

Promoting Effects of Milk on the Development of 7,12-dimethylbenz(a)anthracene (DMBA)-induced Mammary Tumors in Rats

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To assess the effect of milk on the development of 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary tumors, 48 female Sprague-Dawley rats treated with DMBA were divided into 3 groups and given 1 of 3 test solutions for 20 weeks as their drinking liquid: milk, estrone sulfate solution or tap water. The milk group showed a significantly great incidence (75%) in tumor development compared with the water group (38%) and was comparable to the estrone sulfate group (69%). Mean tumor number per rat in the milk group was significantly higher than that in the water group ($p=0.009$). We classified the mammary tumors into three histological types: intraductal papilloma, fibroadenoma, and adenocarcinoma. Although the percent of intraductal papilloma and fibroadenoma was almost same among the three groups, malignant tumor was found only in the milk and estrone sulfate groups. In conclusion, our results indicate that milk as well as estrone sulfate promotes the development of DMBA-induced mammary tumors in rat and could be associated with the occurrence of adenocarcinoma.

Key words: mammary tumor, milk, smooth muscle actin, proliferating cell nuclear antigen, estrogen receptor

I. Introduction

Breast cancer is the most common malignant neoplasm, and is the number one cause of cancer-related deaths in nonsmoking women [3]. The incidence of breast cancer has been increasing in both developed and developing countries [4]. Although epidemiological studies have identified various risk factors, including obesity, menarche at young age, and late menopause, these known risk factors seem to account for only a third of the breast cancer cases [10, 23]. Recently, an epidemiological analysis revealed that the incidence of breast cancer in 42 countries is highly correlated ($r=0.79$) with the consumption of milk and dairy products

[22]. The positive association between milk and breast cancer has been proved by some case-control studies and cohort studies [18, 26].

Recently, Qin *et al.* reported that low-fat milk promoted the development of 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary tumors in rats [17]. But they did not thoroughly observe their natural history and morphology, or perform pathological classification for experimental tumors to assess the effects of milk on morphology and pathology of tumors. It is possible to evaluate tumorigenic response by palpable tumors, but the true nature of palpable lesions can be determined only through histological examination [21]. Moreover, the classification of tumors is very useful for the further interpretation of experimental data [21]. In the present study, we performed haematoxylin-eosin staining (HE) and carefully implemented pathological classification for all experimental tumors. Since the presence of myoepithelial cells is commonly regarded as the hallmark of benignity for human tumors [2, 14] and proliferating cell nuclear antigen (PCNA) is sensi-

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tive enough to differentiate between benign tumors and malignant tumors [20], we also performed smooth muscle actin (SMA) and PCNA immunohistochemistry staining to identify the differences among different histological types of rat mammary tumors. In addition, all tumors were evaluated for estrogen receptor α (ER α) by immunohistochemistry.

II. Material and Methods

Animal experimental procedure and care of laboratory animals followed the Guidelines for Animal Experiments of Peking University. After one week of acclimation to commercial powder chow and water, all of the 48 6-week old female Sprague-Dawley (SD) rats (Beijing Vital River Laboratory Animal Company, Beijing, China) received one intragastric intubation of 5 mg DMBA (Sigma-Aldrich, St. Louis, MO) dissolved in 0.5 ml corn oil. Twenty-four hours after DMBA administration, rats were assigned at random into 3 groups of 16 animals each and given 1 of 3 test solutions: milk, estrone sulfate solution or tap water for 20 weeks. The milk was purchased everyday at local supermarket. Estrone sulfate (Sigma-Aldrich) was dissolved in tap water at a concentration of 100 ng/ml.

Food intake and body weight were recorded weekly. Rats were palpated weekly to monitor tumor development. During the 20th week after DMBA administration, complete autopsies were performed. All organs were examined for gross abnormalities. Visible mammary tumors were rapidly excised and weighed. To carry out histological examination, the tumor with the adjacent normal gland was fixed in 10% neutral buffered formalin and embedded in paraffin. Tissue sections were cut at 2 μ m and stained with HE.

For immunohistochemistry analysis, the following antibodies were used: mouse anti-alpha SMA monoclonal antibody (DAKO Corp., Carpinteria, CA, USA), mouse anti-PCNA antibody (clone, PC10; DAKO Corp.), or mouse anti-human ER α antibody (clone, ID5; DAKO Corp.). Anti-mouse and anti-rabbit, labeled polymer, horseradish peroxidase (DAKO Corp.) were applied to the sections as a second antibody.

For ER α staining, paraffin sections were cut one day prior to immunohistochemistry staining because the immunoreactivity of ER α in paraffin sections can diminish over time [9]. Procedures of immunohistochemistry staining for SMA, PCNA, and ER α have been described elsewhere [9, 12, 15]. Briefly, after removing paraffin wax in three changes of xylene and dehydration in a series of graded

ethanol, sections were autoclaved at 121°C for 10 min in citrate buffer (pH 6.0) for antigen retrieval. Endogenous peroxidase was blocked by immersing the sections in 3% H₂O₂ for 10 min. Sections were incubated for 1 hr at room temperature with the primary antibody. Tissue sections were then reacted with the conjugated second-antibody, horseradish peroxidase, for 40 min at room temperature. Visualization of SMA, PCNA, and ER α was performed with diaminobenzidine for 3 min. After counterstaining with haematoxylin for 10 s, sections were dehydrated in a series of graded ethanol, cleared in 3 changes of xylene and coverslipped for light microscopic examination.

We performed histological diagnosis according to the classification described by Russo and Russo [21].

Statistical analysis

Fisher's exact probability test was used to compare the percentage of animals with tumors and tumor histology type in each group. Mean number of tumors per rat and PCNA labeling index among the three histological types were analyzed by one-way ANOVA followed by LSD multiple comparison tests. Statistical significance was set at $p < 0.05$. Data analyses were performed using SPSS version 11.0 for Windows (SPSS Inc., Tokyo, Japan).

III. Results

Prevalence of mammary tumors

During the period of observation, 48 rats developed 103 mammary tumors. Multiple tumors of different sizes were frequently found in the same rat. Results of mammary tumor development are shown in Table 1. The first tumors were detected at week 6 after DMBA administration in the milk and estrone sulfate groups. Palpable tumors in the water group did not appear until week 9 after DMBA administration. At week 20 after DMBA administration, tumor incidence was 75% (12/16) in milk group, 69% (11/16) in estrone sulfate group, and 38% (6/16) in water group. Milk group showed a significantly greater incidence in tumor development compared with the water group ($p = 0.037$). Tumor incidence of estrone sulfate group was also high but not significantly different from that of the water group. Tumor incidence of milk group is comparable to that of estrone sulfate group. More than one third as many tumors were seen in the milk group as in the water group, and rats in the estrone sulfate group developed almost twice as many tumors as did rats in the water group. No significant difference in number was observed between the

Table 1. Prevalence of DMBA-induced mammary tumors in three groups

Group	No. of rats	Tumor incidence of rats (%)	Total number of tumors	Mean tumor No./rat (mean \pm SE)	p
I	16	75	53	3.3 \pm 0.75	p=0.009
II	16	69	33	2.1 \pm 0.51	
III	16	38	17	1.0 \pm 0.42	

I, milk group; II, estrone sulfate group; III, water group; SE, standard error.

milk and estrone sulfate groups. At autopsy, the cumulative number of tumors in the milk, estrone sulfate, and water groups was 53, 33, and 17, respectively. Mean number of tumors per rat in the milk, estrone sulfate, and water groups was 3.3, 2.1, and 1.0, respectively. Mean tumor number per rat in milk group was significantly higher than that in the water group.

Histology of mammary tumors

Several histological types were recognized in mammary tumors induced by DMBA. According to histological findings, tumors were classified into 3 histological types: intraductal papilloma, fibroadenoma, and adenocarcinoma. Intraductal papilloma is the most common type of tumor in this experiment. Figure 1A shows a typical intraductal papilloma. The papillae containing both epithelial cells and myoepithelial cells are composed of a core of fibrovascular tissue lined by a single layer of cuboidal or low cylindrical epithelial cells. The epithelial cells are homogeneous in size and shape; they have leptochromatic nuclei, and the nucleolus is either absent or inconspicuous; some cellular atypia and mitoses may be present but scarce. Fibroadenoma is a benign tumor composed of mammary epithelium and numerous connective tissues. Ductal and lobular structures are surrounded with layers of fibrous tissue. As with normal rat mammary gland, the epithelium underlies the myoepithelium and basement membrane (Fig. 1B). Adenocarcinoma is characterized by the loss of the tubular-alveolar pattern. Solid sheets of neoplastic epithelial cells are interrupted by secondary lumina of round, oval or irregular shape and variable size, representing a cribriform pattern. No single metastasis was noted, while vascular local invasion can be seen sporadically in many tumors. Individual neoplastic cells are moderately to markedly pleomorphic, the degree of pleomorphism varies from tumor to tumor and even in different areas of the same tumor (Fig. 1C). As in human mammary carcinoma, the different types were not clearly defined and were found in different parts of the same tumor.

The results of histological classification are summarized in Table 2. Most of the tumors were diagnosed as intraductal papilloma or fibroadenoma. There was no significant difference in the percentages of histological types among the three groups. However, the milk group and estrone sulfate group developed adenocarcinoma but the water group did not.

Immunohistochemistry of SMA, ER α , and PCNA

Immunoperoxidase reaction with anti-alpha SMA antibody revealed elongated myoepithelial-like cells at the periphery of epithelial cell nests and glandular structures in all tumors. Appearance and number of myoepithelial cells showing positive for SMA varied among different histological types of rat breast tumors. Most of the epithelial cell nests in intraductal papilloma are lined by myoepithelial cells (Fig. 2A). Adenocarcinoma were also positive for SMA immunostaining and myoepithelial cells were

stretched and scattered at the base of the epithelial cell nests. Additionally, the number of myoepithelial cells in adenocarcinoma was apparently lower than that in intraductal

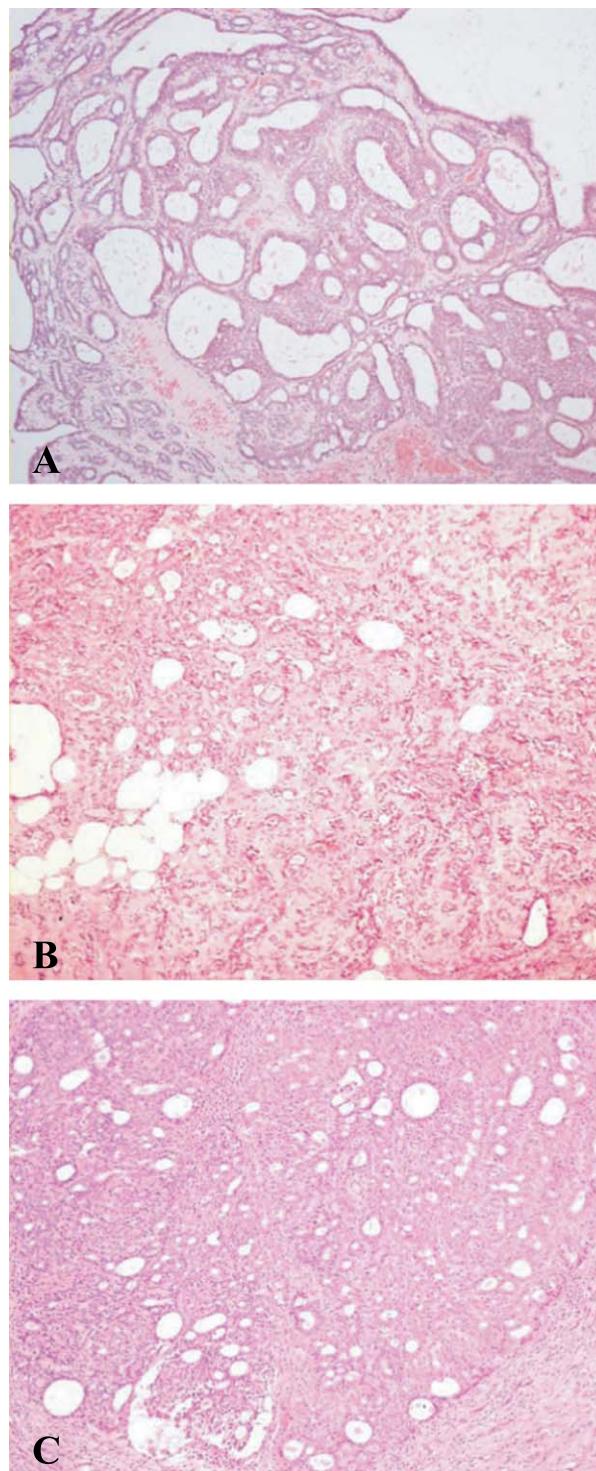
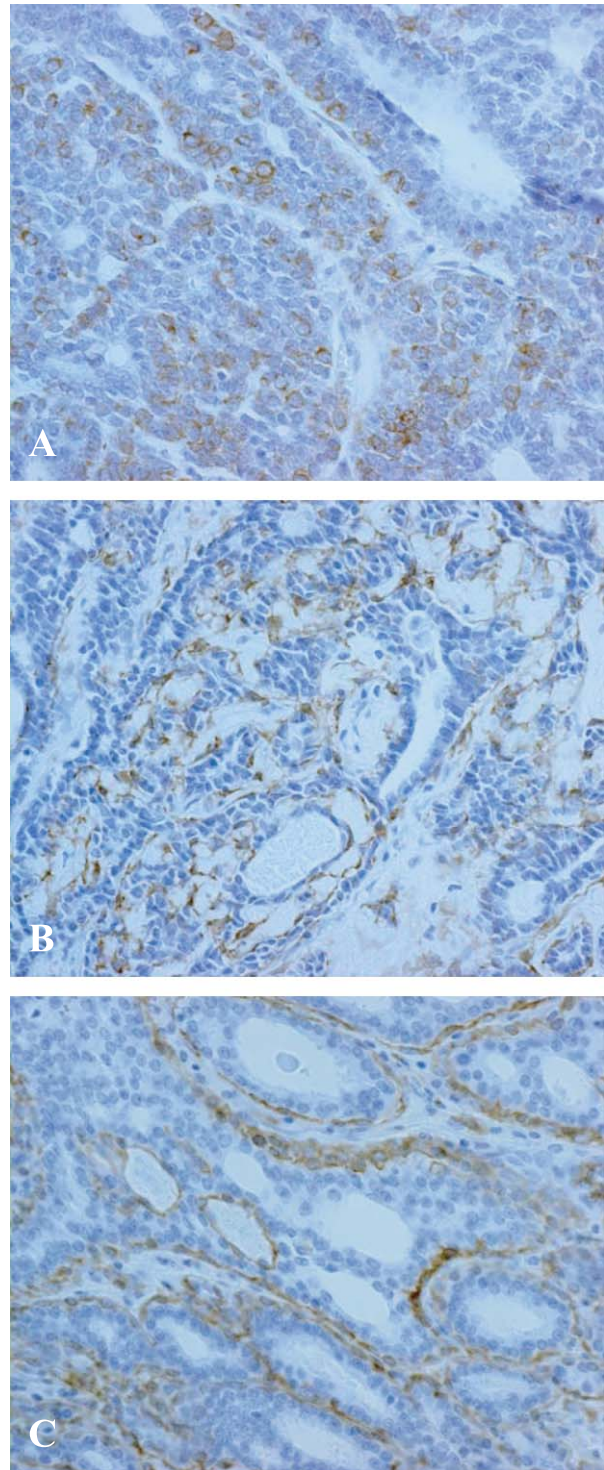


Fig. 1. HE staining of rat mammary tumor. **A)** Intraductal papilloma. Numerous papillary projections sustained by thin connective tissue cores can be seen. HE \times 25. **B)** Fibroadenoma. Fibrous stroma is compressing the tubular alveolar components. HE \times 25. **C)** Adenocarcinoma. Numerous secondary lumina can be seen. HE \times 25.

Fig. 2. Immunostaining for SMA. **A)** Immunoperoxidase reaction with anti-alpha smooth muscle actin antibody reveals myoepithelial cells lining nests of secretory cells in intraductal papilloma. Nuclei counterstained with HE×100. **B)** Immunoperoxidase reaction with anti-alpha smooth muscle actin antibody reveals that myoepithelial cells scattered at the base of nests in fibroadenoma. Nuclei counterstained with HE×100. **C)** Immunoperoxidase reaction with anti-alpha smooth muscle actin antibody reveals that myoepithelial cells scattered at the base of nests and stretched in adenocarcinoma. Nuclei counterstained with HE×100.



papilloma (Fig. 2C). Fibroadenoma showed immunohistochemistry patterns which were intermediate between intraductal papilloma and adenocarcinoma (Fig. 2B).

ER α positive cells were indicated by intensely stained nuclei and observed in all tumors (Fig. 3A–C). There were no significant differences in the appearance and the number of ER α positive cells among the three histological types of rat breast tumors.

PCNA immunostaining was confined to nuclei of tumor cells, suggesting proliferating cells (Fig. 4A–C). We quantified the PCNA-labeled cells in 30 intraductal papillomas, 10 fibroadenomas, and 4 adenocarcinomas using ACT-1 Image Analysis System (Nikon, Japan). The results of PCNA labeling index are shown in Table 3. PCNA labeling index was 24±8% in intraductal papilloma, 36±8% in fibroadenoma, and 56±10% in adenocarcinoma. It was significantly different in the PCNA labeling index among the three histological types of rat breast tumors ($p < 0.001$).

IV. Discussion

Considerable epidemiological studies have shown a positive association between milk and breast cancer [24, 27]. Ganmaa and Sato correlated incidence rates for breast, ovarian, and corpus uteri cancers with food intake in 40 countries [5], and put forth the hypothesis that milk and dairy products have a significant effect on the development of these cancers. However, there were few animal experiments to verify this hypothesis. The present study clearly demonstrated that the tumor incidence in the milk group was significantly higher than that in the water group and comparable to that in the estrone sulfate group. The milk group developed more than one third as many tumors as the water group. These results, therefore, suggest that milk as well as estrone sulfate has a stimulatory effect on the development of DMBA-induced rat mammary tumors.

Table 2. Histological type of tumors

Group	Total number of tumors	Histological type		
		IDP (%)	FA (%)	AC (%)
I	53	44 (83%)	7 (13%)	2 (4%)
II	33	24 (73%)	7 (21%)	2 (6%)
III	17	14 (82%)	3 (18%)	0 (0%)

I, milk group; II, estrone sulfate group; III, water group; IDP, intraductal papilloma; FA, fibroadenoma; AC, adenocarcinoma.

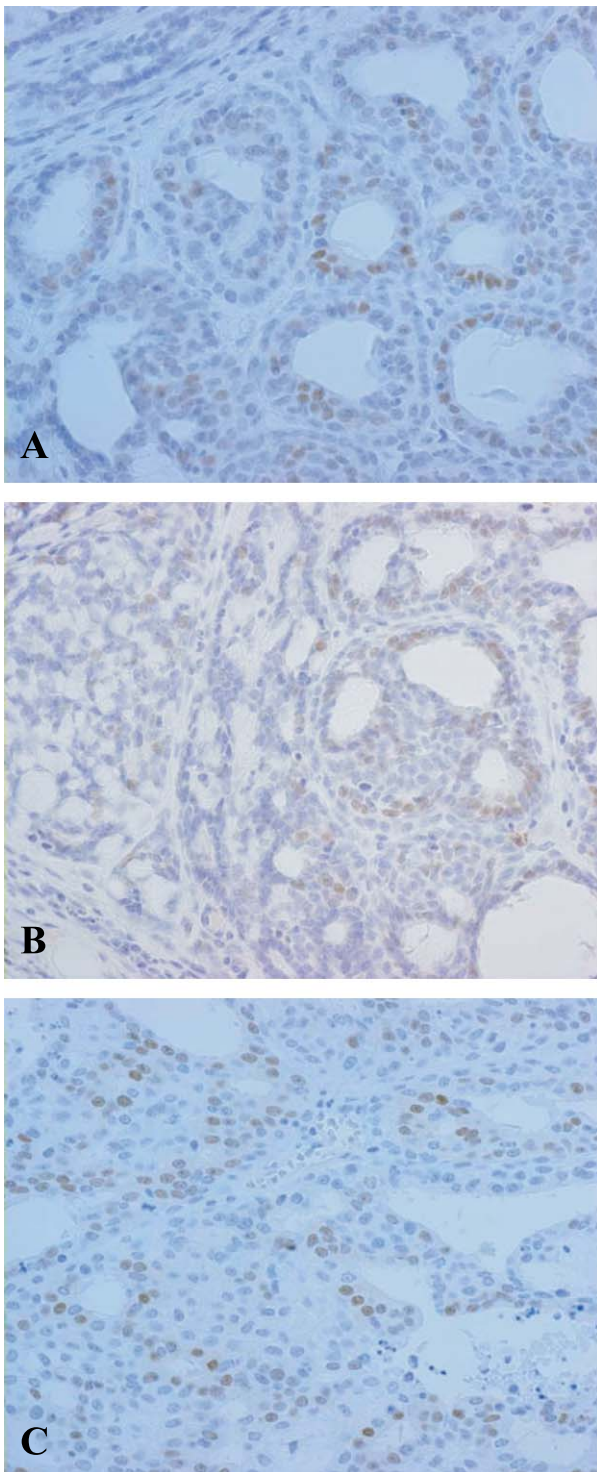


Fig. 3. Immunostaining for ER. Nuclear staining indicates immunoreactive ER. Nuclei counterstained with HE. **A)** Intraductal papilloma, **B)** Fibroadenoma, **C)** Adenocarcinoma. $\times 100$.

Although the mechanisms by which milk could alter tumorigenesis are unknown, there are some hypotheses that have been put forth to suggest an increased risk of breast cancer associated with consumption of milk. Studies of

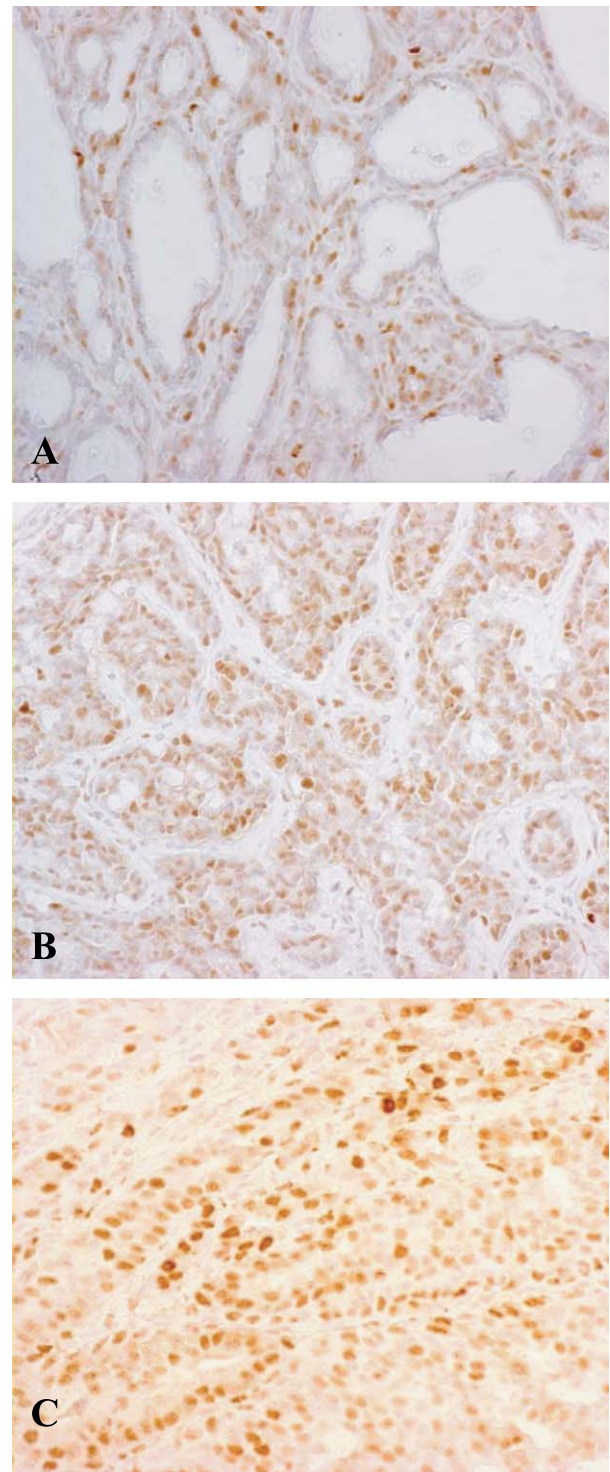


Fig. 4. Immunostaining for PCNA. Nuclear staining indicates immunoreactive PCNA. Nuclei counterstained with haematoxylin. **A)** Intraductal papilloma. PCNA labeling index is low. **B)** Fibroadenoma. PCNA labeling index is moderate. **C)** Adenocarcinoma. PCNA labeling index is high. $\times 100$.

dietary sources of nutrients estimate that the proportion of saturated fat in the diet that comes from dairy products is about 31% in the United States and 50% in Sweden [25, 30].

Table 3. Results of immunohistochemistry staining for PCNA

Histological types	No. of tumors	Mean labeling index \pm SD (%)	P
IDP	30	24 \pm 8	p=0.003
FA	10	36 \pm 8	
AC	4	56 \pm 10	p<0.001

IDP, intraductal papilloma; FA, fibroadenoma; AC, adenocarcinoma; SD, standard deviation.

Some animal studies for breast cancer proved that high fat diets increase the incidence of spontaneous, chemically induced, and radiation-induced mammary tumors [1, 7]. Therefore, a high consumption of milk may result in a high dietary fat intake, particularly saturated fat, which in turn increases breast cancer risk.

Another hypothesis suggesting an increased risk of breast cancer with high milk intake focuses on the insulin-like growth factor I (IGF-I). Some investigators found that bovine growth hormone, which is sometimes administered to dairy cattle to increase milk production, resulted in increased concentrations of IGF-I in milk [16]. IGF-I concentrations in commercial milk have been reported to range from 6 to 162 ng/ml [13]. In a human study, plasma IGF-I concentration increased by 10% when healthy subjects consumed commercial milk [8]. IGF-I has been shown to promote breast cancer cell growth and some experiments found that removing or blocking IGF-I receptors from the cell membrane can abolish viral or cellular oncogene-induced malignant transformation [31]. Therefore, IGF-I in milk may promote the development of tumors induced by DMBA in rat.

Estrogens are breast cancer promoters because of their physiological role in stimulating the mammary gland [28]. Our present experiment also demonstrated that estrone sulfate can stimulate the development of tumors induced by DMBA in rat. Moreover, all tumors induced by DMBA in the present experiment were positive for ER α . Russo *et al.* reported that mammary tumors in the rat are generally strongly hormone-dependent for both induction and growth [19]. Qin *et al.* measured the estrogen concentration of commercial milk and recently reported that the estrogen concentration in commercial milk was about 703.3 pg/ml [17]. Although the concentration of estrogen in milk is not very high, Ganmaa *et al.* have proved that commercial milk has a biologically significant hormonal effect and reported that the uterotrophic effect of commercial milk was almost comparable to that of 100 ng/ml estrone sulfate solution [6]. In this respect, estrogen in milk can be responsible for tumor promotion.

In our present experiment, we classified the mammary tumors induced by DMBA into three histological types: intraductal papilloma, fibroadenoma, and adenocarcinoma. The rates of benign tumors (intraductal papilloma and fibroadenoma) were almost the same among the three groups.

However, malignant tumor (adenocarcinoma) was found only in milk and estrone sulfate groups. Van Zwieten has reported that the administration of estrogen results in an increase in the proportion of rats with malignant tumors, especially cribriform carcinomas [29]. Results of our present experiment suggested that milk as well as estrone sulfate could be associated with the occurrence of adenocarcinoma.

According to histological appearance, we classified four tumors as malignant. However, SMA immunostaining of these four adenocarcinomas also presented positive. Papotti *et al.* studied the cellular composition of rat mammary carcinoma by both light and electron microscopy [15]. They found that mammary myoepithelial cells were a constant component in mammary carcinoma. Evidence of the presence of myoepithelial cells in DMBA-induced rat mammary carcinoma has also been reported in some other studies [11]. Therefore, although the presence of myoepithelial cells is commonly regarded as a hallmark of benignity in humans, myoepithelial cells will present as a constant in rat mammary carcinomas. PCNA is an essential replication factor to initiate cell cycle progression [12]. The number of PCNA-labeled cells generally is higher in malignant tumors than in benign ones. In our present experiment, PCNA labeling index of adenocarcinoma was significantly higher than that of intraductal papilloma and fibroadenoma. Thus, PCNA immunostaining is useful for histological classification of rat mammary tumors.

In conclusion, milk as well as estrone sulfate promoted the development of DMBA-induced mammary tumors in rat and could be associated with the occurrence of adenocarcinoma. Immunohistochemical staining for SMA and PCNA was a useful auxiliary for histological classification of tumors.

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