Expression of Stratified Squamous Epithelia-Type Cytokeratin by Canine Mammary Epithelial Cells during Tumorigenesis: Type I (Acidic) 57 Kilodalton Cytokeratin Could Be a Molecular Marker for Malignant Transformation of Mammary Epithelial Cells

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ABSTRACT. Two monoclonal antibodies (MAbs) were established in 20 clones of MAbs generated against cytokeratin fraction extracted from canine squamous cell carcinoma to investigate the expression of intermediate filament proteins during tumorigenesis. These MAbs were confirmed to react with purified cytokeratin by ELISA. One monoclonal antibody, MAb32 reacted all layers of epidermis except the cornified layer and mammary myoepithelial cells but not any epithelial cells. Another antibody named MAb24 exclusively reacted the basal monolayer of epidermis, the stratum germinativum. Any positive reactions with MAb24 were not detected in normal mammary gland. From the analysis by two-dimensional gel electrophoresis followed by immunoblotting, it was revealed that MAb24-recognizing cytokeratin subunit gave a molecular weight of 57 kilodalton and a isoelectric-pH value of pI 5.1, indicating type I (acidic) cytokeratin. In intraductal papillomas developed in canine mammary glands, most tumor cells were positively stained with MAb32 in addition of myoepithelial cells while no positive reaction with MAb24 was seen. In ductal carcinomas, MAb24-positive cytokeratin was begun to express by tumor cells with positive reaction of MAb32 where these cells showed infiltrative growth into the stroma. We therefore proposed that 57 kilodalton-type I cytokeratin was a molecular marker for malignant transformation in canine mammary tumor and these antibodies could be useful tools to investigate the change of cytokeratin expression during tumorigenesis.—KEY WORDS: canine, cytoskeleton, cytokeratin subunit, mammary tumor, monoclonal antibody.

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Intermediate-type or 10 nm cytoskeletal filaments (IF) are composed of a family of related proteins. This family include cytokeratins (type I and type II), vimentin, desmin, glial fibrillary acidic protein (GFAP) and neurofilament displaying a tissue-restricted distribution [18]. The constituent of IF expressed by epithelial cells is cytokeratins which are a subset of 19 polypeptides in human tissue [13] or 22 polypeptides in bovine and murine tissues [22], and moreover, Moll *et al.* recently reported No. 20 cytokeratin which was restrictedly expressed in human certain carcinomas, e.g., adenocarcinomas of colon, stomach and bile system [14, 15]. The unit of cytokeratin molecule is consisted of the disulfide-bonded heterodimer between type I (acidic) and type II (basic/neutral) cytokeratin polypeptides. Various cytokeratin subunit-specific monoclonal antibodies (MAbs) were established by many workers [3, 6, 7, 11, 15, 16, 19, 20, 24, 27] and it is recognized that these MAbs were useful as the histodiagnostic markers for epithelial cell-originated tumors. As this intermediate filament expression is largely retained even when cells undergo neoplastic transformation, these MAbs has been successfully applied for cell typing and tumor classification. For example, No. 20 cytokeratin was normally detected in gastric and intestinal epithelium and positive reaction of anti-No. 20 cytokeratin MAb with gastric epithelium disappeared during tumorigenesis, while the expression of No. 20 cytokeratin was maintained in colon adenocarcinoma [15]. They proposed, therefore, that cytokeratin No. 20 was a new histodiagnostic marker of colon adenocarcinomas. Weiss *et al.* reported that 48 and 56 kilodalton cytokeratins were molecular markers for hyperproliferative keratinocytes in epidermal diseases, such as psoriasis showing parakeratinization [27]. Furthermore, Ramaekers *et al.* found the MAb to No. 18 cytokeratin could distinguish adenocarcinomas from squamous cell carcinomas (which are cytokeratin No. 18 negative) [19] and the cytokeratin No. 7 distinguish between ovarian carcinomas (cytokeratin No. 7 positive) and carcinomas of gastrointestinal tract (negative) [20].

On the other hand, mammary tumors are the most common tumors in female dogs, and immunohistochemical examination of IF proteins in these tumors were investigated by several workers [9, 23, 26]. It is well known that human mammary epithelial cells express some cytokeratin polypeptides and change the expression pattern of cytokeratins during tumorigenesis [13]. Mammary myoepithelial cells express vimentin in addition of cytokeratin [8]. Certain myoepithelial cells proliferating in mammary [8] or salivary glands [9] can express even GFAP or desmin.

In this study, we established two MAbs reacting with cytokeratin and immunohistochemical and biochemical analyses of different types of canine mammary tumors using these MAbs were performed.

MATERIALS AND METHODS

Surgical specimens: Biopsy specimens from 30 spontaneous canine mammary tumors generated in female

dogs were surgically obtained and immediately fixed in Methacarn solution (60% methanol/30% chloroform/10% acetic acid) [12, 25] or 4% paraformaldehyde/0.1 M phosphate buffer, pH 7.4. Different types of tumors were histologically examined by hematoxylin and eosin staining (Table 1).

Table 1. The number of different types of canine mammary tumors investigated for the expression of cytokeratin

Type of tumor	Number
Fibroadenoma	7
Intraductal papilloma	7
Benign mixed tumor	8
Ductal carcinoma	6
Malignant mixed tumor	1
Squamous cell carcinoma	1
Total	30

Preparation of antigen: Squamous cell carcinoma developed in the skin of old female dog surgically obtained and canine snout epithelia were immediately minced on ice, washed with 0.15 M NaCl/50 mM Tris-HCl, pH 7.6 (Tris-saline) containing protease inhibitor cocktail (1 mM phenylmethylsulfonyl fluoride/2 mM N-ethylmaleimide/5 mM ethylenediamine tetraacetic acid (EDTA)/1 mM p-tosyl-L-arginine methyl ester) and then with high-salt buffer (1 M KCl/1% Triton X-100/Tris-saline) to eliminate non-IF proteins at 4°C, overnight. IF proteins were extracted from the resulting pellets with 8 M urea/20 mM 2-mercaptoethanol/1 mM EDTA/Tris-saline containing the protease inhibitor cocktail at 4°C, overnight, and after centrifugation, the supernatant was used as an antigen.

Production of monoclonal antibodies: The antigen (200 μg) was injected into BALB/c mice subcutaneously with Freund's complete adjuvant and after one booster injection of the antigen, spleen cells were collected and hybridized with myeloma cells (P3x63Ag8U1) in the presence of 50% polyethylene glycol (M.W.: 4000, Merk, Germany). Hybridomas were suspended in 20% fetal calf serum-Dulbecco's modified Eagle's medium (DMEM-20) supplemented with HAT (hypoxanthine, aminopterin and thymidine) solution (Sigma Chemical Co., St. Louis, MO) and antibiotic (penicillin, streptomycin and neomycin) solution (Sigma Chemical Co.), and then seeded in polystylene microtiter plates. Antibody-producing clones were selected by enzyme-linked immunosorbent assay (ELISA) followed by immunohistochemical technique and recloning was performed twice according to the limiting dilution method in DMEM-20 supplemented with hypoxanthine and thymidine. The subclass of MAb was determined by ELISA using a Monoclonal Mouse Isotyping kit (Pharmingen, San Diego, CA). Finally, MAbs reacting with cytokeratin were selected by ELISA against purified human epidermal cytokeratin (Sigma Chemical Co.).

Gel electrophoretic and immunoblot analyses: Sodium dodecyl sulfate-13.5% polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli [10]. For two dimensional electrophoresis, isoelectrofocusing was performed according to the method of O'Farrel [17] using 2.5% Servalyte (pH 2–11, Serva Feinbiochemica, GmbH & Co., Heiderberg, Germany) as the first dimension and then isoelectrofocusing gel was applied on SDS-PAGE as the second dimension [1, 5]. Immunoblotting was performed as previously described [4] with anti-cytokeratin MAb as the first antibody. Alkaline phosphatase-conjugated anti-mouse immunoglobulin rabbit IgG (DAKO patts, Glostrup, Denmark) is used as the second antibody and immunoreactive polypeptide spot was visualized with 0.01% nitro blue tetrazolium (Promega Co., Madison, WI) and 0.005% 5-bromo-4-chloro-3-indolyl-phosphate (Promega Co.).

Immunohistochemistry: Immunohistochemical staining was performed according to the peroxidase-antiperoxidase method [2]. After the inactivation of endogenous peroxidase with 0.5% periodic acid, deparaffinized section was incubated with anti-cytokeratin MAb as the first antibody, then with the second antibody and finally with peroxidase-antiperoxidase complex. The resulting sections were immersed in 0.02% diaminobenzidine/50 mM Tris-HCl, pH 7.6 containing 0.03% H_2O_2 and counterstained with Mayer's hematoxylin.

RESULTS

Immunochemical characterization of monoclonal antibodies: Twenty clones producing interesting MAbs were selected by ELISA followed by immunohistochemical staining using the sections of canine mammary tumor. Of 20 clones, eight clones were confirmed to produce MAbs reacting with cytokeratin by ELISA using purified epidermal cytokeratin (data not shown). Especially, two anti-cytokeratin monoclonal antibodies showed the characteristic immunostaining patterns. MAb32-reactive cytokeratins widely distributed in all layers of canine skin epidermis except the cornified layer (Fig. 1a) and MAb24 restrictedly reacted with the basal monolayer (Fib. 1b). Other stratified squamous epithelia in tangue, esophagus and anus were also stained with both antibodies as same manner as epidermis (data not shown). Cytokeratins prepared from canine snout epithelia were separated by SDS-PAGE and then immunoblot analysis with MAb32 or MAb24 was performed (Fig. 2). Coomasie blue stained-SDS gel showed canine snout epidermal cytokeratins containing 60, 57.5, 57 and 45 kDa polypeptides (Fig. 2; lane 1). Immunoblot analysis revealed that MAb32 recognized 60, 57.5 and 45 kDa bands (Fig. 2; lane 2) and MAb24 exclusively reacted with a 57 kDa single band (lane 3). By two dimensional gel electrophoresis (Fig. 3a) and immunoblotting (Fig. 3b), MAb24-reactive 57 kDa polypeptide gave a isoelectric-pH value of pI 5.1 indicating that the polypeptide belonged to type I (acidic) keratin family, while other 60, 57.5 and 45 kDa polypeptides and

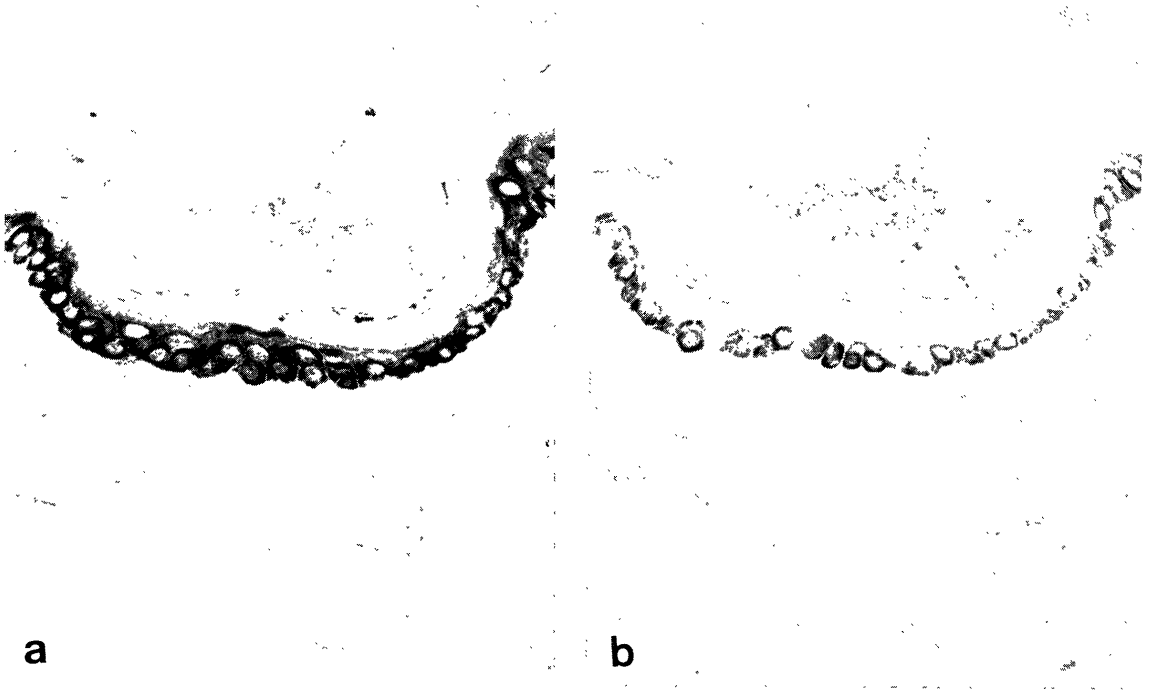

Fig. 1. Immunohistochemical stainings of canine normal epidermis with MAb32 (a) or MAb24 (b). MAb32 immunostained the cell of all layers in epidermis (a), while MAb24 restrictedly reacted with the basal monolayer, stratum germinativum (b). × 250.

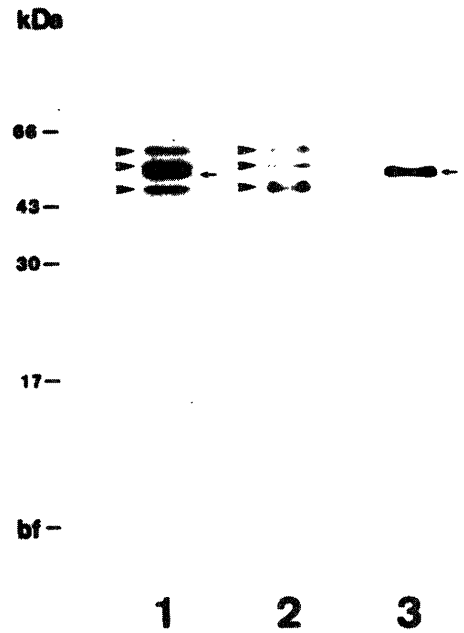

Fig. 2. SDS-PAGE (lane 1) and their immunoblots with MAb32 (lane 2) or MAb24 (lane 3) of epidermal cytokeratin fraction prepared from canine snout epithelia. In epidermal cytokeratin preparation, the arrowheads indicate MAb32-reacting cytokeratin subunits (lane 2) and the arrow indicates MAb24-positive cytokeratin subunit (lane 3), respectively.

type II (basic) keratin family shown in Fig. 3a were not recognized by MAb24 (Fig. 3b). MAb32 was in a subclass of IgG_1, κ-light chain and MAb24 was in a subclass of IgG_{2b}, κ-light chain, respectively.

Immunohistochemical localization of MAb24- and MAb32-positive cytokeratins in canine mammary tumors: In normal mammary glands, MAb32 recognized only myoepithelial cells but not any epithelial cells, lining cells of alveoli and alveolar ducts (Fig. 4a). On the other hand, no positive cells reacting with MAb24 in normal mammary gland was detected (Fig. 4b). In ductal carcinomas (6 cases) raised in canine mammary glands, MAb32 reacted with most of tumor cells in addition of myoepithelial cells (Fig. 5a) and MAb24-reactive cytokeratin was appeared in some tumor cells which showed infiltrative growth into the stroma (Fig. 5b). Especially, in carcinomatous area where neoplastic cells were proliferating with tubular formation in the stroma, MAb24 strongly reacted with some neoplastic cells (Fig. 5d, arrowhead) and mirror-image section analysis revealed that several tumor cells were stained with both antibodies (Fig. 5c and 5d). In well-differentiated intraductal papillomas (Fig. 6) or fibroadenomas (data not shown), MAb32 well stained the adenomatous area (Fig. 6a) and normal myoepithelial cells, while MAb24-positive cells were not seen (Fig. 6b). Squamous cell carcinoma (the source of antigen) was well stained with both antibodies (Fig. 6c and 6d), and MAb32-positive tumor cells (Fig. 6c) more widely distributed than MAb24-positive cells (Fig. 6d) in carcinomatous area. In benign and malignant mixed tumors, only

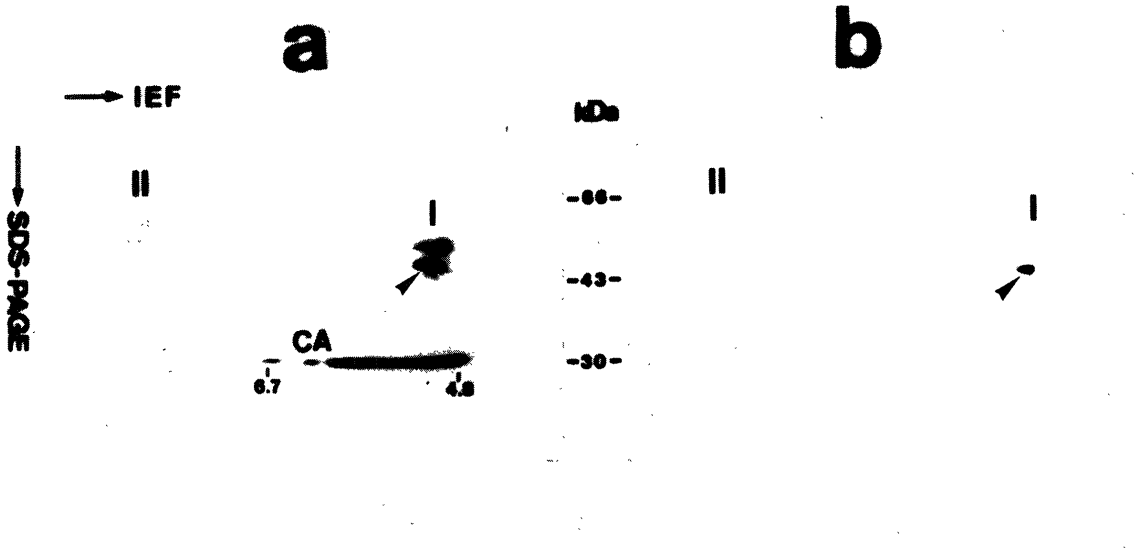

Fig. 3. Two-dimensional gel electrophoresis (a) of purified epidermal cytokeratin and its immunoblot (b) with MAb24. The arrowheads in both (a) and (b) indicate the same cytokeratin subunit spot recognized with MAb24. Numbers written in gothic letter mean type I (I) and type II (II) cytokeratin subfamily. The sample contains an appropriate amount of carbonic anhydrase (CA, 30 kDa and sequentially different isoelectric-pH value, pH 4.8–6.7) as an inner marker.

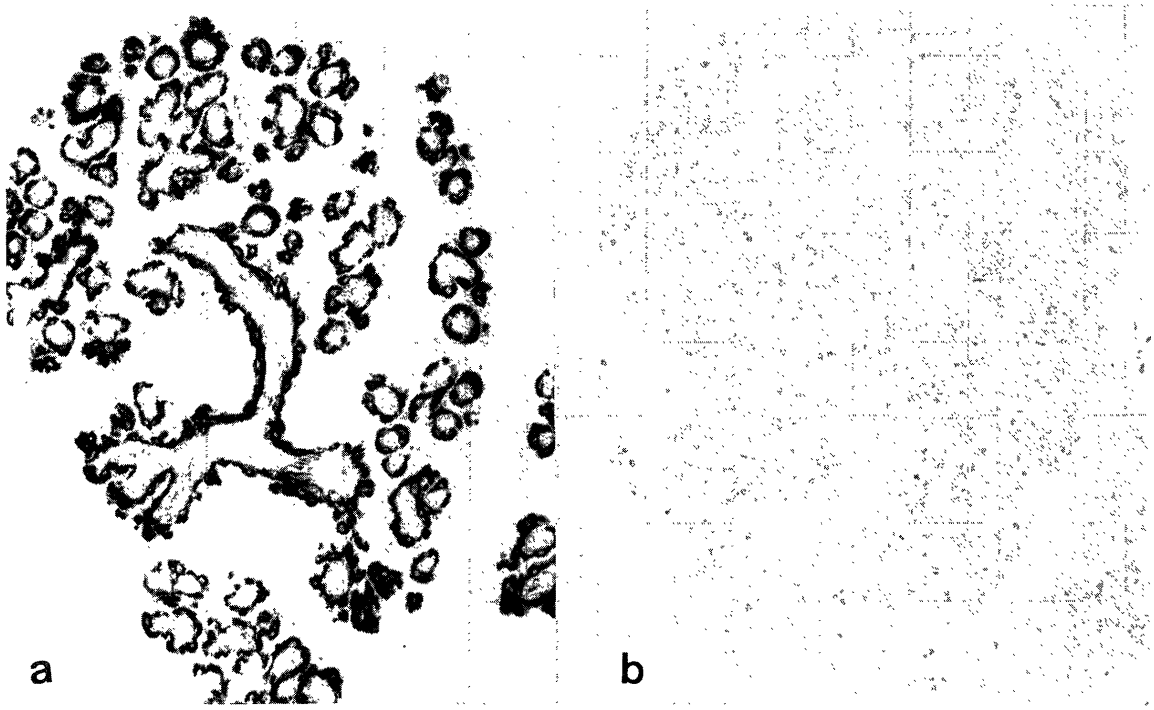

Fig. 4. Immunohistochemical stainings of normal mammary gland stained with MAb32 (a) or MAb24 (b). (a) MAb32 restrictedly reacted against myoepithelial cells in both alveoli and alveolar ducts, while no positive reaction against alveolar cells with MAb32 was detected. (b) No cells were reacted with MAb24 in normal mammary gland. × 125.

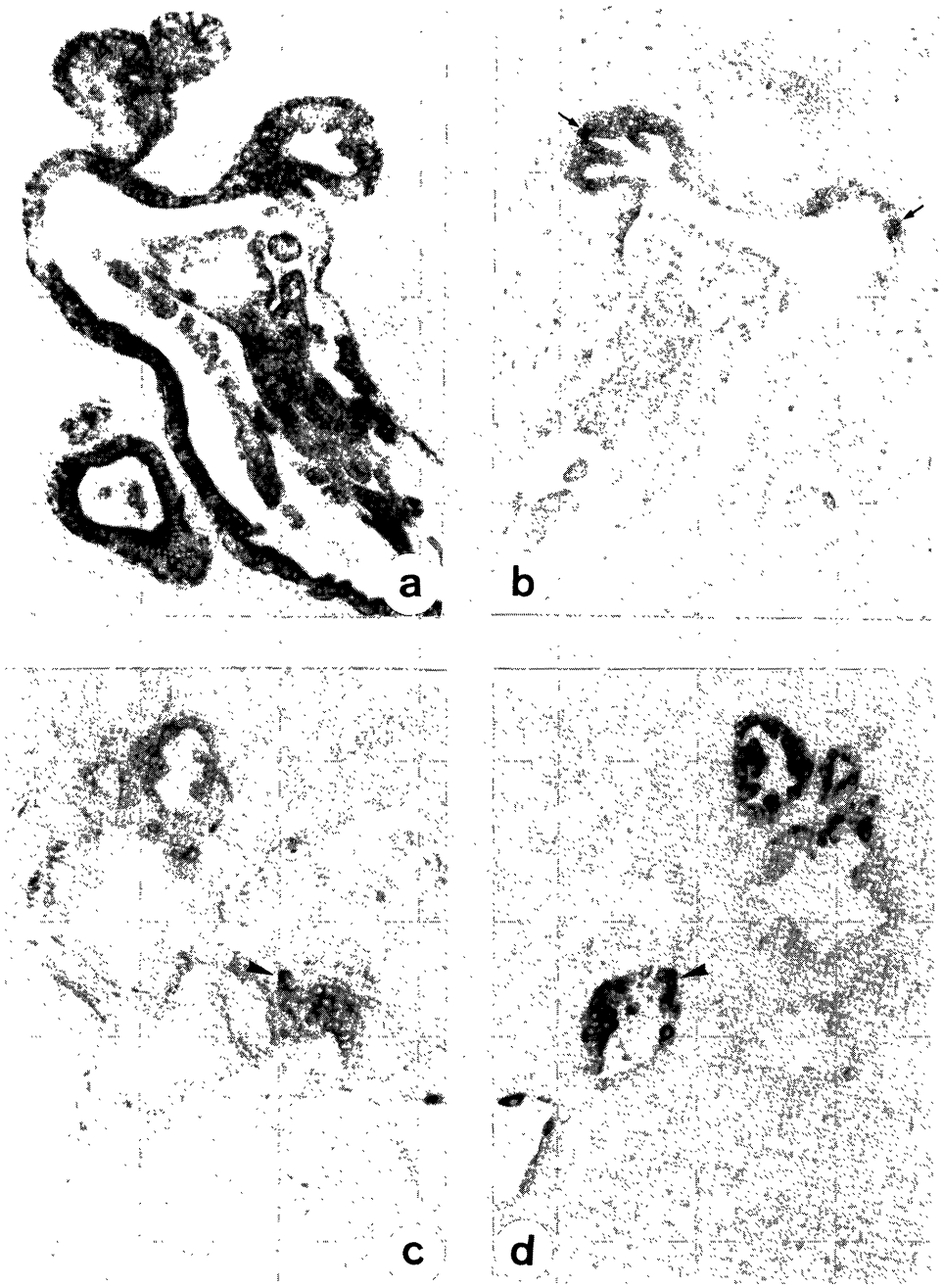

Fig. 5. Immunohistochemical stainings of mammary ductal carcinomas stained MAb32 (a, c) or MAb24 (b, d) on the mirror-image serial sections (a and b, c and d). The arrows in (b) indicate the area positively stained with both MAb32 and MAb24. Moreover, the mirror-image serial sections reveal the same cell positively stained with both MAb32 (c, arrowhead) and MAb24 (d, arrowhead). × 250.

MAb32 immunostained a part of adenomatous area and normal myoepithelial cells, and no MAb24-positive cell was observed same as in intraductal papillomas (data not shown).

DISCUSSION

It is well known that cytokeratin, one of the intermediate-filament proteins, consists of 20 subunits in human tissue [13, 14] and 22 subunits in bovine and murine tissues [22]. Each subunit could be separated into some isoelectric variants by two-dimensional electrophoresis. Various monoclonal antibodies (MAbs) against cytokeratin were established by many workers and these are recognized as molecular markers for tumor diagnosis in human medicine [6–8, 15, 16, 19, 20] and the change of cytokeratin-subunits expression pattern during tumor progression was reported in human transitional cell carcinomas [21].

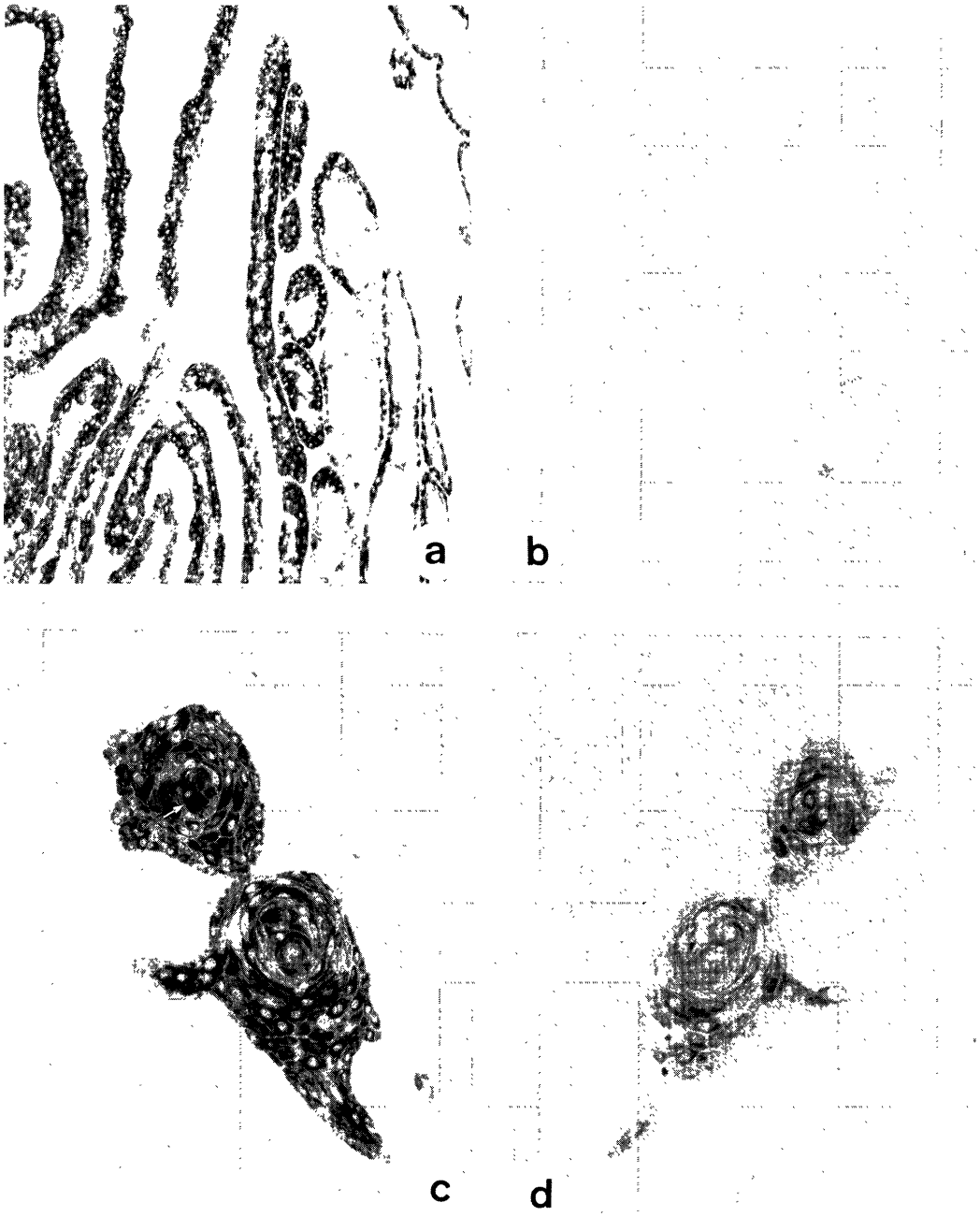

Fig. 6. Immunohistochemical stainings of well-differentiated intraductal papilloma (a, b, × 125) and squamous cell carcinoma (c, d, × 250) stained with MAb32 (a, c) or MAb24 (b, d) on mirror-image serial sections. In intraductal papilloma, MAb32-positive cytokeratins were widely distributed in tumor cells and myoepithelial cells (a), but no positive reaction with MAb24 was seen (b). Squamous cell carcinoma (the source of antigen) was well stained with both MAb32 (a) and MAb24 (b). The minor-image serial sections reveal the same cell positively stained with both MAb32 (c, arrow) and MAb24 (d, arrow).

However, little information of pathobiochemical kinetics concerning cytokeratin polypeptides in domestic animal is available.

In this study, we established two MAbs reacting with cytokeratin in 20 clones of MAbs generated against cytokeratin-enriched fraction extracted from canine squamous cell carcinoma. One antibody (MAb32) immunostained the cells of all layers of epidermis except the cornified layer and mammary myoepithelial cells, and another (MAb24) restrictedly reacted with the basal cells of epidermis, but not with even myoepithelial cells. Both MAbs did not reacted with any epithelial cells in normal mammary gland. In ductal carcinomas, most tumor cells became positive with MAb32 in addition of myoepithelial cells. Some of these cells also expressed MAb24-positive cytokeratin in the area where these cells showed infiltrative growth. It is therefore that mammary epithelial cells obtained the capacity of the synthesis of epidermis-type

cytokeratin during malignant transformation. Especially, MAb24-positive 57 kDa cytokeratin was restrictedly detected in the stratum germinativum of epidermis, and it is thought that MAb24 could recognized the more undifferentiated cells than one recognized with MAb32 in the epidermis. From these aspects, it is suggested that 57 kDa-type I cytokeratin was expressed by more anaplastic epithelial cells also in canine mammary tumors. As Moll *et al.* reported [13], No. 11 cytokeratin only found in epidermis sometimes became detectable in ductal carcinomas of human breast. This molecule gave a molecular weight of 56 kDa and a isoelectric-pH value of pI 5.3. The biochemical characterization of this cytokeratin detected in human ductal carcinomas is extremely resemble to 57 kDa, pI 5.1-cytokeratin described in this paper. On the other hand, the expression of 50 kDa and 58 kDa cytokeratins in stratified epithelia-originated carcinomas was demonstrated by Nelson *et al.* [16] using with AE1 and AE3, anti-cytokeratin monoclonal antibodies and these molecules were not detectable in simple epithelia-originated carcinomas. Their experiments revealed that 50 kDa and 58 kDa cytokeratins could be molecular markers of the neoplastic cells originating from stratified epithelia. In contrast, Debus *et al.* [7] reported No. 18 cytokeratin was found in simple epithelia but not in stratified squamous epithelia and the antibody against No. 18 cytokeratin strongly reacted with the tumor cells in simple epithelia-derived tumor including adenocarcinomas and ductal carcinomas in human breast, indicating a marker protein for simple epithelia. The 57 kDa-type I cytokeratin we described here was not found in normal mammary gland but became detectable in the infiltrating areas of ductal carcinomas. Additionally, the present study showed that simple epithelia could express even stratified squamous epithelia-type cytokeratin during canine mammary tumorigenesis. From the mention above, It was suggested that some cytokeratins were newly expressed or ceased to express and the expression of other cytokeratins was maintained during tumorigenesis or malignant transformation.

Immunohistochemical analysis of canine mammary tumor using two MAbs present in this paper (MAb32 and MAb24) confirmed that neoplastic mammary epithelial cells characterized by infiltrative growth could express stratified squamous epithelia-type cytokeratin and that these two MAbs were very useful markers of neoplastic cells in canine mammary glands. For the investigation of the switching mechanism of cytokeratin molecules expression during tumorigenesis, further molecular biological study such as the analysis of processing mechanism of cytokeratin messenger RNA is needed.

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