IMMUNOHISTOCHEMICAL STUDIES OF MAMMARY TUMORS INDUCED BY THE DIRECT APPLICATION OF DMBA POWDER TO RAT MAMMARY GLANDS

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Abstract: Immunohistochemical studies were performed on locally growing finger tip-sized tumors induced by the direct dusting of 1 mg 7,12-dimethylbenz (a) anthracene (DMBA) powder to the right inguinal mammary tissue of both female and male 30-day-old Sprague-Dawley rats. A variety of benign and malignant tumors of epithelial and/or mesenchymal origin were detected histologically in both sexes of rats. In tumor tissues, as well as in normal mammary glands, immunoreactivity with actin distinctly demonstrated the myoepithelial cells. Type IV collagen clearly separated the parenchymal cells from the surrounding mesenchymal cells. Normal periductal/intralobular mesenchymal cells of the mammary tissues were found only to be vimentin positive, whereas some neoplastic mesenchymal cells were found to express actin. Desmin was found in a few neoplastic mesenchymal cells. This revealed that neoplastic mesenchymal elements were composed of fibroblast, myofibroblast, and muscular nature. (*J Toxicol Pathol* 1: 157~164, 1988)

Key words: Rat, DMBA, Mammary neoplasms, Immunohistochemistry

Introduction

Benign and malignant mammary tumors are common neoplasms in humans, of epithelial and/or mesenchymal origins, are observed¹. Mammary tumors induced in rodents by polycyclic hydrocarbons have been extensively studied as a potential model for human breast neoplasms. In particular, systemic DMBA administration, by means of a single pulse dose administered into either the stomach or caudal vein^{2,3}, is the most effective method, producing mammary tumors in female rats. However, only adenocarcinomas are induced using this method. Sinha and Dao^{4,5} developed a technique to induce mammary tumors by the application of DMBA powder directly to the mammary glands. Using this technique, mammary epithelial tumors developed on the dusted site, arising alone, or in co-existence with mammary

mesenchymal tumors⁶⁻¹⁰. Recently, the morphological and biological studies of these tumors have been investigated¹¹. Accordingly, epithelial, mixed epithelial and mesenchymal, and mesenchymal tumors, comparable to human breast tumors were induced. The present study was carried out in order to characterize directly applied DMBA-induced mammary tumors by means of immunohistochemical techniques.

Materials and methods

Induction of mammary tumors

Sprague-Dawley rats were purchased from Japan Clea, Inc. Osaka, Japan. All rats were housed in an air-conditioned room ($22 \pm 2^\circ\text{C}$), with a daily schedule of 14 hours light and 10 hours darkness. The rats were housed 4 to 6 animals per wire-mesh cage, and fed CMF pellets (Oriental Yeast Co., Tokyo, Japan) and water *ad libitum*.

At 30 days of age, 45 females and 24 males were given approximately 1 mg of DMBA (Eastman Chemical, Rochester, USA) by a single dust-

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ing directly to the right inguinal mammary gland. The direct dusting technique, according to Sinha and Dao^{4,5} is described in detail elsewhere¹¹. For the controls, 10 female and 10 male rats, were treated using the same procedures without DMBA dusting. All rats were palpated once a week to detect mammary tumors. When a tumor appeared, it was measured using calipers, rectangularly crossing 2 diameters of the tumor. The rats were not sacrificed until the tumor became as large as finger tip-size (with a mean diameter of 1 to 2 cm). No rats died during the experiment due to DMBA dusting, but, 2 females and 1 male died of respiratory disease and they were excluded from the present experimental data. All of the rats were sacrificed at 32 weeks of age (28 weeks after dusting). Complete autopsies were performed on all of the rats.

Tissue sections

To carry out histological examination, the tumor, together with adjacent normal mammary tissue, was fixed in methacarn¹² and embedded in paraffin. For the controls, contralateral inguinal mammary glands of the dusted rats, and inguinal mammary glands of the rats which had not undergone DMBA dusting (at 32 weeks of age) were processed in the same manner. Tissue sections were stained routinely with hematoxylin-eosin, alcian blue (pH 2.5), and periodic acid-Schiff.

Immunohistochemical procedures

The ABC method¹³ using peroxidase conjugated antibodies was employed. All immunohistochemical stains were performed on 4 μ m-thick, serially-cut sections from methacarn-fixed, paraffin-embedded tissues. Methacarn gave satis-

factory results in all the antibodies used. Normal goat serum, normal horse serum, biotinylated anti-rabbit IgG, biotinylated anti-mouse IgG, and avidin-biotin complex were purchased from Vector Lab., Burlingame, CA, USA. The primary antibodies used were: polyclonal rabbit anti-actin (Biomedical Technologies Inc., Stoughton, MA, USA), rabbit anti-type IV collagen (provided by Dr. T. Oikawa, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan), rabbit anti-desmin (Lipshaw, Detroit, MI, USA); and, monoclonal mouse anti-vimentin (Dakopatts, Glostrup, Denmark). Sections were stained with DAB and counter stained with Gill hematoxylin. For detection of type IV collagen, tissue sections were digested with pronase before incubation with the antiserum, since no reaction occurred without the pretreatment¹⁴. As for the controls, normal rabbit or horse serum was employed instead of the primary antiserum. For detection of endogenous peroxidase activity, some sections were reacted for DAB only.

Results

Incidence, time of harvest, and type of locally induced tumors

The experiment was terminated 28 weeks after DMBA application. Incidence, types of tumors, and the time of harvest of each finger tip-sized tumor are shown in Table I. The time of harvest was determined as the time interval between the application of DMBA and the withdrawal of palpable tumors with a mean diameter of 1 to 2 cm. With the method of direct application, tumors developed only at the place where the carcinogen

Table I. Incidence, Mean Time of Harvest and Types of Tumors Induced by Direct Dusting of 1 mg of DMBA Powder to 30-day-old Sprague-Dawley Rat Mammary Tissues

	Females	Males	
Number of animals	43	23	
Tumor incidence (%)	100	91	
Types of tumors	mammary	mammary	non-mammary
Number of finger tip-sized tumors	43	16	5
Mean time of harvest (weeks)	15.6	22.9	19.4

Fig. 9. Proliferating periductal cells stained with anti-vimentin serum. The majority of the spindle-shaped cells are vimentin positive. $\times 200$.

Fig. 10. Proliferating periductal cells stained with anti-actin serum. Few cells are labelled with anti-actin antibody. Note the intense staining of myoepithelial cells. $\times 200$.

Fig. 11. Spindle cell sarcomas stained with anti-desmin serum. Some cells are desmin positive, while others are not. $\times 200$.

Fig. 12. Spindle cell sarcoma stained with anti-vimentin serum. The majority of the cells are vimentin positive. Co-expression of vimentin and desmin is visible (compare with Fig. 11). $\times 200$.

was applied. The tumors were designated to be mammary in origin, when mammary parenchymal cells were either atypically proliferating or engulfed by the proliferating mesenchymal cells, the other was designated to be of non-mammary origins, when the tumor tissues were not connected

to periductal/intralobular fibrous stroma. All of the tumors in the females were mammary in origin, while, in the males, some tumors were non-mammary in origin (all spindle cell sarcomas), in addition to those which were mammary in origin.

Mammary tumors induced were heteromor-

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- Fig. 1. Normal mammary gland stained with anti-actin serum. Peripheral myoepithelial cells are contiguous in the ducts and discontinuous in the acini. $\times 200$.
- Fig. 2. Normal mammary gland stained with anti-type IV collagen serum. Basement membranes adjacent to myoepithelial cells and blood vessel walls are stained. $\times 200$.
- Fig. 3. Intraductal papilloma stained with anti-actin serum. Myoepithelial cells are contiguously present along the projections. $\times 200$.
- Fig. 4. Intraductal papilloma stained with anti-type IV collagen serum. Note the basement membranes along the papillary projections. $\times 200$.
- Fig. 5. Adenocarcinoma stained with anti-actin serum. Note the absence of actin-positive cells. The blood vessel wall is stained. $\times 200$.
- Fig. 6. Adenocarcinoma stained with anti-type IV collagen serum. Continuous linear staining is still preserved around the cancerous foci. $\times 200$.
- Fig. 7. Adenoid cystic carcinoma stained with anti-actin serum. Some lumens are surrounded by the actin-positive cells, while others are not. $\times 200$.
- Fig. 8. Adenoid cystic carcinoma stained with anti-type IV collagen serum. Positive staining is seen on the lumens surrounded by actin-positive cells. $\times 200$.

phic with a mixture of various histologies. The histological types of tumors induced are as follows: Epithelial tumors consisted of adenocarcinoma, adenoid cystic carcinoma, and squamous cell carcinomas, as well as adenosis and intraductal papillomas. Mesenchymal tumors consisted of spindle cell sarcomas or fibromatosis. And, mixed epithelial and mesenchymal tumors consisted of carcinosarcomas, phyllodes tumor, and fibroadenoma. The morphology of induced mammary tumors are, in general, the same in both males and females.

Immunohistochemistry

In normal mammary glands, the myoepithelial cells were clearly visualized with the immunoreactivity with actin (Fig. 1). Antibody against type IV collagen delineated the basement membrane as a single continuous line surrounding the mammary parenchyma (Fig. 2). Periductal/intralobular stromal cells were vimentin-positive, but neither actin nor desmin were consistently negative. In benign epithelial lesions (adenosis and intraductal papilloma), the staining patterns obtained, with the antiserum against actin and type IV collagen, were similar to those found in normal mammary glands (Figs. 3 and 4). In the majority of the adenocarcinomas, as seen by the actin stainability, a normal relationship between the glandular epithelial cells and the myoepithelial cells was usually maintained, and the myoepithelial cells continued to remain outside the glandular epithelial cells. But, the stainability of actin in the induced adenocarcinomas was weaker compared to normal mammary glands. And, in some adenocarcinomas, actin-rich myoepithelial cells were not found in any areas (Fig. 5), but such tumor cell nests were always surrounded by a continuous layer of the type IV collagen (Fig. 6). Some lumens in the tumor tissues were surrounded by the actin positive cells (Fig. 7). Staining of type IV collagen was seen within these luminal surfaces surrounded by actin-positive myoepithelial cells (Fig. 8). This indicates their adenoid cystic nature. Squamous cell carcinoma was devoid of actin positive cells and some parts of them lacked continuous linear staining of type IV collagen.

In neoplastic periductal/intralobular stromal

cells, vimentin was diffusely positive (Fig. 9). Areas exhibiting benign proliferation, corresponding to fibromatosis, some cells were actin positive (Fig. 10). In areas corresponding to spindle cell sarcoma, a few selective cells, often with eosinophilic cytoplasm, exhibited marked cytoplasmic staining with desmin (Fig. 11). Co-expression of vimentin and desmin was observed in these cells (Fig. 12). The staining patterns of actin, desmin, vimentin, and type IV collagen, in normal mammary glands and induced tumors are summarized in Table 2.

Discussion

Systemic administration of DMBA induces a toxic effect, e.g. adrenal apoplexy¹⁵, and evokes unexpected tumors, such as ear duct tumors and/or leukemia³. The mortality rate of the experimental animals is increased due to both adrenal apoplexy or tumors. The direct dusting technique eliminated any observable systemic effect and tumors were induced only where the carcinogen was applied. The systemic administration of DMBA could hardly cause sarcomatous change³, while direct dusting affected not only mammary epithelial cells but also mammary mesenchymal cells in the dusted site, leading to variety of histologies in the induced tumors.

Basement membrane as visualized with type IV collagen was usually present in normal, benign, as well as in carcinomas. The benign nature of these lesions may be supported by the well formed basement membrane in cancerous foci¹⁶. Furthermore, myoepithelial cells seen using actin were relatively well preserved. These findings are in contrast to human carcinomas¹⁴. DMBA-induced mammary tumors in rats are of biologically low-grade malignancy. No metastasis was observed in the present study. Mammary tumors in both male and females partly resembled adenoid cystic carcinoma. The adenoid cystic nature was confirmed by electron microscopy^{14,16-18} as well as by immunohistochemistry^{16,18}.

In normal glands, the majority of the periductal/intralobular stromal cells were stained by vimentin, the intermediate type filament characteristic of mesenchymal origins¹⁹, indicating fibroblastic nature. Myofibroblasts can be identified by

their intense staining using actin²⁰. In neoplastic mesenchymal cells, in addition to vimentin positivity, myofibroblasts were clearly identified by anti-actin antibody. In a few spindle-shaped cells with eosinophilic cytoplasm were clearly positive with desmin, the intermediate type filament characteristic of muscle²¹. Vimentin and desmin may co-exist in muscular cells^{22,23}. In humans, periductal/intralobular stroma differ from other stroma by the more frequent occurrence of heterologous malignant tissues, such as bone, cartilage, and muscle²⁴. Even non-malignant mammary stroma express smooth muscle immunoreactivity²⁵. In rats, ultrastructural observation of DMBA-induced mammary mesenchymal components were fibroblast-like, but very rarely myofibroblast-like in character²⁶. In the present study, based on immunohistochemistry, normal periductal/intralobular stroma was composed of fibroblast while myofibroblast appeared in areas exhibiting benign proliferation such as fibromatosis, and some sarcomatous cells such as spindle cell sarcoma expressed muscle immunoreactivity.

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