Immunohistochemical Examination of Neural Cell Adhesion Molecule (NCAM), Tenascin and Fibronectin on the Development of Cartilaginous Tissue in Canine Mammary Mixed Tumors

Katsuhiko ARAI, Masayuki NAOI¹⁾, and Kohkichi UEHARA

Department of Scleroprotein Chemistry and Cell Biology, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183 and ¹⁾Naoi Animal Hospital, Urawa, Saitama 336, Japan

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ABSTRACT. The expression of neural cell adhesion molecule (NCAM), tenascin and fibronectin during cartilage-like tissue formation in canine mammary mixed tumor was immunohistochemically examined. Mirror-image serial sections revealed that proliferating myoepithelial cells simultaneously expressed these adhesion molecules where no cartilage-like tissue and no expression of cartilage-specific type II collagen were observed. With the formation of cartilaginous tissue, positive signals of these adhesion molecules decreased in chondroblast-like cells, the cartilaginous tissue was hyalinized and mature chondrocyte-like cells no longer expressed these cell adhesion molecules. NCAM, tenascin and fibronectin, therefore, may play crucial roles on the proliferation of myoepithelial cells and the subsequent development of cartilaginous tissue in canine mammary mixed tumor.—**KEY WORDS:** canine mammary mixed tumor, cell adhesion molecule, chondrogenesis.

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Recently, several cell adhesion molecules were biochemically characterized and the expression of these molecules during embryogenesis and in cultured cells was investigated suggesting crucial roles on tissue morphogenesis [4, 5, 7, 9]. Neural cell adhesion molecule (NCAM) is an immunoglobulin superfamily membrane protein and includes three isoforms generated by alternative splicing of its messenger RNA. The 180 kilodalton (KDa) and 140 KDa isoforms are transmembrane proteins and the 120 KDa component is known as membrane-anchored isoform [11, 14]. This molecule participates in homophilic binding between neural cells as calcium-independent manner in contrast with calcium-dependent cadherin [8, 15] and also functions as a mesenchymal cell adhesion molecule [3, 9]. Tenascin is an extracellular matrix glycoprotein containing epidermal growth factor (EGF)-like domain and fibronectin type III domain and is expressed in the developmental stage of various tissues. This molecule plays not only as an adhesion molecule but also as differentiation-stimulating factor in some cultured cells, furthermore is involved in mitogenesis using its EGF-like domain [4, 13]. On the other hand, it is well known that fibronectin is an extracellular matrix protein and participates cell-matrix interaction through the integrin-recognition RGDS (Arg-Gly-Asp-Ser) sequences [6]. It is recently reported that these cell adhesion molecules were expressed during chondrogenic differentiation in normal embryogenesis by *in vivo* and *in vitro* studies [6, 12, 13]. In this report, we examined the expression of these cell adhesion molecules in proliferating myoepithelial cells immunohistochemically during cartilaginous tissue morphogenesis in canine mammary mixed tumors.

Eight cases of canine mammary mixed tumors were surgically obtained and were immediately fixed with 4% paraformaldehyde. These tissues were dehydrated and embedded in paraffin. Mirror-image serial sections were obtained and the deparaffinized sections were stained with hematoxylin and eosin or alcian blue (pH 2.5) and were immunostained with monoclonal antibody against NCAM (Sigma Chemical Co., St Louis, MO) and polyclonal antibodies against tenascin (Chemicon International Inc.,

Temecula, CA) and fibronectin (Zymed Laboratory Inc., San Francisco, CA). Anti-NCAM monoclonal antibody recognized 180 KDa and 140 KDa transmembrane isoforms but not 120 KDa membrane-anchored isoform. Anti-type II collagen polyclonal antibody is used as the molecular marker for the expression of cartilage-specific protein [1]. Immunostaining was performed according to the peroxidase-antiperoxidase method and counterstained with Mayer's hematoxylin.

In the early stage of cartilaginous tissue formation, proliferating myoepithelial cells in the nodular mass where cartilaginous matrix was not yet formed or accumulation of type II collagen was not observed (Fig. 1a), were well stained with the antibody against fibronectin (Fig. 1b), NCAM (Fig. 1c) or tenascin (Fig. 1d). The mirror-image serial sections revealed that the proliferating myoepithelial cells in the nodular mass simultaneously expressed these adhesion molecules. In the precartilaginous area, alcian blue-positive matrix was observed (data not shown) and the positive reaction of anti-type II collagen antibody with their matrix was appeared (Fig. 1e). Expression of fibronectin (Fig. 1f), NCAM (Fig. 1g) and tenascin (Fig. 1h) was retained in polygonal chondroblastic cells, however the number of antibody-positive cells was decreased. With the formation of hyalinized cartilaginous matrix, almost chondrocyte-like cells were no longer stained with these antibodies (Fig. 1e-h). The slight deposition of fibronectin and tenascin was also observed in hyalinized cartilaginous matrix.

In normal embryogenesis, it is reported that the expression sequence of these adhesion molecules are distinct in limb bud chondrogenesis [10]. Briefly, fibronectin is expressed by all undifferentiated mesenchymal cells and become enriched in precartilaginous condensation. The following expression of NCAM in precartilaginous condensation and tenascin in chondroblasts are observed [10]. However, in canine mammary mixed tumor, these molecules seem to be simultaneously expressed by proliferating myoepithelial cells in the early stage of the mixed tumor. While, during normal chondrogenesis, tenascin is expressed by chondroblasts and no

Fig. 1. Immunohistochemical stainings of canine mammary mixed tumor with the antibodies against type II collagen (a, e), fibronectin (b, f), NCAM (c, g) and tenascin (d, h) on the mirror-image serial sections (a and b, c and d, e and f, g and h). The large arrowhead in Fig. 1a indicates the fibrous deposition of type II collagen in the intercellular space of proliferating myoepithelial cells. The arrows or small arrowheads indicate the same cells on the mirror-image serial sections. $\times 175$.

positive signal with anti-tenascin antibody in both the undifferentiated mesenchymal cells and differentiated chondrocytes is seen, this molecule was already expressed by proliferating myoepithelial cells in the early stage of the mixed tumor. Tenascin and fibronectin are extracellular matrix proteins and are thought to be involved in cell adhesion against extracellular matrix, e.g. various types of collagen during the cartilaginous tissue formation in this tumor. Transient localization of tenascin in the nodular mass suggested that this molecule also play a role on the proliferation of mammary myoepithelial cells. On the other hand, NCAM was expressed in proliferating myoepithelial cells forming nodular mass suggesting this molecule involved in myoepithelial cell-cell interaction in the early stage of mixed tumor as seen in precartilaginous mesenchymal condensation. These cells which actively expressed NCAM, began to produce cartilage-specific type II collagen around their matrix (Fig. 1a, the large arrowhead) and then differentiated into chondrocyte-like cells. These observation suggested that this molecule might involve in the differentiation into chondrocyte-like cells of proliferating myoepithelial cells. Our previous studies showed that a single myoepithelial cell could automatically differentiated into chondrocyte-like cells cultured in collagen gel suggesting that chondrogenic differentiation in this tumor occurred without cell-cell interaction [1, 2]. The specific roles of NCAM in the chondrogenesis has not been identified. From the immunohistochemical findings above, we speculate that this molecule play an important role in the early stage of chondrogenesis as the mediator for some differentiation-promoting signals not only as cell adhesion molecule. Garin-Chesa *et al.* reported the expression of NCAM in human myxoid chondrosarcoma, one of small round cell tumors, however, they did not discuss about the relationship between the function of this molecule and the histogenesis of cartilaginous tissue [7]. For further study along this line, *in vitro* study using the cell line established from canine mixed tumor which maintained the capacity of chondrogenic differentiation could explain the role of

NCAM during chondrogenesis in the mixed tumor.

In this study, we reported the simultaneous expression of NCAM, tenascin and fibronectin by proliferating myoepithelial cells and suggested that these molecules play crucial roles on the development of cartilaginous tissue in canine mammary mixed tumor.

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