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Expression and Distribution of Kallikrein-related Peptidases 5, 7, 8 and 10 in Normal Apocrine Gland of Canine Skin

Expresión y Distribución de Péptidos Relacionados con la Calicreína 5, 7, 8 y 10 en Glándula Apocrina Normal de Piel Canina

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SUMMARY: Apocrine glands are sweat glands that are located in the skin of the dog. Anal sac apocrine, circunanal apocrine, and mammary glands are considered modified apocrine structures, and there are about nine possible types of neoplasms and other tumors in the apocrine glands of the dog and cat, including cysts, adenoma, carcinoma, and adenocarcinoma. Thus, it is important to provide new markers to characterize these glands to improve the histopathological diagnosis. In this article, we describe the distribution of kallikrein-related peptidases 5, 7, 8, and 10 in the normal apocrine glands of the dog's skin. These proteases have been shown to play a fundamental role in the homeostasis of the human skin barrier but have been scarcely studied in canine skin.

KEY WORDS: Kallikrein-related peptidases; KLKs; Apocrine gland; Dog.

INTRODUCTION

Sweat glands have traditionally been divided into apocrine or eccrine based on their mode of secretion. Apocrine glands are characterized by decapitation secretion, in which the apical part of the cell is pinched off and released into the lumen (Scrivener & Cribier, 2002). In general, apocrine glands are microanatomical epitrichial, and eccrine are atrichial, but this is not always true. Typical epitrichial apocrine glands are present throughout the haired skin in dogs and cats; atrichial eccrine glands are limited to the pawpads. However, ceruminous glands, the specialized apocrine sweat glands of the inner pinna and ear canal, have epitrichial units. Anal sac apocrine, circunanal apocrine, and mammary glands, considered modified apocrine structures, are exclusively atrichial (Goldschmidt & Shofer, 1992).

According to the classification of Lee *et al*. (2005), there are about nine possible types of neoplasms and other tumors in the apocrine glands described in the dog and cat, including cysts, adenoma, carcinoma, and adenocarcinoma. On the other hand, there are 18 types of neoplasms of the mammary gland (Goldschmidt *et al*., 2011). Currently, the diagnosis of these neoplasms is made by histopathology using immunohistochemical markers, i.e., cytokeratin or estrogen-receptor in the case of mammary neoplasm (Goldschmidt *et al*., 2011; Baharak *et al*., 2012). Other cancer biomarkers are being investigated to improve the knowledge about cancer development (Ullah & Aatif, 2009).

Kallikrein-related peptidases (KLKs) are a major family of proteases comprising 15 members encoded by 15 genes clustered on chromosome 19q13.4. KLKs are secreted (chymo)trypsin-like serine peptidases, which are secreted as pro-enzymes and activated through a proteolytic cascade in many tissues, including the skin (Diamandis & Yousef, 2002). To date, at least eleven KLKs (KLK1, KLK4, KLK5, KLK6, KLK7, KLK8, KLK9, KLK10, KLK11, KLK13, and KLK14)

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show varying levels of expression and are located mainly in the stratum granulosum and stratum corneum of the epidermis (Komatsu *et al*., 2013; Williams *et al*., 2017; Zhu *et al*., 2017; Kishibe, 2019). Regarding protease activity, KLK7 and KLK9 exert chymotryptic-like cleavage preference, whereas other KLKs show trypsin-like activity (Kishibe, 2019). In dogs, there is not much evidence regarding the distribution and role of these proteases. However, the expression levels of KLKs in healthy human skin show differences according to age, sex, and anatomical site. In addition, KLKs levels can be affected by stimulation with glucocorticoids, sex hormones, 1,25-dihydroxy vitamin D3, and retinoic acid, among others (Shaw & Diamandis, 2007; Morizane *et al*., 2010). High expression of KLKs has been observed in the upper stratum granulosum (SG), and stratum corneum (SC), where KLK5 and KLK7 have been described as the most abundant KLKs present in the stratum corneum (De Veer *et al*. 2017). In addition, most of the KLKs have been found to be expressed in the epidermis and in associated appendages such as hair follicles, and epithelial and sweat glands of the human skin (Cui *et al*., 2016; De Veer *et al*., 2017; McGovern *et al*., 2017). KLKs participate in various physiological activities such as epidermal cell desquamation, lipid permeability, and antimicrobial defense (Ishida-Yamamoto *et al*., 2004; Eissa & Diamandis, 2008; Pettus *et al*., 2009).

Dysregulation of KLKs has been proposed to have clinical value (Kryza *et al*., 2016; Kishibe, 2019). Thus, upregulation of KLK5 has been reported in many chronic inflammatory skin diseases, including atopic dermatitis (Komatsu *et al*., 2007). The expression of KLKs has also been investigated in neoplasia to expand their use as cancer biomarkers. They have been additionally implicated in carcinogenesis and some of them have been recommended as prognostic biomarkers (Kim *et al*., 2001). In this respect, KLK5 has been proposed as a novel biomarker to distinguish between malignant and benign breast tumors (Avgeris *et al*., 2011). Equally, the abnormal expression of KLK5 in murine and human oral squamous cell carcinoma promotes tumor progression (Pettus *et al*., 2009; Jiang *et al*., 2011; Leusink *et al*., 2015; Johnson *et al*., 2016). We have recently shown the expression and distribution of KLK5 protein in dogs suffering from squamous cell carcinoma and showed that cancer cells that invaded the stroma expressed KLK5 (Ortloff *et al*., 2020).

In canines, many skin diseases involve alterations of skin homeostasis that could be consequence of KLKs dysregulation, an aspect unexplored in disorders affecting the canine skin. (Gross *et al*., 2008). This paper described the immunohistochemical expression and distribution of KLK5, KLK7, KLK8 and KLK10 in the normal apocrine gland of the canine skin.

MATERIAL AND METHOD

Normal skin samples: To perform this study, ten samples were obtained from five cadavers of dogs (aged between 5 and 8 years) without any skin disease, which died of traumatic or cardiovascular etiology and its owner authorized the researchers to obtain a skin samples. Thick skin samples were obtained from the nasal muzzle, whereas thin skin was from the abdomen's ventral portion. Tissue samples were fixed in 10 % neutral buffered formalin processed routinely and embedded in paraffin wax. Five mm thick sections were used for immunohistochemistry.

Immunohistochemical staining: Tissue sections were obtained from all biopsy specimens and stained with hematoxylin and eosin (HE). Immunohistochemistry was performed with aid of the biotin/streptavidin-peroxidase technique as previously described (Ortloff *et al*., 2020). To perform the immunohistochemical procedure 5 µm thick sections were deparaffinized and sequentially incubated with (i) primary antibody for 16 h at 22ºC; (ii) biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) for 30 min; and (iii) Vectastain Elite ABC reagent (Vector Laboratories, Burlingame, CA) for 30 min. Peroxidase activity was developed using diaminobenzidine (Dako, Carpinteria CA). The primary antibodies used were: anti-cytokeratin (ab86734 Abcam); anti-KLK5 (rabbit pAb, ab40951, Abcam); anti-KLK7 (rabbit pAb, ab28309, Abcam); anti-KLK8 (rabbit pAb, ab72800, Abcam); and anti-KLK10 (mouse mAb, ab55623, Abcam); anti-E-cadherin (rabbit polyclonal, sc-7870, Santa Cruz) at 1:100 to 1:200 dilutions. All antibodies were diluted in 50 mM Tris saline buffer (TBS, pH 7.8) containing 0.5 % immunoglobulin-free bovine serum albumin. For anti-KLK antibodies, antigen retrieval with 50 mM Tris-HCl (pH 9.0) was used, and for E-cadherin antibodies, 10 mM citrate buffer (pH 6.0) was used. Pseudoperoxidase activity was inhibited by preincubating tissue sections with 3 % hydrogen peroxide for 30 min before incubation with each primary antibody. The omission of primary antibody during incubation served as a negative control.

RESULTS

Apocrine glands. Apocrine glands comprise a secretory unit and a duct located in the dermis near to the sebaceous gland. At the base of each sweat gland is a structure known as the secretory coil that is surrounded by contractile myoepithelial cells. Histologically, the apocrine gland and its ducts are formed by one layer of cubical epithelial cells (Fig. 1).

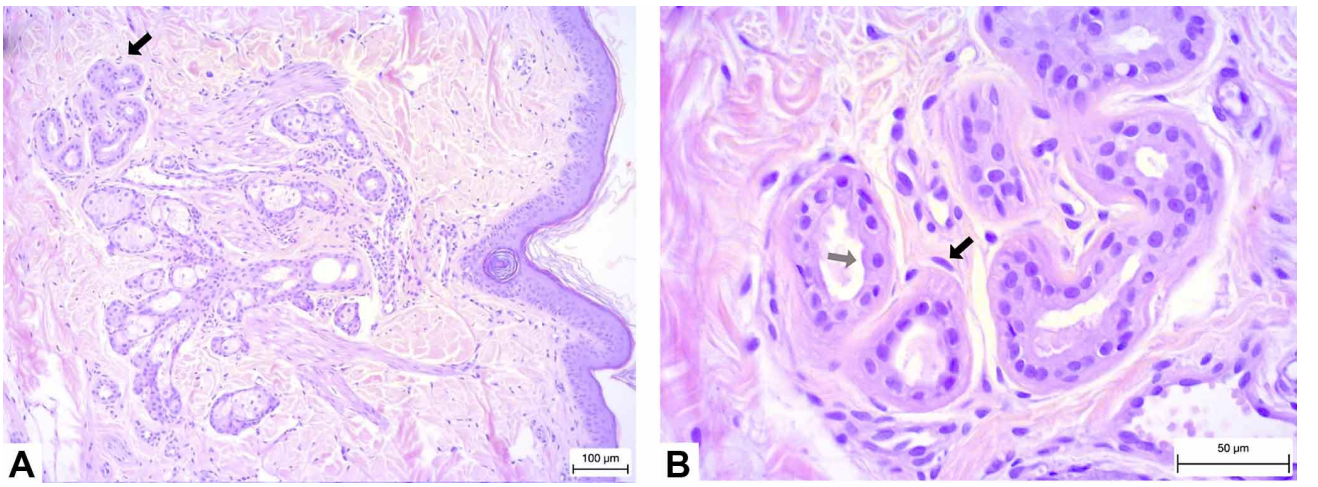

Fig. 1. Normal histology of the dog skin. A. The apocrine glands (black arrow) are located in the dermis, near to the sebaceous glands (black arrow). B. Histological detail of the apocrine gland. The epithelial cells of the gland are simple cubic (gray arrow). Myoepithelial cells are fusiform and surround the group of epithelial cells (black arrows).

The epithelial cells of the apocrine glands stained for cytokeratins, though not all of them were immunoreactive with the same intensity for the pancytokeratins antibody used in this study (Fig. 2A). Regarding E-cadherin expression, we did not observe immunoreaction in the cells forming the glands. Glandular epithelial cells were negative for KLK5 (Fig. 2B), but strongly immunoreactive for KLK7 and KLK10, especially on the apical cell surface (Figs. 2C and 2F, respectively). The glandular cells were negative for KLK8, but the cytoplasm of the myoepithelial cells surrounding the glands was strongly immunoreactive (Figs. 2D and 2E).

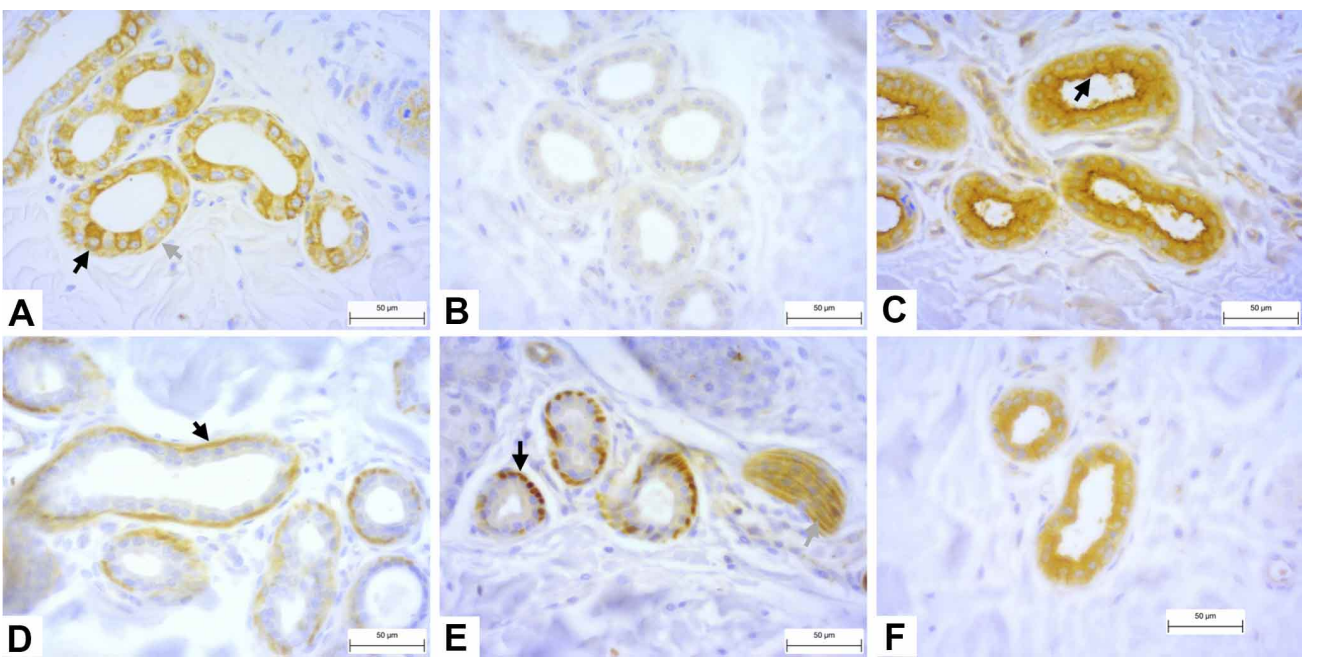

Fig. 2. Immunohistochemical labeling of cytokeratins, KLKs 5, 7, 8 and 10 in normal canine apocrine glands. (A) Cytokeratins. There are cells that show strong immunoreaction (black arrow) anf low immunoreaction (gray arrow). (B) KLK5. (C) KLK7.All the cytoplasm is immunoreactive, but the immunorreaction is stronger in the apical surface (black arrow). (D) KLK8 and (E) KLK8. The myoepithelial cells are positive to KLK8 (black arrows) and is possible to see myoepithelial cells in longitudinal section (gray arrow). (F) KLK10.

DISCUSSION

In human skin, KLKs are mainly involved in the physiological desquamation and development of inflammatory skin diseases with barrier abnormalities, including Netherton syndrome and atopic dermatitis (AD). Indeed, Komatsu *et al*. (2003) determined the mRNA expression of KLK1, KLK4, KLK5, KLK6, KLK7, KLK8, KLK9, KLK10, KLK11, KLK13, and KLK14 in normal human skin using RT-PCR or in situ hybridization. Furthermore, a relationship between KLKs dysregulation and human cancer has been intensively explored in recent years (Nauroy & Nyström, 2020). We have recently reported that KLK5 is differentially expressed in the upper stratum granulosum, stratum corneum, hair follicles, and sweat glands of dogs suffering from cutaneous squamous cell carcinoma (cSCC) (Ortloff *et al*., 2020) and have suggested that KLK5 may represent a potential biomarker for diagnosis and prognosis in veterinary medicine. Although in the present investigation apocrine glands did not express KLK5, its finding in cSCC invites us to consider a possible role of KLK5 in other pathological conditions like apocrine adenocarcinoma.

Only a few studies have addressed the expression and distribution of KLKs in the normal skin and cutaneous adnexal structures of domestic animals (Shaw & Diamandis, 2007; Zhu *et al*., 2017), thus underlining the importance of these proteases in veterinary medicine. In the dog, it is known that AD represents a disease of genetic predisposition caused by the absorption of allergens that provoke specific immunological events (Leung, 1999). In both humans and dogs, skin barrier dysfunction is a major pathogenic event in the onset of AD, leading to water loss and, therefore, to the penetration and sensitization to allergens and/or microorganisms. In this regard, Zhu *et al*. (2017) demonstrated that KLK5 is up-regulated in the skin with AD. In veterinary medicine, deterioration of the skin barrier has been previously related to markers such as ceramides and filaggrin (Marsella *et al*., 2013); so far, the participation of KLKs in the integrity or deterioration of the canine skin has not been described yet. The KLKs function as a proteolytic cascade whose activation has three secondary effects at the human skin level, including: (1) the degradation of corneocytes-related desmosomes that reduces the cohesion of the stratum corneum and promotes desquamation; (2) the reduction of the catalytic activity and degradation of acidic sphingomyelinase and b-glucocerebrosidase, which, in turn, reduces the integrity of the stratum corneum lipids and decreases the cohesion between cells, and (3) the activation of the protease-activated receptor 2 (PAR2) that inhibits the secretion of lamellar bodies from corneocytes, which decreases the content of extracellular lipids and alters the function of the skin barrier (Cui *et al*., 2016). In the case of KLK5 and KLK7, they not only participate in the degradation of proteins that form and stabilize corneodesmosomes but also regulate the processing of profilaggrin at the level of the stratum granulosum (Sakabe *et al*., 2013; Kishibe, 2019), as well as the amount of lipid enzymes both acidic sphingomyelinase and b-glucocerebrosidase at the stratum corneum (Hachem *et al*., 2005; Kishibe, 2019). KLK5 and KLK7 also control skin antimicrobial activity by processing human cathelicidin precursors into shorter inflammatory peptides, which are well-recognized as the primary pathogenic factor in human rosacea (Yamasaki *et al*., 2016; Kishibe, 2019). Even in conjunction with KLK8, these proteases can generate shorter LL-37 peptides with antimicrobial activity (Eissa *et al*., 2011), supporting their importance in mammalian skin physiology as well as their possible implication in skin disorders. Here, for the first time, we show the expression of KLK7 and KLK10 in apocrine glands in the dog.

Our findings reveal that KLKs are differentially expressed in the apocrine glands. Thus, strong immunoreactivity was observed in the epithelial lining the apocrine glands for KLK7 and KLK10, evidencing the absence of immunoreaction for KLK5. Given that some pathological conditions affecting the skin annexes in dogs are primarily diagnosed through histopathology, identifying KLKs and their activators/inhibitors in these structures may be useful in disorders such as apocrine gland carcinoma. Our results show strong immunoreaction for KLK8 in myoepithelial cells that cover the epithelial glandular apocrine cells. Sánchez-Céspedes *et al*. (2015) indicated that in canine mammary tumors is common that myoepithelial cells are involved and take part of the tumor structure, and it is necessary to characterize its immunoreactivity to different antibodies. They found that myoepithelial cells in the mammary gland (a modified apocrine gland) of the dog express cytokeratin 14, calponin and CD10 (Sánchez-Céspedes *et al*., 2015). Despite the role of KLK8 in the myoepithelial cells being unknown, we propose that KLK8 is a good biomarker that must be investigated.

In the human skin, it has been postulated that the transmembrane serine protease matriptase and matrix metalloproteases can activate pro-KLK5, which can also self-activate and then activate KLK8. Once activated, KLK8 can activate in vitro pro-KLK1, pro-LL37 and pro-KLK11 (Eissa *et al*., 2011), whereas other KLKs such as KLK6, KLK7 and KLK14 can also activate KLK5 (Eissa & Diamandis, 2008). In line with this data, we observed that in the normal epidermis of canines, KLK5, KLK7, and KLK8 are highly expressed in the apical pole of spinous cells once they migrate to the stratum granulosum. Although KLK5 and KLK7 are also expressed by granulous cells, this stratum exhibits a predominant immunoreactivity for KLK10. At the

same time, KLK8 does not show reactivity in the granular layer, suggesting that the expression and distribution of these proteases conserve a spatial pattern similar to that observed in humans.

In the human skin, both KLK6 and KLK7 have been involved in the proteolytic cleavage of many extracellular matrix components distributed throughout the epidermis (Nauroy & Nyström, 2020). Although KLK5 did not display immunoreactivity in the dog's thin skin's epidermis, it is well-known that KLK8 can indirectly activate pro-KLK7 through meprin b (Ohler *et al*., 2010), giving a possible explanation for the increase in the activity of KLK7. Future experiments focused on the expression and distribution of natural KLK activators, such as enterokinase, meprin b and/or plasmin, and KLK inhibitors, including LEKTIs and other SPINKs proteins, could give novel insights into the regulation of KLKs in the normal dog thin skin.

Considering the expression and localization of KLKs 5, 7, 8 and 10 described here and the complex mechanisms that regulate their activity in human tissues, further studies in dogs are required to determine how aberrant expression and activity of these KLKs may affect the epidermis and associated glands, inducing inflammation, and a variety of pathological events. Because KLK activity is tightly regulated by endogenous activators and inhibitors, studying their expression patterns may provide novel insights into how this system functions in the maintenance and/or loss of skin homeostasis.

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RESUMEN: Las glándulas apocrinas son glándulas sudoríparas que se encuentran en la piel del perro. Las glándulas apocrinas del saco anal, apocrinas circunanales y mamarias se consideran estructuras apocrinas modificadas, y existen alrededor de nueve tipos posibles de neoplasias y otros tumores en las glándulas apocrinas del perro y el gato, incluidos quistes, adenoma, carcinoma y adenocarcinoma. Por lo tanto, es importante proporcionar nuevos marcadores para caracterizar estas glándulas para mejorar el diagnóstico histopatológico. En este artículo, describimos la distribución de las peptidasas 5, 7, 8 y 10 relacionadas con la calicreína en las glándulas apocrinas normales de la piel del perro. Se ha demostrado que estas proteasas desempeñan un papel fundamental en la homeostasis de la barrera de la piel humana, pero apenas se han estudiado en la piel canina.

PALABRAS CLAVE: peptidasas relacionadas con la calicreína; KLK; Glándula apocrina; Perro.

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