

uPAR EXPRESSION IN CANINE NORMAL PROSTATE AND WITH PROLIFERATIVE DISORDERS

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ABSTRACT

Prostatic lesions such as prostatic intraepithelial neoplasia (PIN) and proliferative inflammatory atrophy (PIA) are studied in human and canine species due to their malignance potential. The plasminogen activator (PA) system has been suggested to play a central role in cell adhesion, angiogenesis, inflammation, and tumor invasion. The urokinase-type plasminogen activator receptor (uPAR) is a component of the PA, with a range of expression in tumor and stromal cells. In this study, uPAR expression in both canine normal prostates and with proliferative disorders (benign prostatic hyperplasia-BPH, proliferative inflammatory atrophy-PIA, prostatic intraepithelial neoplasia-PIN, and carcinoma-PC) was evaluated by immunohistochemistry in a tissue microarray

(TMA) slide to establish the role of this enzyme in extracellular matrix (ECM) remodeling and in the processes of tissue invasion. A total of 298 cores and 355 diagnoses were obtained, with 36 (10.1%) normal prostates, 46 (13.0%) with BPH, 128 (36.1%) with PIA, 74 (20.8%) with PIN and 71 (20.0%) with PC. There is variation in the expression of uPAR in canine prostate according to the lesion, with lower expression in normal tissue and with BPH, and higher expression in tissue with PIA, PIN and PC. The high expression of uPAR in inflammatory and neoplastic microenvironment indicates increased proteolytic activity in canine prostates with PIA, PIN, and PC.

KEYWORDS: CD87; PIA; PIN; prostatic carcinoma; TMA.

EXPRESSÃO DE uPAR NA PRÓSTATA CANINA NORMAL E COM LESÕES PROLIFERATIVAS

RESUMO

Lesões prostáticas como a neoplasia intraepitelial prostática (PIN) e a atrofia inflamatória proliferativa (PIA) são estudadas na espécie humana e canina devido ao seu potencial de malignidade. O sistema de ativador de plasminogênio (PA) tem sido sugerido como um importante mecanismo na adesão celular, angiogênese, inflamação e invasão tumoral. O receptor ativador de plasminogênio do tipo uroquinase (uPAR) é um componente do PA, expresso em células tumorais e

estromais. Avaliou-se, por imunistoquímica em lâmina de microarranjo tecidual (TMA), a expressão de uPAR no tecido prostático canino normal e com desordens proliferativas (hiperplasia prostática benigna-HPB, atrofia inflamatória proliferativa-PIA, neoplasia epitelial prostática-PIN e carcinoma-CP), com o objetivo de verificar o papel desta enzima na remodelação de matriz extracelular (ECM) e no processo de invasão tecidual. Foram obtidos 298 cores e 355 diagnósticos, sendo 36

(10,1%) próstatas normais, 46 (13,0%) com HPB, 128 (36,1%) com PIA, 74 (20,8%) com PIN e 71 (20,0%) com CP. Há variação na expressão de uPAR na próstata canina de acordo com a lesão, com menor expressão nas glândulas normais e com HPB, e maior naquelas com

lesões displásicas e (PIA e PIN) e neoplásicas (CP). A superexpressão de uPAR nos microambientes inflamatório e neoplásico indica aumento da atividade proteolítica em próstatas caninas com PIA, PIN e CP.

PALAVRAS-CHAVE: carcinoma prostático; CD87; PIA; PIN; TMA.

INTRODUCTION

Researchers have studied the canine prostate due to its similarities with the human prostate regarding the natural occurrence of diseases and the hormonal influence in their development, for instance, benign prostatic hyperplasia (BPH) and prostatic carcinoma (PC) (LEROY & NORTHROP, 2009).

Some dysplastic lesions that affect man's prostate are considered premalignant, as the prostatic intraepithelial neoplasia (PIN), because they show morphological similarities to cancer or because they involve potentially carcinogenic factors (DE MARZO et al., 2006). The proliferative inflammatory atrophy (PIA) is another affection that has been constantly investigated due to the controversy regarding its premalignant potential (WATERS & BOSTWICK, 1997; WANG et al., 2009).

In dogs, PIN has been considered a premalignant lesion and it can be observed in cases of prostatic carcinoma (WATERS & BOSTWICK 1997; WATERS et al., 1997; MADEWELL et al., 2004; MATSUZAKI et al., 2010). RODRIGUES et al. (2010) and TOLEDO et al. (2010) mentioned PIA in canine prostates.

The evolution process of PIA and PIN is followed by tumor invasion through the extracellular matrix (ECM). This condition comprises the interaction phases between neoplastic cells and ECM, with hydrolytic destruction by proteolytic enzymes and migration of neoplastic cells through the altered extracellular environment (DEL MAESTRO et al., 1990; AMBIRU et al., 1997).

The plasminogen activator (PA) system is among the proteolytic enzymes involved in tumor invasion. This system is composed of a serine protease urokinase-type plasminogen activator (uPA) and its receptor (uPAR), a serine protease tissue-type PA (tPA), plasminogen and its multiple receptors, besides three inhibitors (plasminogen activator inhibitors PAI-1, PAI-2, and protease nexin 1) (WANG, 2001; BOCK & WANG, 2004).

The coordinated expression of this system has been suggested to play a central role in cell

adhesion, migration, and invasion (PEI et al., 1999; BOCK & WANG, 2004), as well as the degradation of basement membrane and ECM, and the development of cancer metastasis (COHEN et al., 1991; DANO et al., 1994; VASSALLI, 1994; KOBLINSKI et al., 2000).

Components of the PA system and in particular uPAR are suited for routine analysis because of the high levels of antigen found not only within cancer tissue but also within serum, making it readily accessible for measurement (GAO et al. 2001; PLOUGAR et al., 2002; BOCK & WANG, 2004; SEHGAL et al., 2006). The association among high levels of uPAR, higher histological grades, and advanced stages of prostate cancer (STEWART et al., 2004) makes any high uPAR expression in cancer not only possible but also valuable as it is an attractive therapeutic target (MAZAR, 2001; BOCK & WANG, 2004; SEHGAL et al., 2006).

The assessment of uPA and uPAR in prostate cancer was initially performed in primary tumors of this organ in humans and in models of the prostatic disease like rats and mice (GILARDONI et al., 2003; PULUKURI et al., 2007). BAILEY et al. (2006) were the first ones to report the constitutive expression of these proteins only in normal prostates of dogs, without reports on ill tissues.

The expression of uPAR in both normal canine prostatic tissue and with proliferative disorders (BPH, PIN, PIA and PC) was verified in order to evaluate the role of this enzyme in ECM remodeling and tissue invasion processes.

MATERIAL AND METHODS

We evaluated 298 samples of adult canine prostate tissue from files of two Pathology Services. From the paraffin blocks, 3µm sections were stained with hematoxylin and eosin (HE) for microscopic examination. Histomorphological evaluation included normal prostates and with BPH (LEAV et al., 2001), PIA (TOLEDO et al., 2010), PIN (BOSTWICK 1995), and PC (SUGAR, 2006) (Table 1). All histological slides were examined by three investigators. Normal prostatic tissues came from dogs with no lesions in the gland.

The prostate tissue microarray (TMA) was carried out according to criteria described by KONONEN et al. (1998) and BUBENDORF et al. (2001). From the previous defined areas, core biopsies were taken from 298 prostatic paraffin-embedded samples using Tissue Microarrayer (Becher Instruments®, Silver Spring, USA). Tissue cores with a dimension of 1.0 mm from each specimen were punched and arrayed on a recipient paraffin block. Three-µm-sections were obtained from the recipient block and mounted on silanized glass slides for HE and immunohistochemical tests.

Immunohistochemistry was performed in one TMA slide, which was deparaffinized, rehydrated and washed in distilled water. For anti-uPAR mouse monoclonal antibody, clone R4 (Dako M7294), we used a 1:25 dilution and antigen retrieval in water bath at 96°C for 40min, with pre-heated TRIS-EDTA buffer and pH9.0. Endogenous peroxidase activity was blocked and incubation with primary antibody was carried out in a wet chamber, at 4°C, for 18h. Advance HRP signal amplification system (Dako K 4068) was used and the reaction

was visualized by DAB (Diaminobenzidine, Dako, K3468-1). Sections were counterstained with Mayer's hematoxylin, washed, dehydrated, cleared, mounted, and examined by light microscopy.

Dog small intestine was used like positive tissue control for uPAR. The primary antibody was replaced by TRIS buffer, pH7.4, on canine prostate for the negative antibody control.

The intensity of cytoplasmic reactivity of the antibody in epithelial and periacinar stromal cells was scored in 0 = negative, 1 = discrete, 2 = moderate and 3 = intense. Regarding the number of both epithelial and periacinar stromal stained cells the scores were 0 = negative; 1 = 1 - 25%; 2 = 26 - 50%; 3 = 51 - 75% and 4 = 76 -100%.

Kruskal-Wallis and Mann Whitney tests as well as descriptive data were used to compare the scores of percentage of positive cells and the intensity. Data were analyzed using Excel 2007 and SPSS (Statistical Package for Social Science, version 16.0) software. All values were considered at 5% of significance level.

Table 1 - Histomorphological criteria for the prostatic canine tissue classification.

Diagnoses	Architectural features	Cytoplasm	Nucleus	Chromatin	Nucleolus	Basement membrane	Periacinar inflammatory infiltrate
Normal prostate	One epithelial layer with cuboidal or columnar cells and fine fibrovascular stroma	Abundant	Not change	Uniform	None	Intact	None
BPH	Epithelial hyperplasia or hypertrophy	Variable	Not change	Uniform	None	Intact	None
PIA discrete	Atrophic acini, more than one epithelial layer	Reduced and hypochromatic	Discrete changes in size and form	Condensed	One or more evident	Intact	Sparse
PIA moderate	Atrophic acini, more than one epithelial layer	Reduced and hypochromatic	Discrete changes in size and form	Condensed	One or more evident	Intact	Aggregates of mononuclear cells
PIA intense	Atrophic acini, more than one epithelial layer	Reduced and hypochromatic	Discrete changes in size and form	Condensed	One or more evident	Intact	Mononuclear cells forming follicles
PIN	Hypercellular epithelium, stacked, laminated and irregular epithelial cells	Reduced and hypochromatic	Variable in size and form	Coarse	Large and evident	May be ruptured	None
Carcinoma	Cellular proliferation, variation in acini and cell size and form	Variable	Variable in size and form	Coarse	Large and evident	Ruptured	None

Note: Basal cell layer is discontinuous in the dog prostate.

RESULTS

From the 298 TMA cores we obtained 355 different diagnoses and since in 19.1% of the cores presented multiple diagnoses (for example: PIN foci and a PC area). From 355 diagnosis, 36 (10.1%) were normal tissues, 46 (13.0 %) BPH, 128 (36.1 %) PIA, 74 (20.8 %) PIN, and 71 (20.0 %) PC. Concerning PIA (n=128), we observed that 71 (55.5 %) were discrete (PIA-D), 39 (30.5 %) moderate (PIA-M) and 18 (14.0 %) intense (PIA-I).

Immunohistochemistry staining for uPAR was cytoplasmic (Figure 1). In acinar and periacinar compartments there was difference in uPAR protein expression according to the diagnosis, regarding the number and intensity of positive cells ($p < 0.05$).

The number of epithelial cells stained

(NECS) for uPAR was statistically different between all diagnoses, except between PIN and carcinoma. For staining intensity of epithelial cells (SIEC), there was a significant difference between normal prostates and those with PIA, PIN, and carcinoma. Regarding the number of stained stromal cells (NSSC) there was difference between normal glands and those with PIA. Moreover, for the uPAR staining intensity of stromal cells (SISC), there was difference between normal prostates and those with PIA, as well as between those with PIA and carcinoma (Table 2).

Regarding the types of PIA, there was difference between PIA-D and PIA-I in relation to the number of stained stromal cells and between PIA-D and PIA-M in staining intensity of stromal cells for uPAR (Table 2).

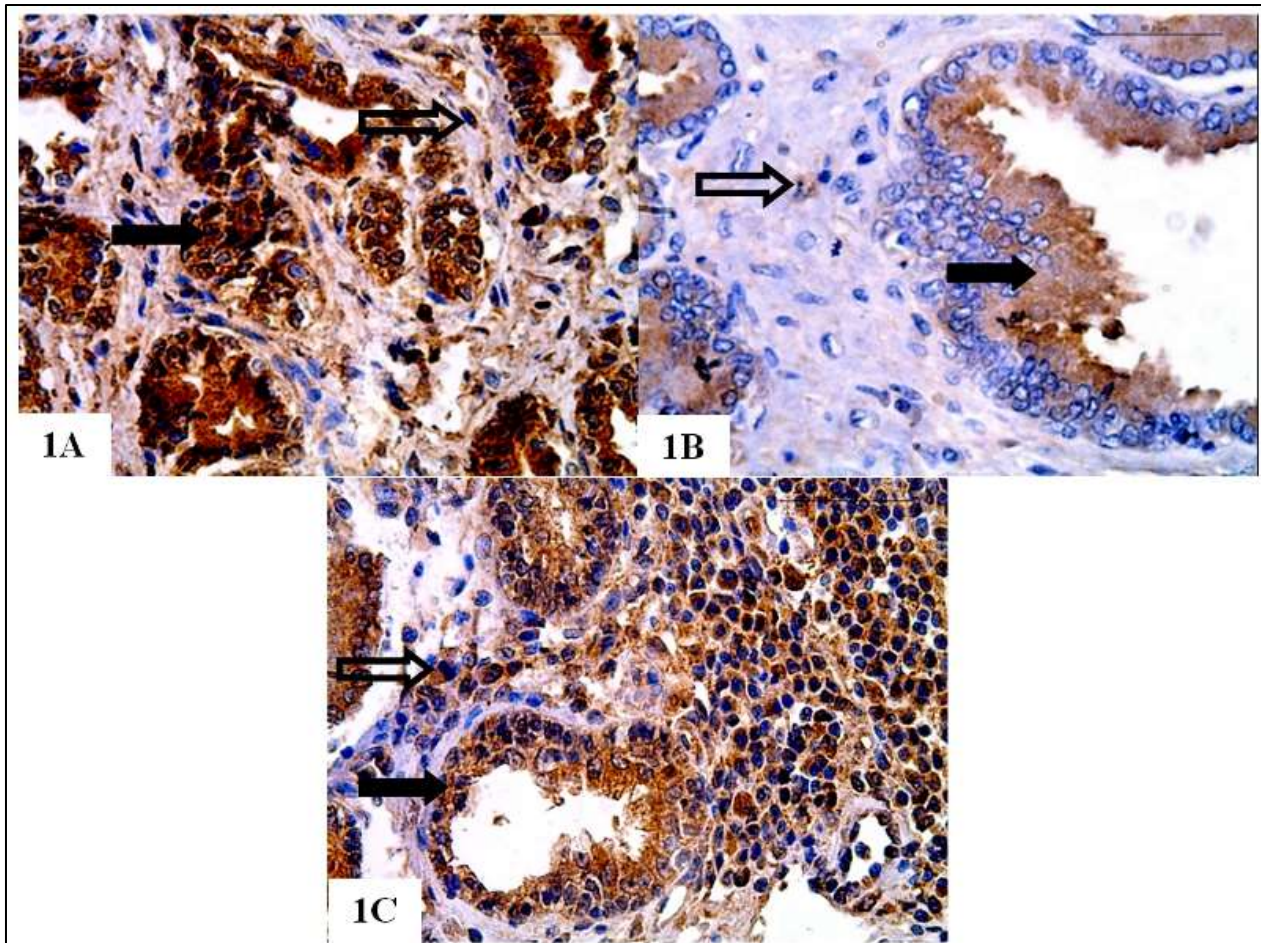


Figure 1 - Photomicrographs of canine prostate. uPAR cytoplasmic staining intensity. A) Prostatic carcinoma. Score three of staining intensity for both epithelial (filled arrow) and stromal cells (hollow arrow). B) PIN. Epithelial cells (filled arrow) with score two and stromal cells (hollow arrow) with score one of staining intensity. C) PIA-I. Score three of staining intensity for both epithelial cells (filled arrow) and stromal cells (hollow arrow). For A, B, and C: objective of 40x and Mayer's hematoxylin counterstaining.

Table 2 - Comparison between diagnoses in relation to the number of stained cells and staining intensity of uPAR in epithelial and stromal cells

	Diagnosis	Cases	NECS	SIEC	NSSC	SISC
uPAR	Normal	36	78.8 ^a	132.4 ^a	150.9 ^a	157.6 ^a
	BPH	46	136.6 ^b	173.8 ^{ab}	177.1 ^{ab}	178.3 ^{ab}
	PIA	128	186.8 ^c	181.5 ^b	193.2 ^b	190.3 ^b
	PIN	74	204.8 ^d	180.1 ^b	168.7 ^{ab}	179.5 ^{ab}
	Carcinoma	71	209.5 ^d	195.9 ^b	178.3 ^{ab}	168.3 ^a
Types of PIA						
	PIA D	71	64.7 ^a	62.3 ^a	57.9 ^a	59.1 ^a
	PIA M	39	68.4 ^a	68.0 ^a	70.2 ^{ab}	69.3 ^b
	PIA I	18	62.0 ^a	72.3 ^a	84.7 ^b	82.3 ^{ab}

Equal letters in the same column do not differ by the Mann-Whitney test ($p < 0.05$). NECS - number of epithelial cells stained; SIEC - staining intensity of epithelial cells; NSSC - number of stained stromal cells; SISC - staining intensity of stromal cells.

DISCUSSION

In different types of human tissues, the PA system has been suggested to play a central role in cell adhesion, migration regulation, wound healing, angiogenesis, inflammation and growth factors regulation (GAO et al. 2001; PLOUGAR et al., 2002; BOCK & WANG, 2004), besides the development of tumor invasion and metastasis (DANO et al., 1994; VASSALLI, 1994; KOBLINSKI et al., 2000).

In this research, we found uPAR expression in normal canine prostatic tissue and increase expression of it in epithelial and stromal cells of canine prostate with benign, dysplastic, and malignant lesions. In contrast, there is minimal information about the activity of this glycoprotein in different tissues of the dogs. Only BAILEY et al. (2006) reported the expression of uPA and uPAR in the urinary tract of healthy dogs, including low expression in the prostate.

The variation in number of stained cells and staining intensity of uPAR in epithelial and stromal cells showed that enzyme presents variable expression in canine prostate tissue according to the pathological process. In this context, there are some controversies for uPAR localization and expression (BAILEY et al., 2006; LI & COZZI, 2007; DASS et al., 2008; KUMANO et al., 2009). In rats, WILSON

et al. (1995) found a significant variation of uPAR expression among the variables age, region of the prostate and castration condition. Moreover, GAVRILOV et al. (2001) reported association between uPAR expressed in prostatic adenocarcinoma cells and prostatic stromal cells.

The number of uPAR stained epithelial cells varied among the diagnoses, excepted between PIN and carcinoma, with low expression in normal cells and high expression in PIN and carcinoma cells as described by GAVRILOV et al. (2001), RIDDICK et al. (2005), SEHGAL et al. (2006) and LI & COZZI (2007) in human and mouse neoplastic prostate. Although they have not studied glands with PIN, the increased expression of uPAR in canine prostates with this injury suggests increased proteolytic activity and possible potential of invasion.

As for the intensity of epithelial cells stained for uPAR among normal prostates and those with PIA, PIN and carcinoma, a significant difference was found with higher expression in prostates with dysplastic and neoplastic lesions. LI & COZZI (2007) believed that the higher expression of this glycoprotein in carcinomas may be related to the degree of cellular differentiation. Thus, the more undifferentiated cells are the higher the uPAR expression, suggesting a greater invasive potential of the tumor. On the other hand, USHER et al. (2005) suggested that, in some cases, uPAR expression in

tumor tissue might be low or negative, which disagrees with this study considering that all samples showed uPAR expression at some degree.

COZZI et al. (2006) reported uPAR expression in eight of fifteen human prostates with PIN, but they emphasized that there was no high expression as in carcinoma with high grade, contrary to the uPAR high expression observed in PIN and carcinoma in this study. This difference can be explained by dissimilarities in the methodology of both studies. In the first one, tumors were classified according to the cellular differentiation degree (Gleason score), with high expression in the undifferentiated ones and intermediate expression in the differentiated ones, similar to what occurred in PIN. In this study, tumors were not graded and showed high expression, but not always, as well as the PIN, which might mean a less aggressive canine tumor pattern.

Samples with PIA presented more staining cells and higher staining intensity for uPAR in the stromal cells than in normal tissues. In this sense, USHER et al. (2005) reported uPAR expression in interstitial leukocytes of neoplastic human prostate, and BAILEY et al. (2006) found accentuated uPAR staining in interstitial inflammatory cells of canine prostate. Therefore, it is likely that the inflammation surrounding the dysplastic epithelial lesion in PIA is responsible for such difference, once the interstitial inflammatory cells showed uPAR high expression. The difference between PIA and carcinoma in the uPAR staining intensity of stromal cells surrounding the tumor supports this idea, once the tumors showed less staining intensity than PIA and they were not surrounded by perineoplastic inflammation.

The comparison among types of PIA confirms what was described considering the inflammatory cells from PIA-M that showed higher staining intensity than PIA-D, and PIA-I presented higher number of stromal cells stained for uPAR than PIA-D, suggesting the role of inflammation in remodeling the ECM that surrounds the dysplastic epithelium of canine prostate, and which possibly can contribute to the invasion of the matrix by transformed epithelial cells. In this context, ANDREASEN, et al. (1997) stated that PA system is important in the process of tissue remodeling due to the ability of the uPA-uPAR complex to degrade the basement membrane in inflammatory and neoplastic diseases.

CONCLUSION

There is variation in the expression of uPAR in canine prostate according to the lesion, with lower expression in normal tissue and with BPH, and

higher expression in prostatic tissue with dysplasia (PIA and PIN) and neoplasia (PC). The high expression of uPAR in inflammatory and neoplastic microenvironment indicates increased proteolytic activity in canine prostates with PIA, PIN, and PC.

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