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Expression of Connexins 43, 26 and 32 in normal, hyperplastic and neoplastic perianal dog glands

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Abstract

Connexin (Cx) expression is reportedly altered in neoplasms. This study aimed to investigate the expression of Cx43, 26 and 32 in normal and pathological canine perianal glands. Thirty perianal glands bearing pathological processes and ten normal canine perianal glands were submitted to immunohistochemistry to search for presence of Cx43, Cx26 and Cx32. Both Cx43 and Cx26 expressions were observed in normal, hyperplastic glands, and in well and moderately differentiated adenomas. However, in poorly differentiated adenomas, expressions were reduced, and they were absent in carcinomas. Cx26 was located in the cytoplasm of normal, hyperplastic perianal gland cells, and in well and moderately differentiated adenomas. Cx32 was not observed in any neoplasm neither in normal or hyperplastic glands. Our results show that Cx43 and Cx26 expressions are altered in more aggressive canine perianal gland neoplasms, and we conclude that they may be related to the perianal gland carcinogenesis process.

Key Words: perianal gland, connexins, neoplasia, hyperplasia, gap junctions

Introduction

The maintenance of metabolic cooperation between cells is crucial for tissue homeostasis. In cells of mammals, homeostasis is controlled by intercellular channels in order to allow cell-by-cell passage of small hydrophilic molecules and ions (<1-2Kda). These structures, known as gap junctions, are formed by protein sub-units, the connexins (Cx), which belong to a multigenic family of at least 20 members (6). Each gap junction channel is formed by 2 hemi channels, also known as conexons, located at each side of the paired cells. Hemi channels are hexamers mounted on the RE, Golgi or on the rear compartment of the Golgi. They are transported to the cellular surface in vesicles and inserted through the fusion of vesicles into the cellular membrane, where they attach. The union of two hemi channels creates the gap junction channel. Gap junctions are widely distributed in several tissues and animal species, and the low communication capacity among cells is frequently associated with tumor progression (4, 17).

Neoplasias in the canine perianal region are frequent. Most originate in the perianal gland and are benign. These perianal gland adenomas occur most often in male dogs and are rarely observed in females and castrated males, showing the androgenic dependency. Perianal adenomas, which represent more than 80% of the neoplasias of this region,, are considered the third most prevalent type of tumor found in male dogs (20, 26). While the cause of the appearance of these tumors is unknown, they can be modulated by sexual hormones (8). .

Adenocarcinomas are generally associated with systemic symptoms, mostly polyuria and polydipsia correlated with hypercalcemia, and hypophosphatemia. Metastasis of perianal carcinomas can occur in the lungs, liver, spleen, iliac and lumbar lymph nodes (2).

In the present study, we examined the expression of Cx43, Cx26 and Cx32 in normal, hyperplastic and neoplastic canine perianal glands.

Material and Methods

Canine perianal glands

Ten normal canine perianal glands were obtained from dogs submitted to necropsy at the Departamento de Patologia da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (FMVZ/USP). Perianal glands presenting lesions were obtained from dogs submitted to surgical ablation and sent to the Animal Pathology Service of the Veterinary Hospital of the FMVZ/USP, where they were routinely processed for paraffin embedding and sectioning. Lesions from 30 altered perianal glands were classified according to Berrocal et al., 1989 (2). A comparison of the classification of canine perianal gland neoplasms by Berrocal et al., 1989 (2), with that of the World Health Organization can be found at Martins et al., 2008 (15). Lesions included in the study were: 6 hyperplasias, 6 type I adenomas, 6 moderately differentiated adenomas, 6 poorly differentiated adenomas and 6 carcinomas. These cases, along with the 10 normal perianal glands, were submitted to the methods described below.

Immunohistochemistry

After surgical removal, representative fragments from perianal glands were fixed in 10% formalin and submited to paraffin embedding; 5µm histological sections were stained with hematoxylin and eosin for histopathology. Additional fragments were fixed in methacarn (70% methanol, 20% chloroform and 10% acetic acid) and embedded in paraffin for immunohistochemistry. Five µm thick histological sections were routinely deparaffinized, rehydrated and incubated with a hydrogen peroxide solution (10%) for 30 min.

The sections were incubated either with antibodies anti-Cx43 (Zymed 71-0700), anti-Cx26 (Zymed-71-0500), or anti-Cx32 (Zymed polyclonal), diluted 1: 100 in blocking buffer (TNB) at 4°C overnight. Mouse biotinylated secondary antibody conjugated to FITC (Dako—E0432) was used as the link (1: 1,000 in phosphate-buffered saline [PBS]) for 2 h. In the case of Cx26, the biotinylated antibody was the anti-mouse (LSAB) for 1 h. The histological sections were analyzed with a confocal microscope (Axiovert 100M—Zeiss, LSM 510 system). Canine liver or heart samples were used as positive controls for the immunohistochemistry of Cx26 and Cx32 or Cx43, respectively. Negative controls omitted the primary antibody in a histologic section while performing all other immunohistochemistry steps.

Results

Immunohistochemistry

The immunostaining of connexins 43, 26 and 32 is presented in Figs. 1, 2 and 3 respectively. Normal canine perianal glands presented positivity to Cx43 and Cx26 immunostaining. The hyperplastic perianal glands presented the same immunohistochemistry pattern for Cx43, 26. We verified the absence of Cx32 in normal, hyperplastic or neoplastic perianal glands (Figs. 1A, 1B, 2A, 2B and 3A, 3B).

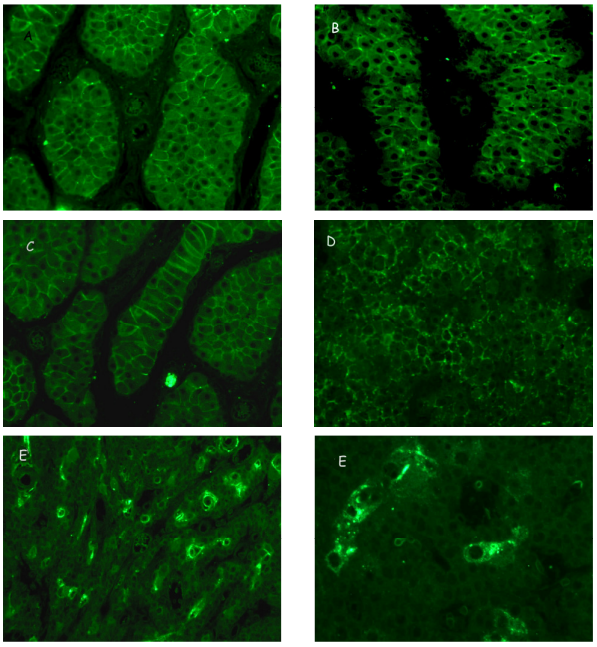

Figure 1. Photomicrograph of Cx 43 immunostaining in canine perianal glands. A. Normal gland (Obj. 20X), B. Hyperplastic (Obj. 20X), C. Adenoma group I (Obj. 20X), D. Moderately differentiated adenoma group II (20X), E. Poorly differentiated adenoma group II (20X) and E". Higher magnification from E. (Obj. 40 X).

Normal and hyperplastic perianal gland cells were highly positive to Cx43 with spots of positivity observed in the plasmatic membrane. (Figs. 1A, 1B). Among the benign neoplasias, the Cx43 pattern was different. Thus, in adenomas of group I and those moderately differentiated of group II, the immunohistochemistry followed the plasmatic membrane normal and hyperplastic glands pattern (Figs. 1 A, B, C and D). In poorly differentiated adenomas of group II, however, localization of Cx43 was mainly cytoplasmic in the majority of cells and absent in the minority. (Fig. 1E).

The immunohistochemistry for Cx26 disclosed it as cytoplasmic in both cases, normal and hyperplastic, and in a very subtle way in the plasmatic membrane (Figs. 2A, 2B). In the adenomas of group I, they showed the same immunohistochemistry pattern of the normal and hyperplastic perianal glands for Cx26, cytoplasmic, and in a very subtle way in the plasmatic membrane (Fig. 2C). However, in moderated and poorly differentiated adenomas of group II, Cx26 was rarely expressed. When Cx26 was expressed, it showed exclusive cytoplasmic localization (Figs. 2D, 2E).

The carcinomas did not show any immunostaining for Cx43 and Cx26, with the

exception of the normal areas present in these tumors where cells expressed intense immunostaining for Cx43 (Fig. 1F). Cx32 was not observed in normal glands, hyperplastic glands, adenomas and perianal glands carcinomas. (Figs. 3A, B, C, D, E and F).

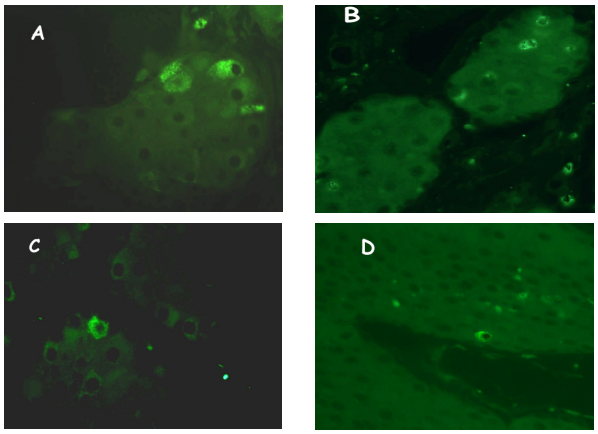

Figure 2. Photomicrograph of Cx 26 immunostaining in the several hyperplastic, neoplastic and normal processes of the perianal gland. A. Normal gland (immersion), B. Hyperplastic (Obj. 40X), C. Adenoma group I (Obj. 100X), D. Adenoma moderately differentiated group II (Obj. 40X).

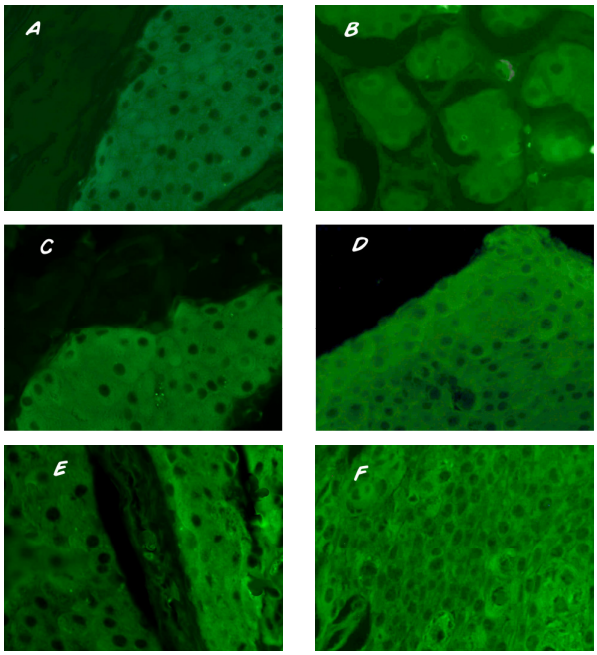

Figure 3. Photomicrograph of Cx 32 immunostaining in the several hyperplastic, neoplastic and normal processes of the perianal glands. A. Normal gland (Obj. 40X), B. Hyperplastic (Obj. 40X), C. Adenoma group I (Obj. 40X), D. Adenoma moderately differentiated group II (Obj. 40X), E. Adenoma poorly differentiated group II (Obj. 40X), F. Carcinoma group III (Obj. 40X).

Discussion

Perianal gland lesions are very frequent in dogs, especially in non-castrated males. (2, 20, 26). Our group recently reported the proliferation and apoptosis indexes in canine perianal gland lesions that were classified according to Berrocal et al.,1989 (2) and Martins et al., 2008 (15). The aim of this study was to examine the expression of connexins 43, 26 and 32 in canine perianal glands and describe their immunohistochemical patterns in hyperplastic and neoplastic perianal glands. To the best of our knowledge, we show, for the first time, that canine perianal glands express Cx43 and Cx26, but not Cx32.

We initially investigated Cx43 in normal, hyperplastic and neoplastic canine perianal glands, since Cx is more distributed and present in various dependent androgynous organs as prostate, testicle, epididymis, during carcinogenesis (11, 12, 16).

The results of our samples using immunohistochemistry for Cx43 were synchronic and coherent with the types and stages of carcinogenesis and showed, for the first time, that expression of Cx43 is reduced in more aggressive perianal gland neoplasms.

We also noticed that, even if Cx43 was expressed in poorly differentiated group II cells, in the majority of the cells, its localization was aberrant, remaining cytoplasmic. Furthermore, Cx43 was not expressed in the minority of the cells of these poorly differentiated neoplasias, Such localization suggests that during perianal gland carcinogenesis, the loss of connexin expression may occur in relatively precocious stages. Several studies demonstrated that Cx expression was decreased or aberrant in pre-cancerous lesions (21, 22). In findings similar to ours, Nishimura et al. 2003 (18), found endometrial carcinomas with aberrant connexin 43 localization. The authors suggested that this abnormal localization was directly connected to cadherin-E expression (cadherin-E is a glycoprotein of 120 KD, which is located in the adherent epithelial cells area, where homophylic calcium-dependent cell adhesion is regulated). In general, the loss of cadherin-E function with consequent inhibition of cell adhesion is an important genetic event in tumor progression. This suggests that 5' CpG methylation islands are responsible for the loss of cadherin-E expression and, therefore, indirectly cause suppression of gap junction intercellular communication capacity. This induces the aberrant localization of connexins in cancerous endometrial cells in earlier stages of carcinogenesis.

More recent data reinforce the idea of a possible Cx localization involvement: for example, the alpha catenin induction favors Cx relocalization in the plasmatic membrane in the cellular ancestry of human prostatic cancer (7).

It is interesting to note that the cytoplasmic localization of connexins was associated to the invasive carcinoma regions: for example, Cx26 and Cx43 in chemically induced cancers in the bladder of rats (1) or Cx43 in human and rat hepatocellular carcinoma (14, 23).

This cytoplasmic localization or Cx43 absence in adenomas of poorly differentiated group II also suggest similarity of the genomic alterations of the

poorly differentiated group II to the group III perianal gland carcinoma. When we observed the slides with normal, hyperplastic and neoplastic perianal gland marked for Cx26 by immunohistochemistry, we noticed that this connexin was present in some cells and, mainly, with cytoplasmic localization in normal, hyperplastic tissues and adenomas of group I. It was rarely present in the plasmatic membrane and, when present, was expressed in a very low intensity In the moderately and poorly differentiated adenomas of group II, it was rarely expressed and always with cytoplasmic localization. The carcinomas did not express Cx26. With the results obtained by immunoblot we observed, in all studied tissues, the expression of the Cx26 dimer (molecular weight between 50 and 50 KDA). We found that large normal areas existed in this tumor which could have had their protein extracted, when we randomly extracted samples for total protein extractions (10).

The exact mechanism, capabilities and reasons for perianal normal cell accumulation of Cx26 in the cytoplasm is not clear. It is known, however, that cytoplasmic localization of connexins is not always associated with abnormal and pathological situations (17). Uterine myocytes tend to accumulate Cx43 before birth (9). Similarly, Cx43 cytoplasmic accumulation is a normal process during spermatogenesis in the ependyme (19), possibly functioning to control proliferation and differentiation of spermatozoa.

Falk et al. 1994 (5) studied the insertion of several connexins in the RE plasmatic membrane of pancreatic cells and other eukaryotic cells, and determined subcellular distribution. The insertion into the membrane of all connexins in the pancreas was accompanied by an efficient proteolytic process which depended on the concentration of microsomes. Connexins that were not endogenically processed were detected in the microsomes indicating that they function as live containers of non-processed connexins. In cells that super express connexins, it was also observed that variable quantities of the products of its cleavage were encountered in the RE membrane. Consequently, a specific factor or condition must be necessary in order to avoid processing connexins that would remain cytoplasmic, as in the case of the canine perianal gland. It would be interesting to know why cytoplasmic Cx26 remains in the normal glands.

Several studies have demonstrated that the Cx26 gene has a tumor-suppression function (16, 27). Cx26 is structurally a minor Cx, not being phosphorylated (due to few amino acids in the C-terminal portion, after the fourth transmembrane domain, which can react with cytoplasmic signalization elements) (25). Little is known about the molecular function of Cx26, only that it lacks the consensual sequence for kinases and is not phosphorylated by the kinase A protein, kinase C protein or by the kinase calmodulin-dependent calcium/protein (3). Tanaka and Grossman, 2004 (24) demonstrated that forced expression of Cx26 with the use of the Cx26 adenovirus vector in prostate cancer induced cell accumulation in the G2/M phase of the cellular cycle, apoptosis and decrease of Bcl-2 expression. In the near future, we aim to study and correlate the Cx26 cytoplasmic expression in normal perianal glands and Bcl2 and Bax gene in order to correlate the physiological function of Cx26 and apoptosis modulation.

The Cx32 expression was not observed in the slides with normal, hyperplastic, neoplastic tissue sections of group I, II (moderately and poorly differentiated) and group III of canine perianal glands by the immunohistochemistry or immunoblot methods. This study demonstrated that alterations exist in Cx26 and Cx43 expressions and in the proliferation index and apoptosis (adjusted growth index = index of the cellular proliferation/apoptosis index) in the canine perianal gland, indices that differ from other androgenous dependent tissues where a parallel increase of both quantifications exist (data not shown). So, although the carcinomas and the hyperplastic glands have a much higher cellular proliferation level than group I adenomas, these have a much higher growth potential, considering the apoptosis. These data are in agreement with Kong and Ringer, 1995 (13), which suggest that the promoters are responsible for the adjusted growth increase potential of pre-neoplastic lesions, thus accelerating carcinogenesis.

In conclusion, these results indicate that Cx43 and Cx26 expression are important for canine perianal gland homeostasis and, as also seen in the literature, both the decrease of connexin expression and the eventual decrease of gap junction intercellular communication capacity coincide with the progress of the carcinogenic process. On the other hand, Cx32 was not observed in normal, hyperplastic or neoplastic glands.

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