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SPONTANEOUSLY ARISING DISEASE

Optimization of an Immunohistochemical Method to Assess Distribution of Tight Junction Proteins in Canine Epidermis and Adnexae

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Summary

Epidermal tight junctions (TJs) have been well characterized in human medicine. Abnormality of these structures is involved in skin diseases such as atopic dermatitis. There is little information about the expression and distribution of TJ proteins in the canine skin. The aim of this study was to develop an optimal immunohistochemical method for assessment of the expression of TJ proteins in the skin of healthy dogs. Formalin-fixed and paraffin wax-embedded skin biopsy samples from healthy human and canine patients were used. Canine skin samples were from the inguinal region and the nasal planum. Immunohistochemistry was used to study the expression of zonula occludens-1 (ZO-1), occludin and claudin-1, -4 and -7. Heat-induced antigen retrieval with EDTA (pH 9.0) yielded the best labelling of TJ proteins. ZO-1 and occludin were expressed in the cytoplasm and along the keratinocyte membrane, while claudin-1 and -4 were mainly membrane in distribution. ZO-1, occludin and claudin-1 were detected in all epidermal layers with the exception of the stratum corneum, while claudin-4 expression was restricted to the stratum granulosum. Expression of claudin-7 was difficult to evaluate. There was no difference in labelling pattern between inguinal and nasal planum skin.

Keywords: dog; epidermis; tight junction

Introduction

In man and in other vertebrates, tight junctions (TJs) are intercellular junctions located at the most apical part of the lateral membranes in a variety of polarized epithelial cells. TJs are composed of transmembrane proteins including occludin (Furuse *et al*., 1993), claudins (Furuse *et al*., 1998), junctional adhesion molecules (JAMs) (Ebnet *et al*., 2004), tricellulin (Ikenouchi *et al*., 2005) and marvelD3 (Steed *et al*., 2009). Scaffolding proteins such as zonula occludens (ZO) protein-1, -2 and -3 (Stevenson *et al*., 1986; Jesaitis and Goodenough, 1994; Haskins *et al*., 1998) and cingulin (Cordenonsi *et al*., 1999) have been identified in the cytosol, where they anchor occludin and claudins to actin and create a link with the cytoskeleton (Fanning *et al*., 1998). In addition, TJs include molecules that participate in vesicular traffic (i.e. sec6/8, syntaxin4, SNAP23 and the Rab13–PKA complex) and in regulation of epithelial cell proliferation and differentiation (Balda *et al*., 2003; Kohler and Zahraoui, 2005; Cereijido *et al*., 2007; Gonzalez-Mariscal *et al*., 2012).

Ultrastructurally, TJs appear as a network of fibrils. They are closely associated with adjacent cells and form anastomosing strands that obliterate the intercellular space. Therefore, their role in cohesion of the epidermal barrier is easily understood. Moreover, TJs allow the selective passage of water, ions and solutes and play an important role in cellular polarity.

The importance of TJs in epithelial barrier function is illustrated by the demonstration that claudin-1-deficient mice die within 1 day of birth, with

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wrinkled skin and increased transepidermal water loss (TEWL) (Furuse *et al.*, 2002). In man, changes in TJs have been identified in dermatological conditions such as psoriasis (Yoshida *et al.*, 2001; Peltonen *et al.*, 2007; Kirschner *et al.*, 2009), neonatal ichthyosis and cholangitis syndrome (Hadj-Rabia *et al.*, 2004; Feldmeyer *et al.*, 2006; Grosse *et al.*, 2012) and atopic dermatitis (De Benedetto *et al.*, 2011). Recently, reduction in claudin-1 expression has been identified in the non-lesional skin of atopic patients (De Benedetto *et al.*, 2011).

Very few studies have been conducted on canine epithelial TJ expression. TJs have been characterized in canine duodenal and colonic mucosa (Ridyard *et al.*, 2007; Ohta *et al.*, 2011), mammary glands (Jakab *et al.*, 2008a) and hepatoid glands (Jakab *et al.*, 2010). The expression of ZO-1, occludin and claudin-1 in the skin has been investigated by immunofluorescence labelling (Bizikova *et al.*, 2011). However, some TJ proteins implicated in human skin diseases (i.e. claudin-4 and -7) have not been studied in the normal canine epidermis.

The aim of the present study was to evaluate immunohistochemically the expression and distribution of TJ proteins in normal canine skin.

Materials and Methods

Samples of Canine and Human Skin

Formalin-fixed and paraffin wax-embedded (FFPE) skin biopsy specimens from healthy people and dogs were used. Specimens were fixed in 10% neutral buffered formalin for at least 3 days and up to 6 months before embedding. Skin samples were obtained from clinically healthy dogs with no history of skin disease. Samples were collected from dogs undergoing an elective surgical procedure ($n = 9$) or that were humanely destroyed for behavioural reasons ($n = 2$). Punch biopsy (8 mm) samples of the skin were collected. Specimens from the inguinal area of each dog were sampled in order to minimize variation. Samples were also taken from the planum nasal of the two dead dogs. All samples were obtained with informed consent of the dog owners and all procedures were approved by the ethical Committee of the National Veterinary School of Nantes (ONIRIS).

Positive controls included samples of healthy human skin for expression of ZO-1, occludin and claudin-1 (Kirschner *et al.*, 2010). Psoriatic human skin was used as a positive control for claudin-4 because in psoriatic skin caudin-4 is expressed in more layers than in normal epidermis, allowing for easier observation (Kirschner *et al.*, 2009). A sample of canine mammary gland adenoma was used as a positive control for claudin-7 (Jakab *et al.*, 2008b). The human skin samples were kindly provided by the Immunology and Dermatology Laboratory of Nantes University Hospital. The specimens were obtained from individuals undergoing abdominoplasty, gynaecomastia surgery or thigh lifting, from the Plastic Surgery Departments of Nantes University Hospital and Nantes Jules Verne Private Hospital (France). All patients provided their written, informed consent. The study was conducted according to the principles of the Declaration of Helsinki and the Medical Ethical Committee of Nantes University Hospital approved all procedures concerning human samples.

Selection of Antibodies

Primary antibodies were selected based on available knowledge of involvement of the corresponding TJ protein in a human or animal dermatological condition (Furuse *et al.*, 2002; Kirschner *et al.*, 2009; De Benedetto *et al.*, 2011). The antibodies targeted human proteins, but have been shown to cross-react with canine TJ proteins (Ando-Akatsuka *et al.*, 1996; Jakab *et al.*, 2008a,b; Bizikova *et al.*, 2011). Moreover, sequence homologies determined with the basic local alignment search tool (BLAST) were high between canine and human proteins and varied from 93% (claudin-1, claudin-7 and ZO-1) to 91% (occludin).

Determination of the Dilution of the Antibodies

For each antibody, at least three dilutions around those recommended by the manufacturer were tested (Table 1) on human and then canine skin samples. The optimal working dilution was that subjectively giving the strongest labelling with the least background.

Comparison of Methods of Antigen Retrieval

Three methods of antigen retrieval were tested on all canine skin samples. Sections (3 μm) were cut onto Superfrost Plus™ slides (VWR, Fontenay-sous-Bois, France). Sections were dewaxed in two changes of xylene and then rehydrated in two changes of absolute ethanol for 10 min each and finally rinsed in distilled water.

After this first step, different methods of antigen retrieval were tested for each antibody. Each sample of canine skin underwent each antigen retrieval method. For each skin sample a positive control and a negative control were tested in parallel.

In the first retrieval method, slides were incubated for 10 min at 37°C with protease type XIV from *Streptomyces griseus* (pronase E; P5147; Sigma, St Louis,