Jpn. J. Cancer Res. 80, 840–847, September 1989

Expression of Type II and Type XI Collagens in Canine Mammary Mixed Tumors and Demonstration of Collagen Production by Tumor Cells in Collagen Gel Culture

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The development of cartilaginous collagen types, II and XI, in canine mammary mixed tumors was studied biochemically and immunohistochemically. In mixed tumor, an alcian blue-positive myxomatous region appeared in the stroma, where round-shaped proliferating myoepithelial cells were scattered. Type II collagen was distributed in metaplastic cartilage matrix, while type XI was located only in the pericellular region, where proliferating cells were positively stained with anti-actin and anti-keratin antibodies. The accumulation of collagen types II and XI in the tumor mass was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by immunoblotting of the extract of the lesion using type-specific antibodies to collagen types II and XI. Tumor cells isolated from metaplastic tumor mass expressed both collagen types II and XI and myoepithelial types of cytoskeleton in gel culture, in which an alcian blue-positive substance became detectable in the pericellular region on day 3 and type II and type XI collagens on day 5. This may be a useful model for studying chondrocyte-type gene expression during tumorigenesis.

Key words: Anti-collagen antibodies — Cartilage — Collagen gel culture — Immunohistochemistry — Pleomorphic adenoma (mixed tumor)

Benign mammary mixed tumor is one of the most common tumors in mammary gland of older female dogs, comprising 50 to 65% of all mammary tumors.[1,2] This mixed tumor resembles the pleomorphic adenoma developed in human salivary glands, based on histopathological findings.[3] In human breast, "mixed" salivary gland-type adenoma is a rare but recognized entity.[4–7] These mixed tumors are characterized by epithelial and myoepithelial growth and by the appearance of myxomatous substance, and cartilaginous and even bony tissues. Cartilage and bone of this sort are recognized to be developed from the metaplasia of proliferated myoepithelial cells, based on light and electron microscopic observations.[8–10] Myoepithelial cells, which surround the alveolar cells, forming a discontinuous basket-like network in mammary and salivary glands, have both epithelial and mesenchymal characteristics. Their morphology and cellular function resemble those of smooth muscle cells and the presence of smooth muscle myosin within the cells has been demonstrated.[10] However, myoepithelial cells are considered to arise from ductular epithelial cells on the basis of developmental studies in rat mammary gland[11] and experimental studies on rat salivary gland tumors.[12] The myoepithelial cell contains keratin, vimentin and myosin as its cytoskeletal constituents, which can serve as markers of the cell.[13,14]

It is well known that hyaline cartilage contains various types of collagen, proteoglycans and other proteins. Among them, collagen types II, IX, X and XI are the main proteinaceous components. Type IX collagen, which has recently been well characterized,[15–18] is known to play a role as a regulatory protein in fibril formation of type II collagen.[19] Type X collagen is strictly localized in the hypertrophic cell zone in the growth plate and functions in calcification of cartilage.[20]

Another collagenous protein, type XI collagen, is known to consist of a heterotrimer with 1α, 2α and 3α chains and behaves much like type V collagen on salt precipitation and in electrophoretic mobility.[21] This collagen retains non-triple helical domains[22] and its 3α chain is closely homologous to $\alpha 1$(II) chain in terms of the cyanogen bromide peptide pattern, but is resistant to interstitial collagenase in contrast to $\alpha 1$(II) chain.[23] Type XI collagen is located around the chondrocyte lacunae and cell surface region as shown by immunohistochemical examinations.[24] The function of type XI collagen is not well understood, but based on its tissue distribution, it seems to play a role in the interaction between the cell and the extracellular matrix.

In mixed tumors (pleomorphic adenoma), cartilaginous tissue is observed. This suggests that cartilage types of collagen are newly expressed by proliferating myoepithelial cells, which originate from ectodermal cells. Therefore, we examined the metaplastic cartilage

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developed in mammary mixed tumor using antibodies to type II and type XI collagens and were able to demonstrate the production of type II and type XI collagens by isolated tumor cells in collagen gel culture.

MATERIALS AND METHODS

Preparation and purification of collagens Canine epiphyseal cartilage was collected and washed with 0.15 *M* NaCl/50 m*M* Tris-HCl, pH 7.6 (Tris-saline) containing a protease inhibitor cocktail of 2 m*M* N-ethylmaleimide (NEM), 1 m*M* phenylmethylsulfonyl fluoride (PMSF), 5 m*M* ethylenediaminetetraacetic acid (EDTA) and homogenized in the same buffer. Proteoglycans and other noncollagenous proteins were extracted with 4*M* guanidine hydrochloride and the residue was digested with pepsin (50 mg/g wet weight, Boehringer Mannheim, FRG). The pepsin-solubilized fraction was neutralized to inactivate pepsin and fractionated by repeated differential salt precipitation as described previously.[25] In the final step of purification, type II and type XI collagens were dissolved in 0.15 *M* NaCl/2 *M* urea/50 m*M* Tris-HCl, pH 7.6, and passed through a DEAE-cellulose column.[26] The purity of both collagens was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Type I, III, IV and V collagens were obtained from canine placenta and purified by differential salt precipitation followed by DEAE cellulose chromatography.[26] Swine type XI collagen was also prepared from epiphyseal cartilage as described above.

Preparation and purification of antibodies to collagens Antigen collagen (2 mg each) was injected into white rabbits with Freund's complete adjuvant and after two booster injections of the antigen (1 mg each) with Freund's incomplete adjuvant, antiserum was collected, treated at 56°C for 30 min and subjected to affinity chromatography. Antiserum against type II collagen was first passed through a type I collagen-coupled CH-Sepharose 4B column (Pharmacia, Uppsala, Sweden), then adsorbed on a type II collagen-coupled Sepharose column equilibrated with Tris-saline and eluted with 3 *M* NaSCN/Tris-saline. Antiserum against type XI collagen was first passed through sequentially connected type I collagen-coupled and type II collagen-coupled Sepharose columns and finally adsorbed on a swine type XI collagen-coupled Sepharose column and eluted. Anti-type I collagen antibodies were also purified by affinity chromatography after passage through type II and type III collagen columns. The purity of antibodies against individual collagens was examined by both enzyme-linked immunosorbent assay (ELISA) and western blotting.

Immunochemical analysis ELISA was performed as previously described.[27] Polystyrene microtiter plates (Becton Dickinson and Co., Lincoln Park, NJ) were coated with 100 μl/well of 10 μg/ml purified collagen. One percent bovine serum albumin was used as a blocking agent. The plate was washed with phosphate-buffered saline containing 0.02% Tween 20 five times, then incubated with 100 μl/well of purified type-specific anti-collagen antibodies overnight at 4°C and with alkaline phosphatase-labeled anti-rabbit $F(ab')_2$ (Dako, Copenhagen, Denmark) for 1 h at 37°C. Finally, *p*-nitrophenyl phosphate/diethanolamine buffer was added and incubated at room temperature for 30 min. The reaction was stopped with 50 μl of 5 *M* NaOH and the absorbance was read at 405 nm.

SDS-PAGE was carried out with a 3% stacking gel, pH 6.8, and a 7.5% separating gel, pH 8.8, according to the method of Laemmli.[28] Immunoblotting was performed as follows.[29] Type I, II, III, IV, V and XI collagens were subjected to SDS-PAGE and the separated protein bands were electrophoretically transferred to a cellulose nitrate membrane. After blocking with 5% normal swine serum, the membrane was incubated with purified antibodies to individual types of collagen overnight at 4°C, then with horseradish peroxidase-labeled second antibody (Dako) for 30 min at 37°C, and immersed in 0.02% diaminobenzidine/50 m*M* Tris-HCl, pH 7.6, containing 0.03% H_2O_2 (DAB solution).

Tissue specimens Benign mammary mixed tumor tissues which had developed in six old female Japanese domestic dogs were surgically obtained and each specimen of the isolated tumor tissues was divided into three parts under sterile conditions. A part of the tumor tissue was immediately fixed in 4% paraformaldehyde/0.1 *M* phosphate buffer, pH 7.4. The tumor tissue was dehydrated and embedded in paraffin. When the tissue was partly mineralized, pretreatment with 0.5 *M* EDTA, pH 7.6, was performed before dehydration. The sections were routinely stained with hematoxylin and eosin, alcian blue (pH 2.5) and periodic acid-Schiff (AB-PAS), and Mallory's AZAN. The second part of the tissue was used for isolation of tumor cells and the remaining part was stored at −80°C for biochemical analysis.

Immunohistochemistry Immunoperoxidase staining was performed according to the peroxidase-antiperoxidase method.[30, 31] Anti-keratin rabbit antibody (Advance Co., Tokyo) and anti-actin rabbit antibody (Dako) were employed as markers of myoepithelial cells in tumor tissue. To examine the development of cartilaginous and osseous metaplasia, type-specific antibodies to type I, type II and type XI collagen were employed. Deparaffinized sections were pretreated with 0.05% protease (Type III, Sigma Chemical Co., St. Louis, MO) in Tris-saline for 10 min at 20°C, incubated with the first antibody overnight at 4°C, then with the second antibody (Dako) diluted to 1:100 for 30 min at 37°C and finally

with peroxidase-antiperoxidase complex (Dako) diluted to 1:100 for 30 min at 37°C. The section was immersed in DAB solution for 5 min at room temperature and counterstained with methyl green or hematoxylin.

Biochemical analysis Tumor tissue manifesting cartilaginous metaplasia was minced under a light microscope, washed with Tris-saline containing a protease inhibitor cocktail and treated with 4 *M* guanidine hydrochloride. The residue was digested with pepsin and the supernatant was dialyzed against 0.9 *M* NaCl/0.5 *M* acetic acid to separate type II collagen from type XI collagen. After centrifugation, the precipitate and supernatant fractions were dialyzed separately against 5 m*M* acetic acid and lyophilized. These samples were subjected to SDS-PAGE followed by immunoblotting with anti-type II or anti-type XI collagen antibodies. As a control, a non-mixed type adenoma specimen was solubilized with pepsin and subjected to SDS-PAGE followed by immunoblotting.

Cell culture The surgically obtained tumor mass was washed with sterilized phosphate-buffered saline, minced and explanted. Tumor cells were obtained by outgrowth of the explants and subcultured in Dulbecco's modified Eagle's medium (DMEM, Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, KC Biologicals, Lenaxa, KS) and antibiotic and antimycotic solutions (Gibco Lab). The cells were cultured in a 0.15% purified canine acid-soluble type I collagen gel/10% FBS/DMEM in a humidified atmosphere of 5% CO_2-air at 37°C. At predetermined periods of incubation, gels were fixed with 4% paraformaldehyde and embedded in paraffin. Thin sections were prepared; some were stained with alcian blue, pH 2.5, and others were examined immunohistochemically using type-specific anti-collagen antibodies.

RESULTS

Specificity of antibodies prepared to collagen types II and XI The antibodies to type II collagen reacted only with type II collagen but not with type I, III, IV and V collagens, except for 3α chain of type XI, which was reactive with the antibodies due to the homology of α1(II) chain to 3α chain (data not shown). On the other hand, the anti-type XI collagen antibodies purified by affinity chromatography using a swine type XI collagen-Sepharose column reacted exclusively with 1α chain, although a slight reaction with 2α chain was seen at a high titer of the antibodies (Fig. 1). No cross-reactions were observed with 3α chain or with type I, II, III, IV and V collagens. Our preliminary experiments on the purification of anti-type XI collagen antibodies using an antigen (canine type XI collagen)-Sepharose column showed that the antibodies reacted with both 1α and 2α chains.

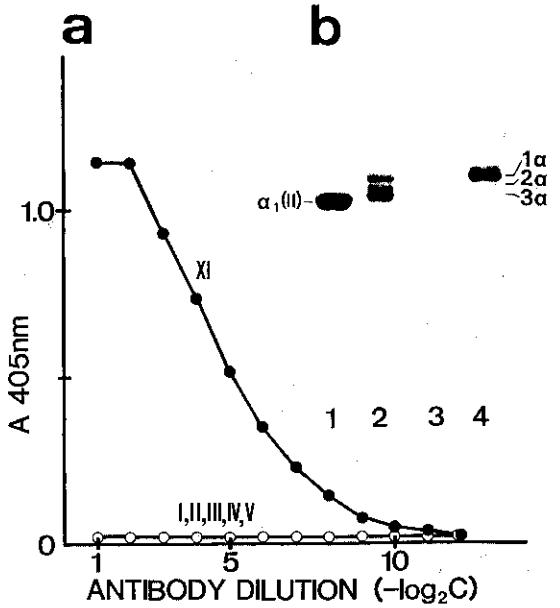

Fig. 1. ELISA of anti-canine type XI collagen antibodies. The cross-reactivity of the anti-type XI collagen antibodies with other types of collagen was tested by ELISA (a) and immunoblotting (b). (a), type XI (●), type I, II, III, IV and V collagens (○). (b) SDS-PAGE of type II (lane 1) and type XI collagens (lane 2) and immunoblotting of type II (lane 3) and type XI collagens (lane 4) by anti-type XI collagen antibodies.

Type-specificity of these antibodies was further confirmed by immunohistochemical stainings of epiphyseal cartilage. The anti-type II collagen antibodies reacted widely with the cartilage matrix or interterritorial region, while the anti-type XI antibodies reacted with territorial matrix in the epiphysis proper, showing quite a contrast to the type II collagen distribution.

Localization of type II and type XI collagen in cartilaginous metaplastic lesion In the earliest stage of mammary mixed tumor, epithelial cells showed a variety of proliferations, from a light hyperplasia to papillomatous growth. In the stroma, on the other hand, an alcian blue-positive myxomatous region first appeared (Fig. 2a). In addition, proliferated myoepithelial cells were scattered in the myxomatous mass and a variety of cell shapes from spindle type to round type were observed. Intercellular ground substance looked like hyaline cartilage (Fig. 2b), and sometimes bone formation with osteoblasts and osteoclasts was observed (Fig. 2c).

Immunohistochemical analysis of cartilaginous metaplasia showed that anti-type II collagen antibodies reacted with metaplastic cartilage matrix (Fig. 3a), but not with adenomatous regions or normal glandular tissues. In addition, the distribution patterns of type II

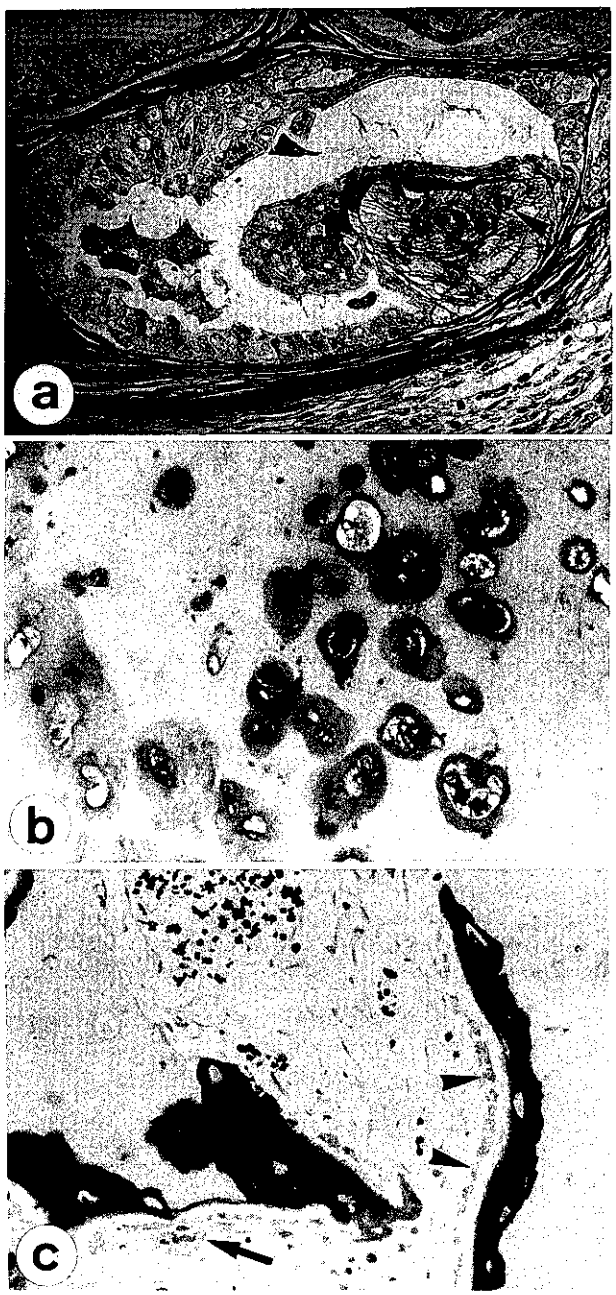

Fig. 2. Histological features of mammary mixed tumor. (a) In the earliest stage, an alcian blue-positive myxomatous region appeared between proliferating epithelium and basement membrane. Myoepithelial cells (arrowhead) were scattered in the myxomatous area (alcian blue, pH 2.5, and periodic acid-Schiff stain, ×200). (b) Irregularly arranged chondrocyte-like cells were seen in the chondroid region (alcian blue and periodic acid-Schiff stain, ×200). (c) Osteoid was seen in the cartilaginous tissue with osteoblasts (arrowhead) and osteoclasts (arrow) (Mallory's AZAN stain, ×200).

collagen were not homogeneous but fibrous, in contrast to normal hyaline cartilage (Fig. 3b). Anti-type XI collagen antibodies reacted strongly with a pericellular region called the territorial matrix, but reacted weakly with metaplastic cartilage matrix (Fig. 3c). In addition, the osteoid developed in metaplastic cartilage was positively stained with antibodies to type I collagen (Fig. 3d). When we examined nodular masses at the early stage of cartilaginous metaplasia which were hardly recognized to be normal hyaline cartilage, however, the region was not stained with anti-type II collagen antibodies, but was positively stained with anti-type XI collagen antibodies. Proliferating cells were positively stained with anti-actin and anti-keratin antibodies, indicating myoepithelial cell origin (data not shown).

Biochemical evidence for cartilage-type collagens in mammary mixed tumor Biochemical analysis of collagen isolated from the tumor mass was performed by SDS-PAGE and immunoblotting with type-specific anti-collagen antibodies. Figure 4 shows SDS-PAGE patterns of two collagenous fractions separated after solubilization of the tumor mass with pepsin treatment: the 0.9 *M* NaCl/0.5 *M* acetic acid precipitate (lanes 1, 3, 5) and the supernatant (lanes 2, 4, 6). The presence of type II collagen in the precipitate and type XI collagen in the supernatant was confirmed by immunoblotting analysis using purified antibodies to type II and type XI collagen (lane 3–6). Neither type II nor type XI collagen was detected in the pepsin extracts of normal mammary gland and non-mixed type adenoma tissues (data not shown).

Cartilaginous collagen expression in tumor cells in collagen gel culture To further analyze the switching mechanisms of gene expression of cartilaginous-type collagen in affected cells of the mixed tumor, tumor cells were isolated and cultured both in monolayer and in collagen gel. Tumor cells subcultured in monolayer were spindle-shaped and contained keratin and actin as cytoskeletal proteins. The cells were shown by immunohistochemistry to produce type I collagen but not type II or type XI collagen (data not shown). However, when cultured in collagen gel, the cells began to produce type II and type XI collagens and no indication of type I collagen production was observed. This strongly suggests that the interaction between tumor cells and matrix collagen affects collagen phenotype expression. The collagen gel began to contract on the third day after tumor cells were inoculated. The gel contraction continued for about two weeks then ceased, although the contraction rate varied depending on the cell populations and collagen concentrations employed. The cells in collagen gel retained a round shape, forming a cavity similar to a chondrocyte lacuna which stained positively with alcian blue on day 3 (see Fig. 5a), while type II collagen (Fig. 5b) and type XI collagen (Fig. 5c) became detectable on day 5.

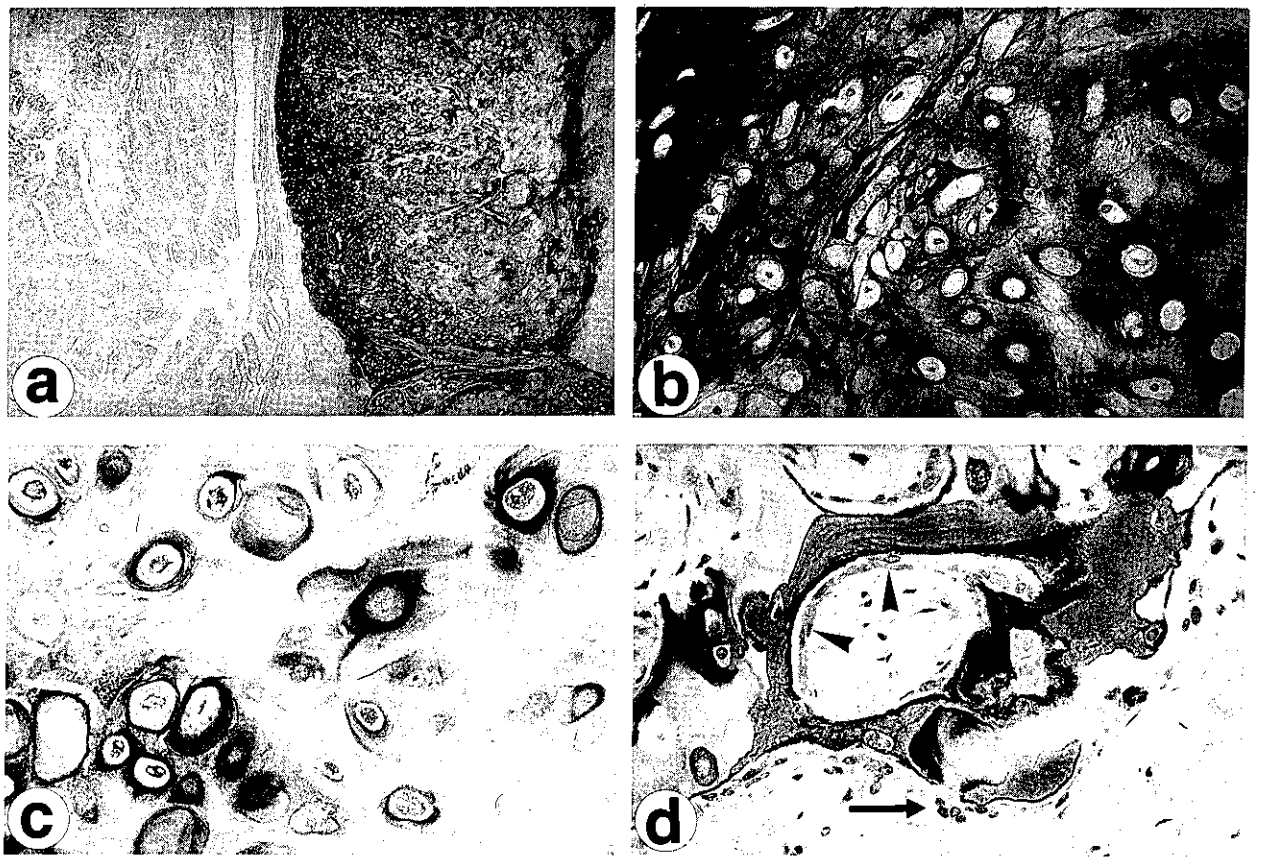

Fig. 3. Peroxidase-antiperoxidase stainings of cartilaginous and osteoid regions. (a) Cartilaginous region was stained with anti-type II collagen antibodies. No positive reaction was seen in the adenomatous region (×20). (b) Higher magnification revealed fibrous distribution of type II collagen in contrast to normal hyaline cartilage (×100). (c) Anti-type XI collagen antibodies strongly stained the territorial matrix region of chondrocyte-like cells in the cartilaginous region (×200). (d) The osteoid developed in the cartilaginous region was positively stained with anti-type I collagen antibodies. Osteoblasts (arrowhead) and osteoclasts (arrow) (×200).

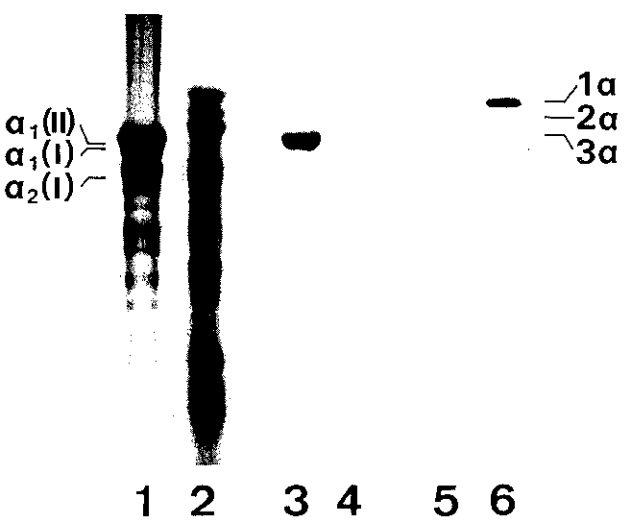

Fig. 4. SDS-PAGE and immunoblotting of pepsin-solubilized material obtained from tumor tissue. The solubilized fraction was dialyzed against 0.9 *M* NaCl/0.5 *M* acetic acid to separate precipitable collagen (lanes 1, 3, 5) from soluble collagen (lanes 2, 4, 6). SDS-PAGE (lanes 1, 2). Immunoblotting was performed with anti-type II collagen antibodies (lanes 3, 4) and anti-type XI collagen antibodies (lanes 5, 6).

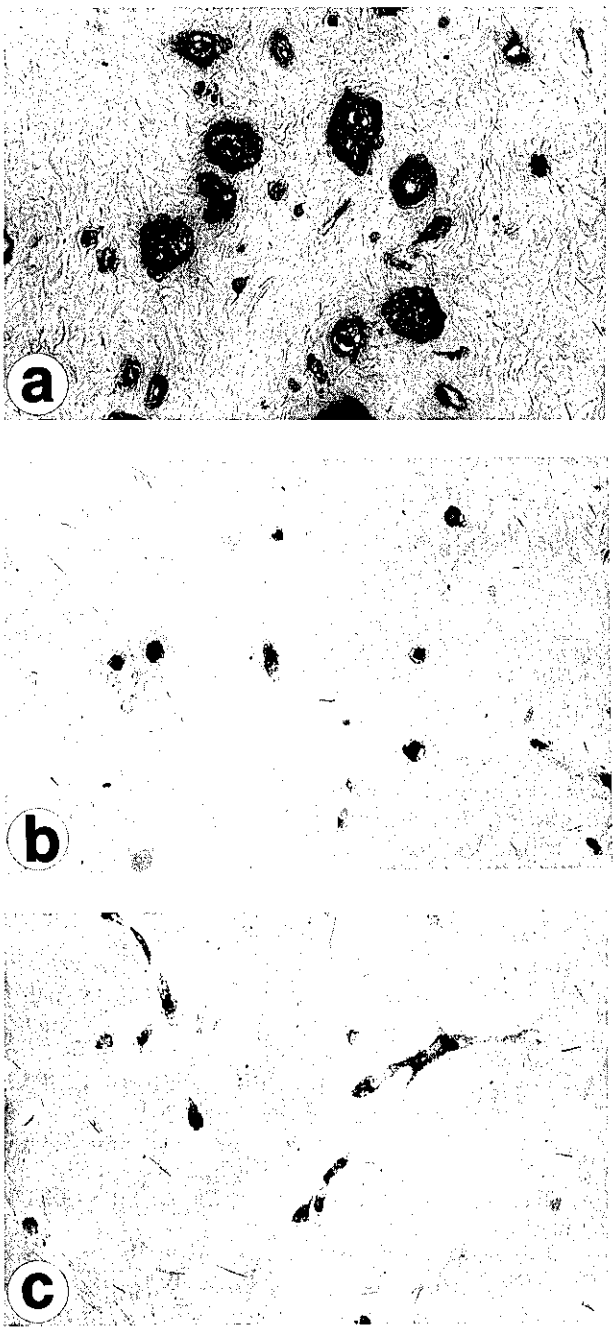

Fig. 5. Alcian blue and peroxidase-antiperoxidase stainings of tumor cells cultured in collagen gel for 12 days. (a) Alcian blue-positive material accumulated around the pericellular region (×200). (b) Cells stained well with both anti-type II collagen antibodies (×200) and (c) anti-type XI collagen antibodies (×200).

DISCUSSION

The cartilaginous tissue developed in canine mammary mixed tumor (pleomorphic adenoma) is known to be of myoepithelial origin based on histopathological observations[8,9] and is not distinguishable from normal hyaline cartilage based on physical characteristics and histochemical stainings for chondroitin sulfate and alkaline phosphatase.[9] However, little information is available on collagen types in the mixed tumor mass so far, although the presence of type IV collagen in the myxoid area of pleomorphic adenoma in human salivary glands was observed immunohistochemically.[10]

In this study, we could demonstrate immunohistochemically the presence of type II and type XI collagens in the cartilaginous tissue developed from metaplasia of proliferated myoepithelial cells. These results were confirmed by extraction of the two types of collagen from the tumor mass. In addition, active production of both type II and type XI collagens by isolated tumor cells which carried actin and keratin was demonstrated in collagen gel culture.

In an earlier stage of cartilaginous metaplasia, where myoepithelial cells were proliferating in the myxomatous area, the cells reacted only with antibodies to type XI, but not with antibodies to type II collagen. Subsequently the intercellular matrix became positive to staining with anti-type II collagen antibodies. In the late stage of cartilaginous metaplasia, which is indistinguishable from normal hyaline cartilage, the cartilaginous matrix reacted strongly with anti-type II collagen antibodies, but type XI collagen was exclusively located in the territorial region of scattered cells. There observations suggest that type XI collagen may play a crucial role in tissue morphogenesis of cartilaginous metaplasia.

Terada *et al.*[32] reported the development of cartilage-like tissue from androgen-dependent mouse mammary carcinoma, an undifferentiated medullary carcinoma, when it was inoculated into castrated male mice. The cartilage-like tissue showed close similarities in electron-microscopical and histochemical features to normal cartilage tissue. This suggests that the tumor cells may change into chondrocyte-like cells under androgen-depleted conditions, and that development of cartilaginous metaplasia is closely related to a balance of sex-steroid hormone levels in the recipient animal. Mixed tumors in canine mammary tissue are reported to develop with high incidence in the estrus state.[1] Taking the findings described above into account, the development of cartilaginous metaplasia in a mixed tumor may also be related to the disturbance of sex hormone levels.

The tumor cells in our case, however, expressed cartilaginous types of collagen in collagen gel culture without addition of any exogenous hormones or growth factors

except for 10% FBS, under which conditions the cells in monolayer culture failed to produce type II collagen, but did produce type I collagen. The expression of myoepithelial cytoskeletons, actin and keratin, in the tumor cells was maintained in either culture condition. These observations strongly suggest that the isolated tumor cells had acquired a potential to differentiate into chondrocytes at an early stage of tumorigenesis, under which conditions proliferating mammary epithelial cells may induce adjacent myoepithelial cells to differentiate. It should be noted that cartilaginous metaplasia observed in the mixed tumor is an atypical type of metaplasia, in that it develops from cells of ectodermal origin (myoepithelium) which change into cells that can also express the function and morphology of cells of mesenchymal origin (chondrocytes).

Of most interest in this study was the finding that cells isolated from the mixed tumor produced either type I collagen or cartilage types of collagen, type II and type XI, depending on the culture conditions. The cells carrying this switching capacity, together with actin and keratin expressions, have been cloned recently (Arai *et al.*, manuscript in preparation). This cell culture system seems to be a useful tool for elucidation of switching mechanisms of chondrocyte-type gene expression including collagen phenotypes during pathomorphogenesis. Further studies along this line are in progress.

ACKNOWLEDGMENTS

The authors are grateful to Dr. T. Katoh (Small Animal Surgeons Association) for the supply of surgical specimens and Mr. R. Fukuda for technical assistance in the preparation of anti-collagen antibodies. Thanks are also due to Dr. T. Nakamura for valuable discussions throughout this study.

(Received May 6, 1989/Accepted July 6, 1989)

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