Rat mammary carcinoma cells (C3, C11 and C17),[21] which were established from DMBA-induced tumors in Hras128 rats, were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS). Cells were seeded in 96-well plates, and then incubated with PCC, C3-G (Extrasynthese, Genay Cedex, France) or PC (Wako Pure Chemicals, Osaka, Japan) for 1 day. Cell viability was measured using a CellTiter-Glo Luminescent Cell Viability Assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

In some experiments, the cells were grown to confluency, then rendered quiescent by incubation in DMEM containing 0.5% FCS. After serum starvation for 48 h, the cells were treated with 10% FCS to initiate signaling cascades.

Western blot. Cells or tissues were lyzed in a minimal volume of lysis buffer (50 mM Tris-HCl [pH 7.4], 1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM Na_3VO_4, 1 mM NaF and protease inhibitors [protease inhibitor cocktail, Sigma, Saint Louis, MO, USA]). Total proteins were resolved by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Billerica, MA, USA). The blots were incubated with primary antibodies after blocking with 3% non-fat milk. The blots were then washed and incubated with horseradish peroxidase-conjugated antirabbit and antimouse immunoglobulin G (IgG) antibodies (Southern Biotechnology Associates, Birmingham, AL, USA). The bound antibodies were detected using enhanced chemiluminescence (ECL) plus Western blotting detection system (GE Healthcare Bio-sciences, Piscataway, NJ, USA). Detection of activated Ras protein was performed using a Ras activation assay kit (Upstate, Lake Placid, NY, USA) as described previously.[22,23]

The following antibodies were used: pan-Ras (1/4000, clone RAS10; Upstate), extracellularsignal- regulated kinase (Erk) 1/2 (1/50 000, 06–182; Upstate), phospho-ERK 1/2 (1/1000, #9106; Cell Signaling Technology, Danvers, MA, USA), Akt (1/1000, #9272; Cell Signaling Technology), phospho-Akt (Ser473) (1/1000, #4051; Cell Signaling Technology), caspase-3 (1/1000, #9662; Cell Signaling Technology), cleaved caspase-3 (1/1000, #9661; Cell Signaling Technology), caspase-8 (1/1000, H-134; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and β-actin (1/10 000, A5441; Sigma, Saint Louis, MO, USA).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) of apoptotic cells. Apoptotic cells were detected by TUNEL assay using an *In situ* Apoptosis Detection Kit (Takara Bio, Otsu, Japan) according to the manufacture's instructions. Fluorescent nuclear staining was performed with TO-PRO-3 iodide (Molecular Probes, Eugene, OR) at a dilution of 1/1000. A confocal microscope FLUOVIEW FV300 (Olympus, Tokyo) was utilized for imaging.

Real-time polymerase chain reaction (RT-PCR). Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA), and the RNA was reverse-transcribed using Superscript III Reverse Transcriptase with Random primers (Invitrogen) according to the manufacturer's instructions. PCR amplification was carried out using SYBR *Premix Ex Taq* and the Smart Cycler II System (Takara Bio, Otsu, Japan) according to the manufacturer's instructions. All PCR amplifications were done for 40 cycles and a melt curve analysis was used to examine the specificity of the amplified products. The following primers were used: H*ras*, 5′-CAGTACAGGGAGCAGATCAA-3′ and 5′-AGCACACACTTGCAGCTCAT-3′; K*ras*, 5′-GCGTAGGCAAG AGTGCCTTGA-3′ and 5′-GACCTGCTGTGTCGAGAATATCCA-3′; N*ras*, 5′-AGCAGTGAGGATGGCACTCAAG-3′ and 5′-GATGTC AGAACCAGGGCATCAG-3′; β-*actin*, 5′-CCGTAAAGACCT CTATGCCAACA-3′ and 5′-CGGACTCATCGTACTCCTGCTT-3′.

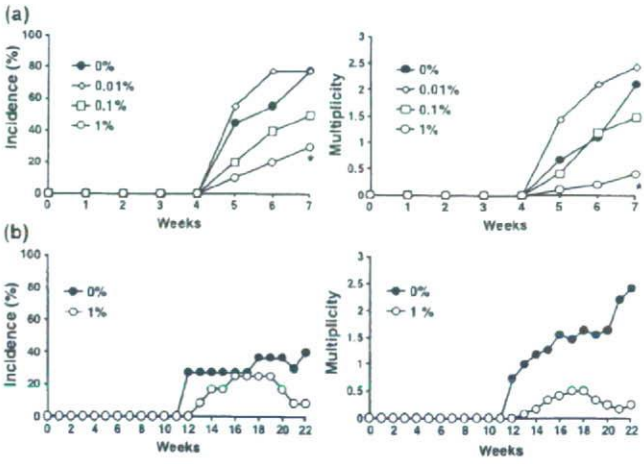

Fig. 1. Periodic observation of palpable mammary tumors after 7,12-dimethylbenz[a]anthracene (DMBA) treatment in female (a) transgenic (Tg) and (b) non-Tg rats fed purple corn color. The *x*-axis indicates weeks after DMBA treatment. $^*P < 0.05$ as compared with the 0% group.

Statistics. All the average values are expressed as the means ± SD. Analysis was performed using the JMP software package (SAS Institute, Cary, NC, USA). Fisher's exact test was conducted for tumor incidence data. Dunnett's *t*-test was conducted for body and organ weight. Mann–Whitney tests were conducted for tumor weight data and multiplicity data.

Results

General observations in the animal experiments. Modifying effects of PCC on DMBA-induced mammary carcinogenesis in female Tg and non-Tg rats were examined. Two rats died in the non-Tg 0% group before the experiment was terminated. There were no consistent significant differences noted in the intake of food or the weights of body, liver or kidneys (data not shown). Coloring of black feces, considered to be related to PCC treatment, was noted in rats fed 1% PCC.

Effects of PCC on mammary tumor induction. In the Tg rats, palpable mammary tumors were first observed at 5 weeks after administration of DMBA. After 5 weeks, the incidence increased rapidly. Tumor incidence and multiplicity increased with time, but was suppressed in a dose-dependent manner in animals fed PCC (Fig. 1a). Final mammary tumor incidences and multiplicity data determined by histological examination are summarized in Table 1. Most of the mammary tumors were diagnosed as adenocarcinomas. The weight of the mammary tumors was significantly lower in the 1% group compared with the 0% group ($P < 0.05$). PCC significantly decreased the incidence of middle-sized (0.5–2.0 g) mammary tumors in Tg rats ($P < 0.05$). It was not statistically significant, but the number of large-sized (>2.0 g) mammary tumors was also decreased by PCC. On the other hand, the number and incidence of smaller-sized (<0.5 g) mammary tumors was not suppressed by PCC, indicating that PCC is not able to inhibit the emergence of mammary tumors in Tg rats.

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Table 1. Inhibitory effects of purple corn color on mammary tumor induction

	Dose (%)	No. of rats	Tumor incidence and multiplicity								Weight of tumor (g)
			~0.5 g†		0.5 g ~2 g†		2 g~†		Total		
			Incidence (%)	No./rat	Incidence (%)	No./rat	Incidence (%)	No./rat	Incidence (%)	No./rat	
Tg	0	9	8 (88.9)	3.4 ± 2.1	8 (88.9)	1.8 ± 1.7	4 (44.4)	1.0 ± 1.4	8 (88.9)	6.2 ± 4.2	1.01 ± 1.34
	0.01	9	9 (100)	5.3 ± 4.4	8 (88.9)	2.9 ± 2.4	6 (66.7)	1.2 ± 1.1	9 (100)	9.4 ± 5.5	0.99 ± 1.52
	0.1	10	6 (60.0)	3.1 ± 4.0	6 (60.0)	1.9 ± 3.1	4 (40.0)	0.8 ± 1.1	8 (80.0)	5.4 ± 5.4	1.06 ± 1.68
	1	10	10 (100)	3.9 ± 3.5	2 (20.0)**	0.6 ± 1.3	3 (30.0)	0.4 ± 0.7	10 (100)	4.9 ± 4.6	0.69 ± 1.64*
non-Tg	0	10	6 (60.0)	4.1 ± 8.6	4 (40.0)	2.6 ± 5.1	3 (30.0)	0.9 ± 1.9	7 (70.0)	7.6 ± 15.3	0.90 ± 1.17
	1	12	2 (16.7)	1.0 ± 2.7	1 (8.3)	0.16 ± 0.58	0 (0)	0	2 (16.7)*	1.2 ± 3.2*	0.24 ± 0.31***

*$P < 0.05$, **$P < 0.01$, ***$P < 0.005$, as compared with 0% group; †tumor weight. Tg, transgenic; non-Tg, non-transgenic.

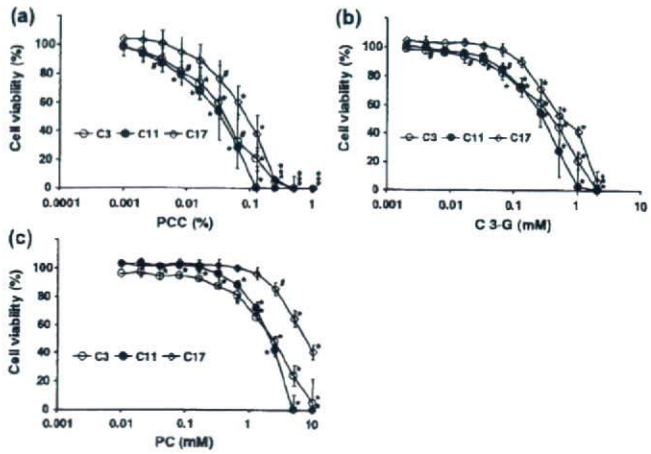

Fig. 2. Cytotoxicity induced by purple corn color (PCC) in mammary tumor cells. Rat mammary tumor cells (C3, C11 and C17) were established from 7,12-dimethylbenz[a]anthracene (DMBA)-induced tumors in a transgenic (Tg) rat. These three cell lines have mutated human Ha-*ras* but not rat Ha-*ras*. Cells were incubated with each compound for 2 days. Dose dependent inhibition of cell proliferation by (a) PCC, (b) cyanidin 3-*O*-β-D-glucoside (C3-G) and (c) protocatechuic acid (PC). #$P < 0.05$, * $P < 0.01$ as compared with no treatment cells.

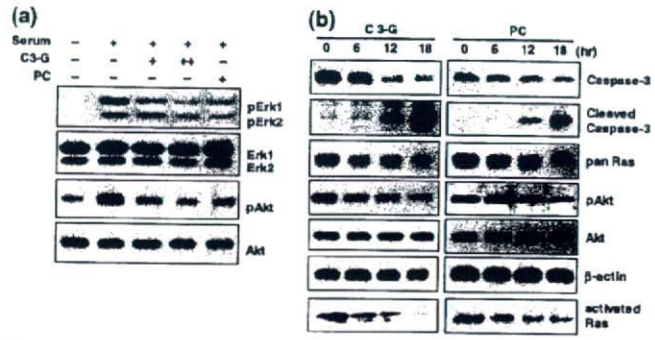

Fig. 3. Cyanidin 3-*O*-β-D-glucoside (C3-G) and protocatechuic acid (PC) inhibit Ras signaling and induce caspase-3 activation. (a) Effect of C3-G and PC on the activation of Erk1/2 and Akt in mammary tumor cells. C11 cells were serum starved for 48 h (0.5% FCS). The cells were incubated in the absence or presence of C3-G (+; 0.25 mM, ++; 0.5 mM) or PC (5 mM) in 0.5% FCS for 24 h prior to serum stimulation. After incubation in media with 10% FCS for 30 min, proteins were extracted. (b) C3-G and PC induced caspase-3 activation by degradation of Ras protein in mammary tumor cells. C11 cells were treated by C3-G (0.5 mM) or PC (5.0 mM) for the indicated time. The protein levels were assessed by Western blot. Activated Ras was precipitated by Raf-1 agarose and detected by antipan-Ras antibody.

Since PCC inhibited mammary carcinogenesis in Tg rats, we tested the inhibitory effect of 1% PCC on mammary carcinogenesis in non-Tg female rats. In non-Tg rats, palpable mammary tumors were first observed at week 12 and the incidence increased with time. PCC reduced mammary tumor incidence and multiplicity (Fig. 1b). Surprisingly, some palpable tumors regressed and became undetectable in rats fed 1% PCC. This suggests that PCC exhibits chemopreventive and chemotherapeutic activity. In non-Tg rats, the incidence and weight of total mammary tumors (Table 1) was significantly lower in the 1% group compared with the 0% group ($P < 0.05$ and $P < 0.005$ respectively). Importantly, not only was the overall number of mammary tumors decreased by PCC in non-Tg rats, but there was complete suppression of the formation of large tumors (2.0 g <) in the 1% PCC group. Most of the mammary tumors in the control group and all of the mammary tumors in the 1% PCC group were diagnosed as adenocarcinomas in non-Tg rats. There was no histological difference between the 0% and the 1% PCC group.

C3-G, and PC inhibit Ras signaling and reduce cell viability in mammary cancer cells. We examined the effects of PCC on the viability of rat mammary cancer cells. Data for the effects of PCC, C3-G, and PC on the viability of mammary cancer cell lines C3, C11, and C17 are summarized in Fig. 2. PCC, C3-G and PC decreased the viability of the cells dose dependently. PCC, C3-G and PC caused a 50% reduction in cell viability at concentrations of 0.051%, 0.395 mM, and 4.37 mM respectively. On the basis of these results, a concentration of 0.5 mM for C3-G and 5 mM for PC were selected for further analysis.

To examine possible effects of C3-G and PC on the Ras signaling pathway, Ras protein levels and phosphorylation of Erk and Akt were examined. Total Ras and activated Ras protein levels were decreased by treatment with C3-G or PC (Fig. 3b). Serum stimulation of serum-starved C11 cells led to activation of Erk and Akt after 30 min. When the cells were pretreated with C3-G or PC for 1 day before serum stimulation, activation of Erk and Akt by growth stimuli was suppressed (Fig. 3a). Although C3-G and PC reduce Ras protein levels and suppress Ras signaling, the gene expression level of *ras* was not changed by treatment with C3-G or PC (data not shown). This suggests that C3-G and PC reduce Ras protein levels by a post-transcriptional mechanism.

To determine whether C3-G and PC induce apoptosis, C11 cells were treated with C3-G or PC for 6, 12 and 18 h, and apoptotic cells were detected using TUNEL. TUNEL staining showed that C3-G and PC induced apoptosis in these mammary tumor cells (Fig. 4).

Caspase-3 is a key protease associated with DNA fragmentation and apoptosis. C3-G and PC induced activation of caspase-3 after

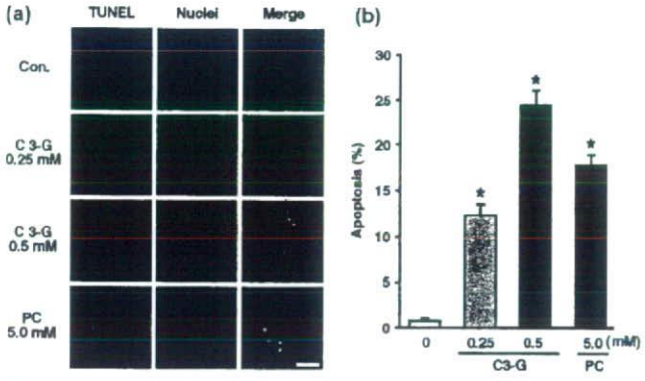

Fig. 4. Cyanidin 3-*O*-β-D-glucoside (C3-G) or protocatechuic acid (PC) induced apoptosis in mammary tumor cells. (a) Terminal deoxynucleotidyl transferase dUTP nick end (TUNEL) staining of mammary tumor cells treated with C3-G or PC. Bar = 50 μm. (b) The percentage of apoptotic cells was determined by counting TUNEL-positive cells from at least 3 fields. $*P < 0.01$ as compared with control.

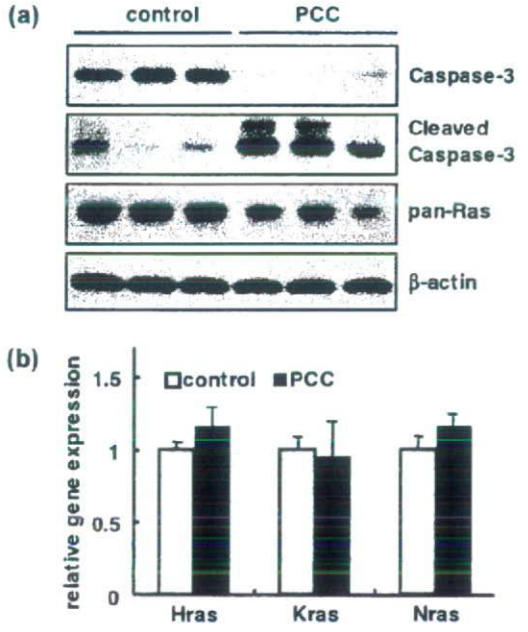

Fig. 5. Purple corn color (PCC) induced degradation of Ras protein in mammary tumors *in vivo*. Non-transgenic (Tg) rats were fed 0 or 1% PCC for 22 week after receiving 7,12-dimethylbenz[a]anthracene (DMBA). The tumors were removed, and proteins and RNA were extracted. (a) Western blot analysis: PCC reduced Ras protein level and activated caspase-3 in mammary tumors *in vivo*. (b) Quantitative real-time PCR analysis: The expression level of *ras* genes, H*ras* (the endogenous gene and the transgene), K*ras* and N*ras*, was not significantly changed in mammary tumors by PCC.

12 h (Fig. 3b). The level of cleaved caspase-3 correlated well with suppression of Ras protein expression and suppression of phosphorylation of Akt. C3-G and PC did not induce activation of caspase 8 (data not shown). This suggests that the TNFα/Fas signaling pathway is dispensable for C3-G and PC apoptotic activity.

PCC inhibits Ras and activates caspase-3 *in vivo*. In determining the true biological significance of a novel pathway, it is always important to confirm *in vitro* results in an *in vivo* context. We evaluated the effect of PCC on the stability of the Ras protein and activation of caspase-3 *in vivo*. As shown in Fig. 5a, PCC suppressed the level of Ras protein and induced the activation of caspase-3 in non-Tg rats. In line with *in vitro* data, total H*ras* (the endogenous gene and the transgene), K*ras* and N*ras* mRNA level was not changed by PCC (Fig. 5b).

Discussion

In the present study, we showed that PCC inhibits mammary tumor development in both cancer prone transgenic rats and their non-transgenic counter parts, and that this inhibition was clearly in line with induction of apoptosis in mammary cancer cell lines derived from the transgenic rats. A previous study reported that 5.0% PCC in the diet did not show any evidence of adverse effects.[4,24] Cyanidin 3-*O*-β-D-glucoside (C3-G) is the major anthocyanin in PCC. C3-G treatment decreased the number of skin tumors induced by 12-0-tetradecanoylphorbol 13-acetate (TPA) in DMBA-initiated mouse skin.[25] C3-G also reduced the size of A549 tumor xenograft growth and significantly inhibited metastasis in nude mice.[25] C3-G is converted to protocatechuic acid (PC) *in vivo*,[11] and PC possesses chemopreventive activities on liver, colon, oral and urinary bladder carcinogenesis.[26–30] Consistent with these observations, PCC inhibited development of mammary tumors in Tg and non-Tg rats.

We also demonstrated that Ras protein levels were decreased by treatment with PCC, C3-G and PC. One mechanism by which this could occur is via reduction of reactive oxygen species (ROS) levels. High ROS levels in conjunction with activation of ERK1/2 stabilize Ha-Ras protein by inhibiting proteasome degradation,[31] and anthocyanins show strong free radical scavenging and antioxidant activities.[32–34]

Down-regulation of Ras levels protects primary cells from inappropriate growth factor signaling, which may result in DNA damage, oxidative stress, and ultimately in apoptosis. However, Ras can activate seemingly contradictory intracellular pathways and in certain conditions Ras has antiapoptotic effects.[35–38] ROS generation is common in cancer cells experiencing oncogenic stimulation by factors such as Ras and Myc,[39,40] and, as noted above, high ROS levels stabilize the Ha-Ras protein. Therefore, PCC, C3-G, and PC might induce apoptosis in cancerous cells by decreasing Ras via reducing ROS levels.

Interestingly, it has been shown that Ha-Ras proteins are modified by mono- and diubiquitination which targets them to endosomes,[41] however, there are no reports of Ras polyubiquitination, which would target Ras proteins for proteasomal degradation. Therefore, it is likely that the effect of PCC on Ras protein expression is mediated through the proteasomal degradation of a factor that affects the stability of Ras.

Ras is found mutated in 30% of all tumors. Pancreatic cancer is the tumor type with the highest incidence of Ras mutations (90%), followed by colon (40%), thyroid (50%), and lung adenocarcinomas (30%).[42] Ras signaling to the PI3 kinase–Akt pathway is an important contributor to tumor cell survival. Aberrant Ras activation can occur as the result of several different cellular abnormalities, not only from mutation of the *ras* gene itself, but, for example, overexpression of the epidermal growth factor receptor. Amplification and/or overexpression of proto-oncogenes such as H-, K-, and N-ras, and neu/erbB-2/HER-2 are frequent events in mammary malignancies of humans,[43,44] and mice.[45] The level of active Erk is clearly elevated in the terminal end buds in the mammary glands of Hras128 transgenic rats.[46] Altogether, it is believed that 80% of all tumors have aberrant Ras pathway activation.[47]

Previous studies have shown that anthocyanidins inhibit TPA-induced Erk, AP-1 activation, and cell transformation.[48] Another flavonoid, silymarin, inhibited both ligand-induced and constitutive activation of erythroblastosis B1/epidermal growth factor receptor(erbB1) [Correction added 1 August 2008: in the preceding sentence erB1 has been corrected to erbB1] and its downstream signaling events.[49] These studies suggest that flavonids including anthocyanin have inhibitory effects on the Ras signaling

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cascade. Previous studies showed that treatment with limonene, an inhibitor of Ras protein isoprenylation, inhibited mammary tumor development,[50,51] and limonene is effective in preventing Ras-initiated mammary carcinomas. Therefore, inhibitors of the Ras signaling cascade might be good candidates for cancer preventive/therapeutic agents.

In the present study, we showed that the activation of Erk was inhibited by C3-G and PC. Our finding suggests that PCC induces apoptosis in mammary tumors by decreasing Ras protein levels. Withdrawal of oncogenic Ras results in regression of tumors.[52] The initial stages of regression involved marked apoptosis in the tumor cells and the surrounding endothelial cells. Apoptosis has been reported to play an important role in elimination of seriously damaged cells and tumor cells by chemopreventive agents.[53] Therefore, apoptosis-inducing agents are expected to be ideal anticancer drugs. Consequently, PCC and it constituent anthocyanidin C3-G are promising candidate chemopreventive and chemotherapeutic agents for cancers that have abnormally high Ras activity.

In summary, dietary administration of PCC significantly suppressed the development of DMBA-induced rat mammary adenocarcinomas. Such cancer protective effects mediated by PCC most likely relate to the modulation of cell proliferation and apoptosis in the mammary neoplastic lesions by reducing Ras protein levels. Furthermore, our *in vivo–in vitro* system, which utilizes Hras128 transgenic rats, non-transgenic rats and mutant Ras-expressing mammary cancer cell lines, can be used for screening for chemopreventive agents that act via suppressing the Ras signaling pathway.

Acknowledgments

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RESEARCH COMMUNICATION

Lack of Chronic Oral Toxicity of Chemopreventive Bovine Lactoferrin in F344/DuCrj Rats

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Abstract

Studies were undertaken to determine whether bovine lactoferrin (bLF) and related compounds, shown to prevent carcinogenesis in the colon and other organs in rats, have any toxic effects in long-term feeding studies. In experiment I, male F344/DuCrj rats received a basal diet containing 0.2% bLF for 40 weeks. No adverse findings were noted, furthermore, serum triglyceride level was significantly decreased to 72% of the control level, suggesting preventive effects against the metabolic syndrome. In experiment II, male and female F344/DuCrj rats were fed a basal diet containing 0.02, 0.2, 2.0 and 5.0% bLF, 2.0% bLF hydrolysate (bLF-H) or 0.1% lactoferricin (LFcin), a peptide derived from bLF, for 60 weeks in males and 65 weeks in females. No toxicological effects, including carcinogenicity, were evident in either sex. The results of the studies provide subjective support for safety of clinical studies of bLF for supplement use.

Key Words: Chemopreventive agent - bovine lactoferrin - *in vivo* toxicity - F344/DuCrj rat

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Introduction

Lactoferrin is a multi-functional iron-binding glycoprotein, which is present at high concentration in mammalian colostrums, as well as neutrophilic leukocytes (Masson et al., 1969), which release the compound in response to inflammatory stimuli (Lash et al., 1983). The primary biological function of lactoferrin is related to its anti-bacterial, anti-viral, anti-fungal and immune-modulating effects (Bullen, 1975; Arnold et al., 1980; Brock, 1995; Lönnerdal and Iyer, 1995). Furthermore, lactoferricin (LFcin), a pepsin digested peptide of lactoferrin, has been shown to possess potent and wide-spectrum anti-microbial effects (Tomita et al., 1991; Bellamy et al., 1992), as confirmed by extensive analyses of the mechanisms (Wakabayashi et al., 2003). Recently, bovine lactoferrin (bLF) was shown to exert cancer preventive effects in various organs of rodents including the colon, lung and esophagus (Sekine et al., 1997; Tsuda, et al., 2000b, 2004). Furthermore, bLF was also demonstrated to have anti-metastatic effects in mice (Iigo et al., 1999; Tsuda et al., 2000a).

However, for application of bLF in human trials of its ability to prevent carcinogenesis and tumor metastasis with long-term ingestion, it is obviously necessary to conduct chronic feeding studies to detect any adverse effects. Prior to the current studies, acute and subchronic exposure indicated no obvious toxicological effects after 4-weeks and 13-weeks feeding with doses up to 2,000 mg/kg/day (Yamauchi et al., 2000a). Lack of mutagenicity was also reported (Yamauchi et al., 2000b). However, chronic toxicology studies have hitherto not been performed, promoting the present studies of long-term toxic effects of dietary bLF in F344 rats of both sexes.

Materials and Methods

Test Chemical

Bovine lactoferrin (bLF), lactofferin hydrolysate (bLF-H), generated by acid-pepsin hydrolysis (Tomita et al., 1991), and lactoferricin (LFcin), identified as an antimicrobial peptide derived by pepsin digestion of lactoferrin (Bellamy et al., 1992)(Morinaga Milk Industry Co., Ltd., Zama, Japan) were used.

Diet preparation and analysis

The compounds were mixed at the designated levels into powdered basal diet MF (Oriental Yeast Co., Ltd., Tokyo, Japan) after being previously confirmed to be stable in diet for 3 months when stored in a cold room controlled at the Food Science and Technology Institute, Morinaga Milk Industry Co., Ltd.. Therefore, the diets were prepared at intervals of 3 months and stored in a cold room. Amounts of bLF in the diet preparations were

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within ± 5% of the target concentrations. Homogeneity was confirmed as satisfactory.

Animals and their maintenance

Male and female F344/DuCrj (F344) rats, 5 weeks of age, were purchased from Charles River Japan (Atsugi, Japan) and allowed a 7 days quarantine and acclimation period. After confirmation of normal health status, they were used for the studies.

Animals of the same sex were housed two or three to a polycarbonate cage, with wood chip (Oriental Yeast Co., Ltd., Tokyo, Japan) for bedding. They were placed on powdered MF basal diet (Oriental Yeast Co., Ltd., Tokyo, Japan) with or without test compounds and tap water ad libitum. The room temperature and relative humidity were controlled at 21~25°C and 50~60%, respectively, and the room air was changed 15 or more times per hour. Fluorescent tube lighting was employed to provide a 12-hr light/dark cycle.

Experimental procedures

Experiment I: Starting at 6 weeks of age, groups of 15 male rats were given diet containing 0% (control) or 0.2% of bLF for 40 weeks. Diet and drinking water were available ad libitum. The animals were observed for general conditions every day and weighed once weekly for the initial 4 weeks and once every 4 weeks thereafter. Determination of food consumption by cage was performed at the same time as body weight measurement. Test material intake (mg/kg body weight/day) was calculated for each group from mean food consumption and body weight data and the nominal dietary levels.

At the end of the treatment, all animals were fasted overnight and then killed in the next morning under deep ether anesthesia. Whole blood samples were collected from the all rats via the inferior vena cava and blood biochemistry determinations were performed with an Automatic Analyzer Model 7070 (Hitachi Co., Ltd., Tokyo, Japan). Parameters were aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyltranspeptidase (γ-GTP), alkaline phosphatase (ALP), blood urea nitrogen (BUN), creatinine (CRE), glucose (GLU), total cholesterol (T-CHO), trigryceride (TG), total protein (TP), albumin (ALB), serum iron. Gross inspections for any lesions were made at autopsy and the findings recorded. The liver, kidneys and spleen were weighed and the organ-to body weight ratios were determined.

Experiment II: A total of 100 F344 rats each sexes were used. Starting at 17 (in males) or 11 (in females) weeks of age, groups of 25 rats (groups 1 and 5) and 10 rats (groups from 2 to 4, and 6 and 7) of each sex were given powdered diet as in Experiment I containing 0% (control), 0.02, 0.2, 2.0 and 5.0% bLF, 2.0% bLF-H or 0.1% bLFcin for 60 weeks in males and 65 weeks in females. The animals were observed for general conditions every day and weighed 8 times during the experiment. Measurement of food consumption and water intake by cage were performed once every 2 weeks for the first 16 weeks and once every 4 weeks thereafter. Test material intake (mg/kg body weight/day) was calculated for each group from mean food consumption and the nominal dietary levels. Careful gross examinations were made at autopsy. The following organs, the liver, kidney, spleen, adrenal and pituitary were weighed for each animal. Samples of these organs, thymus, lungs (including trachea), salivary glands, esophagus, stomach, duodenum, jejunum, ileum, cecum, pancreas, urinary bladder testes, prostate, seminal vesicle, ovaries (including oviducts), uterus, vagina, spinal cord and grossly visible lesions were fixed in 10% buffered formalin solution. Tissues were routinely processed for histopathological examination.

Statistical analyses

For body weight, blood biochemistry and organ weight data, the significance of inter group differences was assessed using the Bartlett's test (Bartlett, 1937). If homogeneous, the data were analyzed with the Dunnett's multiple comparison test (Dunnett, 1955) and if not then with the Steel's test (Steel, 1959). For the incidences of histopathological lesions, the significance of differences observed between the control and treated groups was evaluated with the Fisher's exact probability test (Fisher, 1955). The Mann-Whitney U test was employed for comparison of degrees of change (Gad and Weil, 1989). The levels of significance were set at $P<0.05$ and $P<0.01$.

Results

Experiment I

Neither clinical signs related to bLF treatment nor deaths were observed throughout the 40 weeks of the study. No alteration in body weights related to bLF treatment was noted (data not shown). However slight, but significantly decreased relative liver weights (but not absolute weights) were noted in the 0.2% bLF treated animals (data not shown). No treatment-related macroscopic changes were observed in the bLF fed animals (data not shown). Selected blood biochemistry data are given in Table 1. AST, ALT, ALP, BUN and TG were significantly lowered in the 0.2% bLF group. No treatment-related histopathological lesions were observed.

Experiment II

Neither clinical signs related to bLF treatment nor deaths were observed throughout the study period. No significant alteration in body weights related to bLF treatment was noted. Average food consumption values were comparable to the targeted doses of test compound in both sexes, exhibiting dose-dependent increase in total bLF intake. Average water intake values in bLF-treated groups were not different from controls in either sex. There were no significant differences in final body, liver, kidneys, spleen, adrenal and pituitary weights between treated groups and control groups in either sex. No treatment-related macroscopic changes were found (data not shown).

No treatment-related histopathological changes were observed in either sex of the treated and control groups. All incidences of histopathological alterations observed in the present study were within the ranges for spontaneously occurring lesions in F344 rats (Goodman et al., 1979; Haseman et al., 1990).