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Simultaneous Expression of Type IX Collagen and an Inhibin-related Antigen in Proliferative Myoepithelial Cells with Pleomorphic Adenoma of Canine Mammary Glands

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To identify an alcian blue-positive component expressed in the early stage of mammary mixed tumor and to pursue the possible involvement of a tumorigenic inducing factor, monoclonal antibodies to type IX collagen were generated and used to investigate the immunohistochemical kinetics of type IX collagen expression by the myoepithelial cell-derived chondrocyte-like cells during the development of chondrometaplasia. We also examined the expression of inhibin-related antigen using antibodies to a synthetic peptide spanning amino acids 1–30 of the inhibin α chain. At the earliest stage of chondrometaplasia, where myoepithelial cells began to proliferate inside the basement membrane, the cells expressed type IX collagen together with an inhibin-related antigen which was immunoreactive with the anti-inhibin peptide antibodies. The expression of the inhibin-related antigen was also demonstrated in normal embryonic chondrocytes and myoblasts, but was much less strong in mature chondrocytes and myotubes, strongly suggesting that the inhibin-related antigen is involved in the development of chondrocytes and myoblasts from undifferentiated mesenchymal cells as well as proliferating myoepithelial cells as a chondro-progenitor cell in the mammary mixed tumor. The pathophysiological significance of type IX collagen expression as a possible cell marker of the progenitor cell in myoepithelial cell-related chondrometaplasia is also discussed.

Key words: Anti-collagen antibody — Anti-inhibin antibody — Immunohistochemistry — Myoepithelial cell — Pleomorphic adenoma (mixed tumor)

In previous papers, we reported the localization of type II and type XI collagens in canine mammary mixed tumor (pleomorphic adenoma)[1] and the expression of these two types of collagen by tumor-derived cells in collagen gel culture.[2] Proliferating myoepithelial cells first formed a nodular mass with alcian blue-positive glycosaminoglycan, and then cartilage-like matrix, where type II and type XI collagens were accumulated. Type XI was densely localized in the territorial capsule of chondrocyte lacunae and type II in the interterritorial matrix in metaplastic cartilage, as observed in normal hyaline cartilage. The tumor cells produced type I collagen, but not type II in monolayer culture. However, when these cells were placed in collagen gel, the cells began to produce an alcian blue-positive glycosaminoglycan and type II and type XI collagens, which are all characteristic of the chondrocyte.

To pursue the nature of the alcian blue-positive component(s) produced in the early stage of the mixed tumor and its inducing factor(s), monoclonal antibodies to type IX collagen or proteoglycan PG-Lt,[3] were elaborated. In addition, antibodies to inhibin and to a 30-residue synthetic peptide covering the amino-terminal region of its alpha subunit[4,5] were used to explore a possible candidate for the inducing factor.

Here, we present immunohistochemical evidence for expressions of type IX collagen and inhibin-related antigen by the myoepithelial cell at the initial stage of its proliferation inside the basement membrane. The expression of this inhibin-related antigen by chondrocytes in normal fetal cartilage as well as in the nodular mass of mixed tumor is also described.

MATERIALS AND METHODS

Preparation of type IX collagen Hyaline cartilage tissues obtained from canine epiphysis and porcine femur were separately washed three times with chilled Tris-saline (0.15 *M* NaCl/50 m*M* Tris-HCl, pH 7.6) containing a protease inhibitor cocktail (2 m*M* N-ethylmaleimide/1 m*M* phenylmethylsulfonyl fluoride/5 m*M* ethylenediaminetetraacetic acid/10 m*M* benzamidine hydrochloride). The tissue specimen was homogenized and digested with pepsin (50 mg/g wet weight, Boehringer Mannheim GmbH, Mannheim, Germany) in 0.5 *M* acetic acid. The pepsin-solubilized material was neutralized to

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inactivate pepsin and fractionated by differential salt precipitation as described previously.[6] Briefly, type II and type XI collagens were precipitated at a concentration of 1.2 *M* NaCl in 0.5 *M* acetic acid and removed by centrifugation. Type IX collagen was precipitated at a concentration of 1.8 *M* NaCl, and the precipitate was dissolved in 5 m*M* acetic acid, dialyzed, and lyophilized.

To prepare the intact form of type IX collagen, CsCl gradient ultracentrifugation was performed.[7] Hyaline cartilage tissue was extracted with 4 *M* guanidine hydrochloride/Tris-saline containing protease inhibitor cocktail at 4°C overnight and centrifuged. To the solubilized material, CsCl was added to give an initial density of 1.47 g/ml. A dissociative gradient was established by ultracentrifugation in a Hitachi RP-45T rotor (100,000*g*, 15°C, 72 h), then fractionated from the bottom of the tube and immediately dialyzed against 5 m*M* acetic acid. Hexuronate and protein contents in each fraction were determined by the carbazole method of Bitter and Muir[8] and measurement of the absorption at 280 nm, respectively.

Glycosaminoglycan chains of type IX collagen were analyzed by two-dimensional electrophoresis on a cellulose acetate membrane as previously described.[9]

Chondroitinase ABC-treated type IX collagen was dialyzed against 5 m*M* acetic acid and ultracentrifuged. The supernatant was gently mixed with an equal volume of glycerol and sprayed onto freshly cleaved mica, dried *in vacuo*, then rotary-shadowed with platinum-palladium and carbon. Electron micrographs were taken on a JEOL 100C electron microscope.

Production of monoclonal antibodies against type IX collagen Purified swine type IX collagen (HMW component, 50 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 (P3x63Ag8Ul) in the presence of 50% polyethylene glycol (MW: 4,000, Merck, Darmstadt, Germany). Hybridomas were suspended in serum-free medium (Hymedium-606, Kojin Co., Tokyo) 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 seeded in microtiter plates. Antibody-producing clones were selected by enzyme-linked immunosorbent assay (ELISA), followed by immunohistochemical detection on canine fetal cartilage sections. Recloning was performed twice according to the method of limiting dilution in serum-free medium supplemented with hypoxanthine and thymidine. The medium containing monoclonal antibody was collected, concentrated by ammonium sulfate precipitation and used in the following experiments. The subclass of the monoclonal antibodies was determined by ELISA using a Monoclonal Mouse Isotyping Kit (Pharmingen, San Diego, CA).

Immunochemical analysis ELISA was performed as previously described.[1] Microtiter plates were coated with 1 μg of antigen or purified canine/porcine collagen type I, II, III, IV, V, IX or XI. Each fraction obtained by CsCl density gradient centrifugation was treated with chondroitinase ABC (Chase ABC; protease-free, Seikagaku Kogyo Co., Tokyo) at 4°C overnight. One percent casein (Hammarsten, Merck) was used as a blocking agent. The plates were washed with phosphate-buffered saline containing 0.02% Tween 20 (PBS-Tween), incubated with 100 μl/well of cultured medium at 4°C overnight and then with alkaline phosphatase-conjugated anti-mouse IgG (Dakopatts, Glostrup, Denmark) at 37°C for 1 h. The plates were washed 5 times, and the wells positive for alkaline phosphatase were selected by adding *p*-nitrophenyl phosphate/diethanolamine-HCl, pH 9.8, and measuring the absorbance at 405 nm. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with a 3% stacking gel, pH 6.8 and a 5% separating gel, pH 8.8, according to the method of Laemmli.[10] Type IX collagen fraction obtained by CsCl density gradient centrifugation was treated with Chase ABC (protease-free). The resulting sample was subjected to SDS-PAGE and the separated protein bands were electrophoretically transferred onto a nitrocellulose membrane. After blocking with 1% casein, the membrane was incubated with the first antibody, and then with alkaline phosphatase-conjugated second antibody (Dakopatts) and finally immersed in 0.01% nitro blue tetrazolium (Promega Co., Madison, WI) and 0.005% 5-bromo-4-chloro-3-indolyl phosphate (Promega Co.) diluted with 100 m*M* Tris-HCl, pH 9.5, containing 100 m*M* NaCl and 5 m*M* $MgCl_2$.

Immunohistochemistry Adult canine femoral epiphysis was obtained at autopsy of dogs with no apparent joint disorders, and fetal femur was obtained at artificial abortion at a late-stage pregnancy. Benign mammary mixed tumor tissues developed in eight female Japanese domestic dogs were surgically obtained and immediately fixed in 4% paraformaldehyde/0.1 *M* phosphate buffer, pH 7.4. These tissue specimens were dehydrated and embedded in paraffin. Immunoperoxidase staining was performed according to the peroxidase-antiperoxidase method.[1] Polyclonal anti-cytokeratin rabbit antibodies (Dakopatts) were employed as a marker of the myoepithelial cell in normal and tumoral mammary glands. Deparaffinized thin sections were pretreated with 0.05% protease (type III, Sigma Chemical Co.) in Tris-saline for 10 min at 20°C or with Chase ABC (0.5 unit) in Tris-saline, pH 7.6 at 37°C overnight, incubated with the first antibody or culture medium overnight at 4°C, then with the second antibody (Dakopatts) diluted to 1:100 for 30 min

at 37°C and finally with peroxidase/antiperoxidase complex (Dakopatts) diluted to 1:100 for 30 min at 37°C. Each section was incubated with 3,3′-diaminobenzidine (DAB)/Tris-saline containing 1% cobalt chloride and 1% nickel ammonium for heavy metal intensification,[11] then immersed in DAB solution supplemented with 0.03% H_2O_2.

Rabbit antiserum generated against a synthetic peptide spanning amino acids 1–30 of porcine inhibin alpha subunit (TAPLPWPWSPAALRLLQRPPEEPAVHADCY)[12] and rabbit antiserum generated against bovine native inhibin (alpha and beta subunits heterodimer) were generous gifts from Prof. S. Sasamoto, Department of Veterinary Physiology, Tokyo University of Agriculture and Technology. These antisera were used for immunohistochemical examinations of canine mammary mixed tumor. Prior to this experiment, we confirmed that ovarian granulosa cells, which are known to be the main inhibin-producing cells, were positively stained with these two antisera.

RESULTS

Specificity of monoclonal antibody to type IX collagen

The monoclonal antibody (1308E) to the HMW component of type IX collagen belonged to the subclass of mouse IgG2a and kappa-light chain, based on ELISA. The reactivity of 1308E to intact type IX collagen could be detected only after treatment with Chase ABC. 1308E reacted with both canine and swine type IX, but not with type I, II, III, IV, V or XI from either of the animals (Fig. 1). However, immunoreactivity of 1308E with swine type IX was much higher than that with canine type IX, and markedly diminished under either denaturing or reducing conditions, suggesting that 1308E recognized a conformational epitope on the molecule. A typical CsCl density gradient pattern, showing the contents of hexuronate and protein and the reactivity of each fraction with 1308E, is given in Fig. 2. Two major peaks containing hexuronate were observed in both the bottom and top layers, while the protein components were mostly concentrated in the top layer. Fig. 2 also shows that type IX collagen was exclusively present in the top layer based on ELISA after Chase ABC treatment. Immunoblotting analysis using 1308E (Fig. 3a) showed definite reaction with unreduced Chase ABC-treated type IX collagen (apparent molecular weight, 350 kDa), but the reactivity was markedly suppressed under denaturing and reducing conditions, unless immunoblotting was carried out at 4°C (see the 150 kDa band on lanes 2 and 4 in Fig. 3a). This is consistent with the results obtained by ELISA described above.

The purified intact type IX collagen, which was bacterial collagenase-sensitive, contained a hybrid structure component of chondroitin sulfate and dermatan sulfate as reported[7] (data not shown).

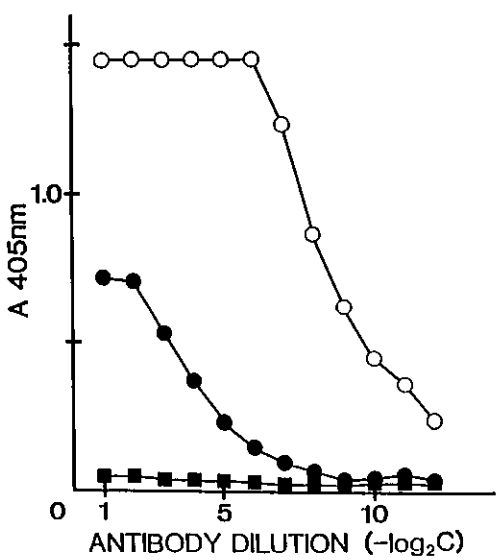

Fig. 1. ELISA of anti-swine type IX collagen monoclonal antibody. Swine (antigen) type IX collagen (○), canine type IX collagen (●), swine and canine type I, II, III, IV, V and XI collagens (■).

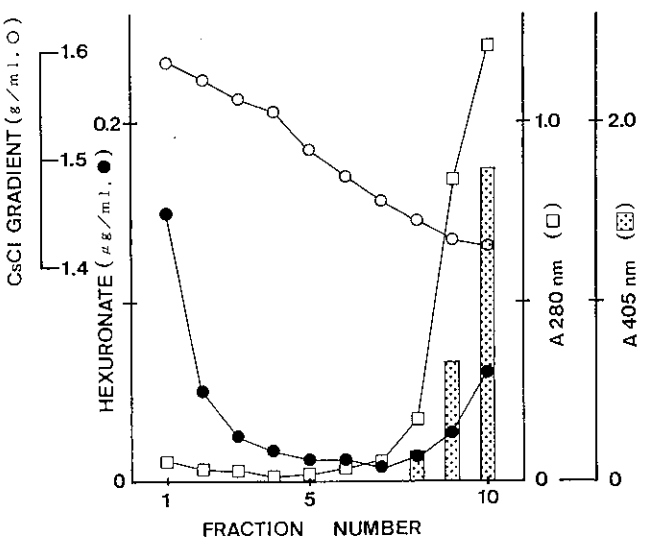

Fig. 2. Fractionation of type IX collagen (PG-Lt) by means of CsCl gradient (○) ultracentrifugation. Protein (□) and hexuronate (●) contents in each fraction were determined by measuring the absorbance at 280 nm and by the carbazole method, respectively. Immunoreactivity with anti-type IX collagen monoclonal antibody is shown by the dotted bars (ELISA).

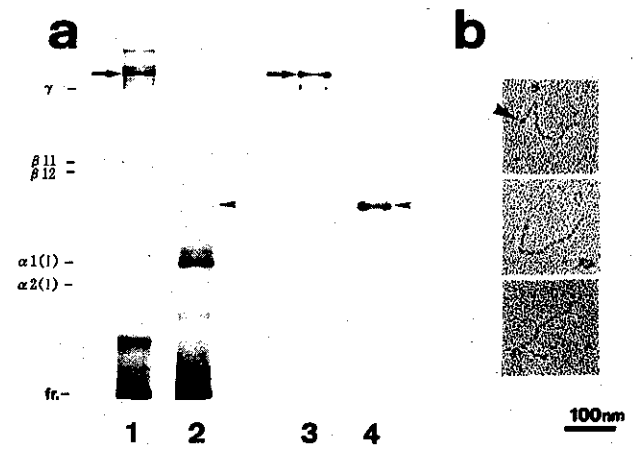

Fig. 3. Purified type IX collagen as observed by immunoblotting and rotary-shadowing. (a) SDS-PAGE (lanes 1, 2) and immunoblot with anti-type IX collagen monoclonal antibody (lanes 3, 4) of swine type IX collagen before (lanes 1, 3; arrow) and after (lanes 2, 4; arrowhead) reduction. (b) Rotary-shadowed replicas of swine type IX collagen after treatment with chondroitinase ABC, showing the globular domain (arrowhead) and kink structure.

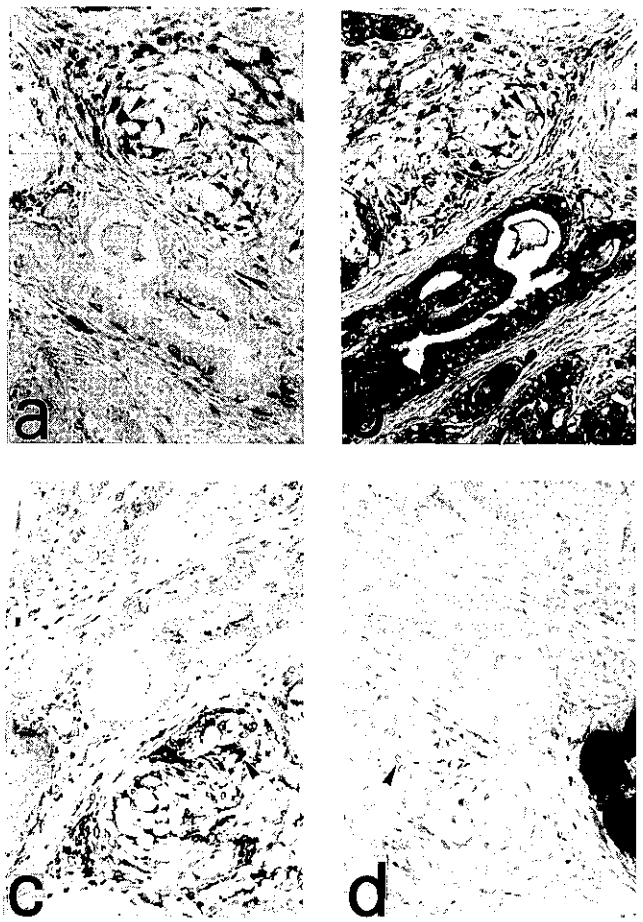

Fig. 4. Immunohistochemical stainings of a precartilaginous region of mixed tumor on mirror-image sections (a vs. b and c vs. d) with antibodies to vimentin (a), cytokeratin (b), type IX collagen (c) and type II collagen (d). The arrowheads shown in (a) and (b) as well as (c) and (d) indicate identical cells, respectively. a, b, c and d: ×150.

Rotary shadowing of the type IX collagen molecule showed a characteristic kink structure with a globular domain at one end, as reported[3] (Fig. 3b).

Differential expressions of type II and type IX collagen by proliferating myoepithelial cells 1308E strongly stained the chondrocyte-like cells in metaplastic cartilage mass, but weakly stained the cartilage matrix, whereas anti-type II collagen antibodies strongly reacted with the cartilage matrix. The proliferating myoepithelial cells in the nodular mass were demonstrated by the positive staining with anti-vimentin antibodies (Fig. 4a), but not by anti-cytokeratin antibodies (Fig. 4b), suggesting that the intermediate-type cytoskeleton had changed from cytokeratin to vimentin with tumorigenesis. While these proliferating cells were well stained with 1308E (Fig. 4c), type II collagen (Fig. 4d) and type XI collagen (data not shown) were not yet detected. These results indicate that proliferating myoepithelial cells first expressed type IX collagen as a cartilaginous type of collagen. Furthermore, in the very early stage of the tumor where myoepithelial cells did not show apparent proliferation, type IX collagen was already being produced by some myoepithelial cells *in situ* (Fig. 5a) and the intermediate-type cytoskeleton of these cells was cytokeratin (Fig. 5b). Even at the stage where type IX-expressing myoepithelial cells began to proliferate inside the basement membrane (Fig. 5c) and glandular cells showed independent adenomatous growth, expressing a large amount of keratin-intermediate filament, the proliferating myoepithelial cells were still expressing cytokeratin (Fig. 5d).

These observations indicate that some myoepithelial cells programmed as the progenitor cells of chondrocytes first express type IX collagen before proliferation *in situ* and switch their intermediate-type cytoskeleton from cytokeratin to vimentin.

Expression of inhibin-related antigen by normal embryonic chondrocytes and proliferating myoepithelial cells during chondrogenesis Immunohistochemical stainings using two different anti-inhibin antisera generated against inhibin α chain synthetic peptide and native inhibin showed quite similar positive reactions. These antibodies strongly stained immature chondrocytes in

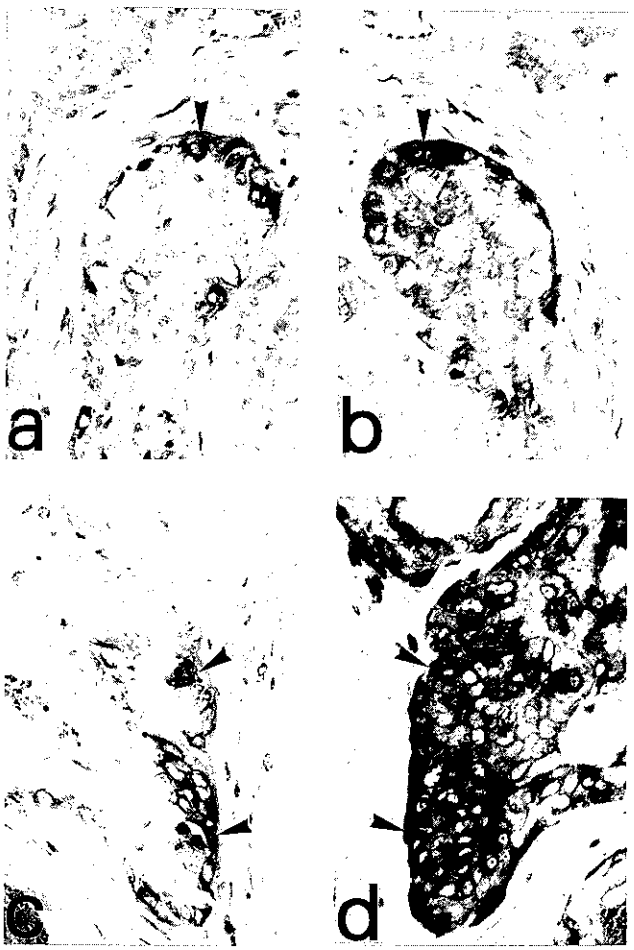

Fig. 5. Immunohistochemical stainings of proliferating myoepithelial cells inside the basement membrane (arrowheads) with anti-type IX collagen (a, c) and anti-cytokeratin (b, d) antibodies on mirror-image sections (a vs. b and c vs. d). a, b, c and d: ×320.

normal rat (Fig. 6a) and canine (Fig. 6b) fetus, although a much smaller population of chondrocytes was reactive in adult cartilage (Fig. 6c). Proliferating myoepithelial cells and chondrocyte-like cells in metaplastic cartilage of canine mammary pleomorphic adenoma were strongly stained with these antibodies (Fig. 6d). In the early stage of cartilaginous metaplasia, where type II collagen and type XI collagen were not detectable, proliferating myoepithelial cells were positively stained with anti-inhibin antibodies (Fig. 6e) and 1308E (Fig. 6f). These mirror-image sections revealed simultaneous expression of inhibin-related antigen and type IX collagen in the same cell. Chondrocyte-like cells in the cartilaginous region were also stained with both antibodies.

DISCUSSION

Our previous studies on canine mammary mixed tumor (pleomorphic adenoma) demonstrated the expression of cartilaginous types of collagen in the tumor tissue[1] and in isolated tumor cells in collagen gel culture.[2] The distributions of collagen type II and type XI in the metaplastic cartilage were quite similar to those in normal hyaline cartilage, and the dense distribution of type XI in the territorial matrix surrounding chondrocytes suggests its biological function in providing an appropriate microenvironment distinct from the cartilage matrix for chondrocytes to maintain homeostatic expression.

In this study, we investigated the immunohistochemical kinetics of the third cartilaginous collagen, type IX, during the development of chondrometaplasia in the canine mammary mixed tumor. Type IX collagen was detected in mammary myoepithelial cells starting to proliferate inside the basement membrane. At this earliest stage, proliferating myoepithelial cells did not show characteristics of the chondrocyte or expression of any other cartilaginous collagen type, II or XI, expressed by immature chondrocytes in normal cartilage development or by myoepithelial cell-derived chondrocyte-like cells in the metaplastic cartilage. Only type IX collagen was detected in their cytoplasm, but it was not secreted into the extracellular matrix, where few histological characteristics of cartilaginous tissue were observed. This type of collagen, therefore, could be an excellent cell marker of the progenitor cell in myoepithelial cell-related chondrometaplasia and may have an important role(s) in the earliest stage of chondrometaplasia, such as promotion of cell proliferation and/or cell movement, as a proteoglycan. In this connection, it should be noted that there is a correlation between expressions of type II and type IX collagen and a tumor-suppressor gene phenotype in some cell lines. Furthermore, the cDNA and predicted amino acid sequences of its suppressor gene are highly homologous to those of the chondrocyte-specific collagens type II and type IX.[13] Therefore, the distinctive structural properties of type IX collagen and the homology to the tumor-suppressor gene make type IX collagen a candidate marker of cartilaginous tumors, as reported.[14]

Fitch *et al.*[15] reported independent deposition of type II and type IX collagen in the developing chicken notochord and indicated that type II became detectable prior to type IX, while in the chondrogenic mesenchyme these collagens became detectable simultaneously. In addition, they found that the expression of subepithelial type II collagen in chick hind limb was not associated with subsequent chondrogenesis, and type IX collagen is not involved in the induction of mesenchymal chondrogenesis. However, our present data, with special reference to chondrometaplasia developed in the mammary

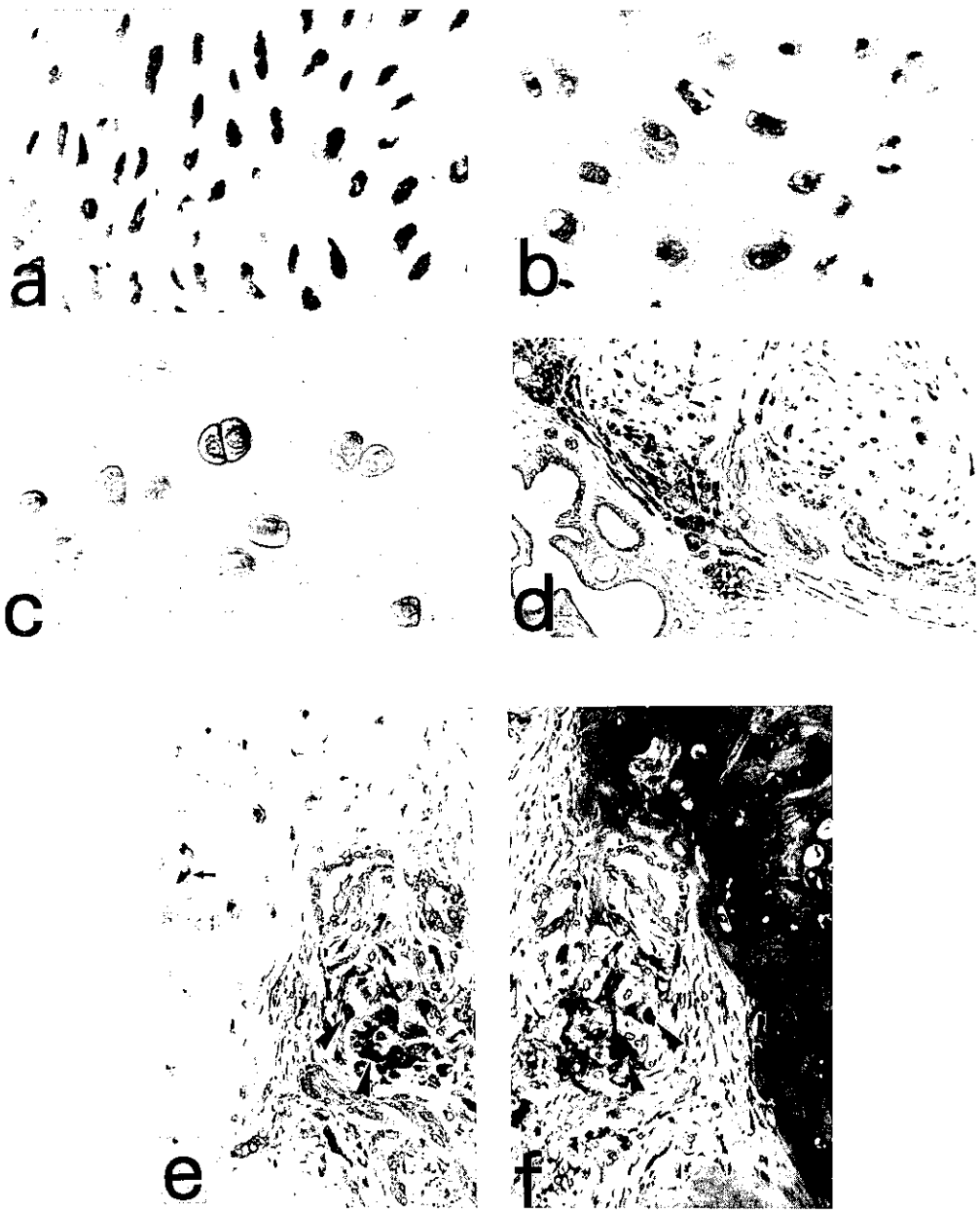

Fig. 6. Immunohistochemical stainings of normal cartilage and mixed tumor tissues with anti-inhibin antibodies. Rat embryonal (a), canine embryonal (b) and adult (c) cartilage and canine mammary mixed tumor (d) were stained with the antibodies. The mirror-image sections immunostained with anti-inhibin (e) and anti-type IX collagen (f) antibodies are also shown. Identical cells positively stained with anti-inhibin and anti-IX collagen are indicated by small arrows (chondrocyte-like cells in cartilaginous tissue) and by arrowheads (proliferating myoepithelial cells in nodular mass). a, b and c, ×360; d, ×40; e and f, ×84.

mixed tumor, suggest that type IX collagen expression is involved in the induction of chondrogenesis.

To our surprise, polyclonal anti-inhibin antibodies strongly stained normal embryonic chondrocytes, myoblasts (data not shown) and proliferating myoepithelial cells in mammary mixed tumor. This reaction was diminished or disappeared in mature chondrocytes and myotubes. Normal myoepithelial cells in mammary gland were not stained with the antibodies. However, when myoepithelial cells began to proliferate, these cells

became simultaneously positive to both anti-inhibin and anti-type IX collagen antibodies. Furthermore, antibodies generated against a synthetic peptide corresponding to amino-acid residues 1–30 of the inhibin α chain also strongly stained immature chondrocytes and myoblasts, suggesting that the inhibin-related antigen is involved in the development of chondrocytes and myoblasts from undifferentiated mesenchymal cells. This hypothesis was supported by the positive reaction with proliferating myoepithelial cells as chondro-progenitor cells in the mammary mixed tumor. It is well known that the inhibin molecule is an α and β chain heterodimer and functions as a down-regulatory factor of the follicular stimulating hormone (FSH).[16] A homology was present between inhibin β chain and transforming growth factor-β (TGF-β),[17] and the activin consisting of inhibin β chain homodimer is known to be an FSH secretion-stimulating factor.[16] Both TGF-β1 and TGF-β2 are capable of inducing chondrogenesis and osteogenesis in the rat femur.[18] Furthermore, Rosa *et al.*[19] reported that TGF-β had a mesoderm-inducing activity in *Xenopus* oocytes. Other members of the TGF family such as bone morphogenetic proteins,[20–22] including osteogenin[23] and osteogenic proteins,[24] were recognized to be cartilage- and/or bone-inducing factors based on *in vivo* studies. However, the antibodies we used this study were generated against inhibin α chain peptide, which does not belong to the TGF-β family, and were not crossreactive with TGF-β1 by radioimmunoassay. Therefore, inhibin-related antigen may function at some stage(s) in the differentiation of mesenchymal cells to chondrocytes, including chondrometaplasia. Although the myoepithelial cell is known to originate from the ectoderm, Palmer *et al.* reported that the cell has smooth muscle myosin as a cytoskeletal protein.[25] They also demonstrated smooth muscle myosin in the myoepithelial cells in both normal tissue and pleomorphic adenoma. Taking all these data into account, we suggest that ectoderm-originated myoepithelial cells would retain the characteristics of the mesoderm-originated smooth muscle cell and the differentiating capacity to chondrocytes during tumorigenesis. We have recently generated several monoclonal antibodies against inhibin-related antigen. Immunoblot analysis revealed that the monoclonal antibodies recognized a 200 kDa (apparent molecular weight) component, which dissociated into 52 kDa and 26 kDa proteins under reducing conditions (data not shown). Molecular cloning as well as characterization of the biological function of this antigen are in progress.

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