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Expression of Claudin-1, -2, -3, -4, -5 and -7 Proteins in Benign and Malignant Canine Mammary Gland Epithelial Tumours

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Summary

Claudins are tight junction proteins expressed by epithelial and endothelial cells. The present study has evaluated the expression of claudin-1, -2, -3, -4, -5 and -7 in 115 hyperplastic and neoplastic lesions of the canine mammary gland and compared this expression with that of normal mammary epithelium. The lesions studied included lobular hyperplasia ($n = 15$), simple adenoma ($n = 20$), non-infiltrating carcinoma *in situ* ($n = 20$) and infiltrating carcinomas of histological grades I, II and III ($n = 20$ of each). There was strong expression of claudin-1, -3, -4, -5 and -7 by epithelia within examples of lobular hyperplasia and simple adenoma, and strong expression of claudin-3 and -4 by non-infiltrating carcinomas and all three grades of infiltrating carcinoma. By contrast, there was reduced expression of claudin-5 and -7 by non-infiltrating and infiltrating carcinomas and the expression of these two molecules was inversely correlated with histological grade. Claudin-1 was expressed focally within carcinoma *in situ*, but this molecule was not detected in any invasive carcinoma. Claudin-2 was weakly expressed within areas of lobular hyperplasia and by simple adenomas, but was not expressed by any carcinoma cells. These results suggest that loss or reduction of expression of claudin-1, -2, -5 and -7 may lead to cellular disorientation, detachment and invasion in canine mammary neoplasia.

Keywords: claudin; dog; immunohistochemistry; mammary tumour

Introduction

Tight junctions (TJs) are one of the means by which vertebrate epithelial and endothelial cells adhere to one another to form structure. These regions of intercellular adhesion are generally found in the most apical region of the lateral membranes of adjacent cells (Schneeberger and Lynch, 1992; Gumbiner, 1993; Anderson and van Itallie, 1995). TJs were first identified by electron microscopy (EM) (Farquhar and Palade, 1963) and on freeze-fracture EM, TJs are visualized as a continuous anastomosing network of intramembranous particle strands and complementary grooves (Staehelin, 1974). The TJs are critical for establishing epithelial cell polarity and for the control of paracellular transport between the luminal and basolateral fluid compartments (Cereijido *et al.*, 1998; Madara, 1998; Heiskala *et al.*, 2001; Furuse *et al.*, 2002). Epithelial TJs are dynamic structures and subject to modulation during epithelial tissue remodelling (Peralta Soler *et al.*, 1996), wound repair (McCartney and Cantu-Crouch, 1992), inflammation (Madara *et al.*, 1992; Richl and Stenson, 1994) and neoplastic transformation (Mullin, 1997).

TJs are multimolecular structures comprising a series of proteins including occludin, members of the claudin family of proteins, the junctional adhesion molecule (JAM) and several associated peripheral membrane proteins, such as zonulae occludens (ZO-1,-2 and -3), cingulin, symplekin, plit, MAGI-1 and

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afadin (AF-6). Occludin was the first of these TJ proteins to be identified (Furuse *et al*., 1993; Ando-Akatsuka *et al*., 1996). The TJs are connected to the actin cytoskeleton by the TJ-associated proteins such as ZO-1,-2 and -3 (Stevenson *et al*., 1986; Fanning *et al*., 1998; Furuse *et al*., 1998; Haskins *et al*., 1998; Kawabe *et al*., 2001; Tsukita *et al*., 2001).

Claudins (CLDNs) are a relatively large family of 17–27 kDa integral membrane TJ tetraspanin proteins that are classified on the basis of the size of the molecules that pass through the paracellular spaces between epithelial and endothelial cells. Claudin proteins are crucial for TJ formation and function. They have four transmembrane-spanning domains, with the N-terminus and the C-terminus both located in the cytoplasm (Furuse *et al*., 1998; Morita *et al*., 1999). The C-terminus of claudin, in turn, binds to the second PDZ domain (named after Postsynaptic density protein 95, Disc-large, Zonula occludens-1) of ZO-1, linking the complex to a cellular scaffold (Tsukita *et al*., 1999). In humans, 24 members of the claudin family have been described. The association of abnormal TJ function and epithelial tumour development has been suggested by studies showing alterations in the TJ structure in human epithelial cancers (Martinez-Palomo, 1970).

The aim of the present study was to characterise the expression pattern of claudin-1, -2, -3, -4, -5 and -7 proteins in proliferatve diseases of the canine mammary gland including lobular hyperplasia, simple adenoma, non-infiltrating carcinoma *in situ* and infiltrating carcinomas of histological grades I, II and III. The expression of claudins within these lesions was compared with that of normal canine mammary tissue (Jakab *et al*., 2008a).

Materials and Methods

Tissue Samples

Biopsy samples of canine mammary gland lesions were submitted for diagnosis to the Szent István University, Faculty of Veterinary Science, Department of Pathology and Forensic Veterinary Medicine. The samples were fixed in 8% neutral buffered formalin for 24 h at room temperature, dehydrated in a series of ethanol and xylene baths and embedded in paraffin wax. Sections (3–4 μm) were stained with haematoxylin and eosin (HE). Lesions were classified by the World Health Organisation system (Misdorp, 1976; Misdorp *et al*., 1999) as lobular hyperplasia ($n = 15$), simple adenoma ($n = 20$), non-infiltrating carcinoma *in situ* ($n = 20$), grade I infiltrating simple carcinoma ($n = 20$), grade II invasive simple carcinoma ($n = 20$) and grade III solid infiltrating simple carcinoma ($n = 20$).

Immunohistochemistry

Serial sections (3–4 μm) were initially dewaxed in xylene and graded ethanol. After treatment with appropriate antigen retrieval (Target Retrieval Solution, pH 6.0; DAKO, Glostrup, Denmark; 30 min in 880 W microwave oven), the sections were incubated with primary antibodies against claudin-1 (rabbit polyclonal, diluted 1 in 100), claudin-2 (mouse monoclonal, diluted 1 in 80), claudin-3 and claudin -7 (both rabbit polyclonal and diluted 1 in 80), claudin-4 and claudin-5 (both mouse monoclonal and diluted 1 in 100) for 60 min at room temperature. All primary antibodies were from Zymed Inc., San Francisco, CA, USA. Immunohistochemical labelling was performed using the streptavidin-peroxidase procedure. Antigen-bound primary antibody was detected using standard avidin-biotin immunoperoxidase complex (DAKO, LSAB2 Kit). The chromogen substrate was 3, 3′-diaminobenzidine tetrahydrochloride (DAB substrate-chromogen, DAKO). Sections were counterstained with Mayer's haematoxylin.

Negative controls were performed by omission of the primary antibody and positive controls were normal human skin (stratum spinosum and sebocytes) for claudin-1, normal human colonic mucosa for claudin-2, -3 and -4, endothelial cells of vessels in normal human skin for claudin-5 and human fibroadenoma for claudin-7 (Jakab *et al*., 2008a; 2008b). Claudin labelling patterns were compared with adjacent normal epithelium. The internal positive controls were the peritumoural normal mammary tissue and epidermal layer, hair follicles, sebocytes and apocrine glands for claudin-1, the peritumoural normal mammary tissue and apocrine glands for claudin-2, -3, -4 and -7 (Jakab *et al*., 2008b) and the endothelial cells of the peritumoural lymphatic vessels, small arteries and veins for claudin-5 (Jakab *et al*., 2008c). Peritumoural fibroblasts and adipocytes served as internal negative controls since these cells do not express claudin proteins.

In each case, two independent observers (CSJ and JK) recorded the distribution and intensity of labelling. The percentage of positively labelled cells was determined by counting 100 cells in 10 randomly selected fields per slide (×20 objective). Numerical scores were assigned to the lesions on the basis of these counts: 0 (0–5% positively labelled cells), 1 (5–20% positively labelled cells), 2 (20–40% positively labelled cells), 3 (40–60% positively labelled cells), 4 (60–80% positively labelled cells) and 5 (80–100% positvely labelled cells).