イヌの分泌型炭酸脱水酵素(CA-VI)の鼻腔における組織局在と遺伝子発現

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Immunohistolocalization and Gene Expression of Secretory Carbonic Anhydrase Isoenzyme CA-VI in Canine Nasal Cavity

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ABSTRACT. Immunolocalization of the secretory form of carbonic anhydrase isoenzyme, CA-VI were studied using a specific canine CA-VI antiserum, and CA-VI mRNA signals were also investigated using the reverse-transcriptase polymerase chain reaction (RT-PCR) in canine nasal mucosal epithelia and glands. Immunoreactivity to CA-VI was positive throughout the mucosal epithelial cells and in the cytoplasm of serous acinar and ductal epithelial cells of the nasal mucosa and glands, including the vestibule of the nose, but the mucous acinar cells of the glands were immunonegative. We detected CA-VI gene transcripts in the same regions as the CA-VI immunoreactivity. The physiological roles of CA-VI in the nasal mucosal epithelium and glands might maintain bicarbonate levels in nasal secretions and protect the mucosa against acid.

KEY WORDS: canine nasal cavity, CA-VI, immunohistochemistry, mRNA.

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Carbonic anhydrase (CA; EC4.2.1.1) is a zinc-containing metalloenzyme that rapidly catalyses bicarbonate hydration and carbonic acid dehydration. Carbonic anhydrase also plays important roles in the stabilization of macromolecules, gas exchange, acid-base balance, ion transport, and bone resorption [33, 35, 36]. At least 14 distinct active isoenzymes of CA are widely distributed throughout mammalian organs [11]. Of these isoenzymes, only CA-VI occurs in secreted form, and it localizes to the digestive organs, specifically the parotid, submandibular and esophageal, lingual serous von Ebner's glands in sheep [8, 9, 31], rats [7, 18, 23], humans [13, 18, 20, 24, 29] and dogs [2, 14, 21]. Furthermore, the CA-VI isoenzyme has been identified in the lacrimal and mammary glands of rats [26], sheep [25] and cattle [12, 16]. The CA-VI secreted in mammary glands regulates acid-base balance of upper alimentary canal [12]. Highly concentrated buffer ions are important for mucosal protection against an excessively acidic milieu. Together with other cytosolic carbonic anhydrases in humans, CA-VI might work to neutralize the excessive organic acidic milieu produced by microbial flora in dental and epithelial surface cells [17]. In the respiratory tract, immunohistochemistry and real-time PCR have detected CA-VI in the nasal mucosa, lower airways and lungs of guinea pigs, humans and mice [15, 19, 28, 35]. The suggested roles of CA-VI in these areas include the maintenance of pH homeostasis, protecting the upper respiratory tract against excessive acidity, and transporting electrolytes via epithelial cells.

The present study examined the immunohistolocalization and gene expression of secretory CA-VI in canine nasal mucosa and glands using anti-canine CA-VI antiserum and CA-VI mRNA expression as an initial step towards exploring the physiological roles of CA-VI in the respiratory system.

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MATERIALS AND METHODS

Animals: The dogs were supplied by the Department of Surgery, Azabu University. The dogs were sacrificed by an overdose of pentobarbital. The animal facility at Azabu University has animal care and use programs that are accredited by Office of Laboratory Animal Welfare.

CA-VI antibody: Canine salivary CA-VI was purified as described [14] and then used to determine antiserum specificity by means of double immunodiffusion [29].

Tissue: Nasal mucosa samples obtained from the vestibule of the nose, respiratory region, olfactory region, lateral nasal glands and parotid glands (positive control) of 5 adult beagles were immediately fixed in neutralized 10% formalin or Bouin's solution, and then cut into 4-μm-thick sections.

Immunohistochemical staining: Deparaffinized and rehydrated sections were immersed in methanol containing 0.3% H_2O_2 to block endogenous peroxidase activity, followed by normal goat serum (2% in PBS) for 20 min to block fragment crystallizable receptors. Then, sections were incubated with specific antisera against CA-VI (diluted 1:100) for 1 hr at room temperature. Immunoreactive sites were visualized using the Vectastain Elite avidin-biotin-peroxidase complex kit (ABC-POD reagent kit; Vector, Burlingame, CA, U.S.A.) and 3,3'-diaminobenzidine, tetrahydrochloride (DAB). Sections were counter stained with hematoxylin, dehydrated through a graded alcohol series and mounted on coverslips for light microscopic

observation and photography.

RNA isolation, cDNA preparation and polymerase chain reaction: Total RNA was prepared from the tissues described above using the RNeasy Mini Kit (Qiagen, Tokyo, Japan), and then cDNA was synthesized from the total RNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster city, CA, U.S.A.) according to the manufacturer's instructions for both procedures. EX Taq Hot Start Version (TaKaRa, Otsu, Japan) enzyme was used for PCR. The primer sequences for canine CA-VI (GenBank accession number AB080972) were 5'-CAGCGGCTCGGAGCATAC-3' and 5'-GGCTGGATCCTGCGGTAGTCA-3', which generated a 441-bp fragment. The primer sequences for canine β-actin (GenBank accession number AF021873), a constitutively expressed housekeeping gene, were 5'-GGGAGATCGTGCGTGACAT-3' and 5'-TCAGCAATGCCAGGGTACAT-3', which generated a 316-bp fragment. Canine CA-VI was amplified using 35 cycles of denaturation (95°C, 30 sec), annealing (64°C, 30 sec) and extension (72°C, 30 sec) in a thermal cycler (TP650; TaKaRa). Amplification of β-actin involved 35 cycles of denaturation (95°C, 30 sec), annealing (56°C, 30 sec), and extension (72°C, 30 sec). Aliquots (5 μl) of the PCR products were resolved on 1.5% agarose gels and stained with ethidium bromide for observation under UV irradiation at 254 nm.

RESULTS

Table 1 summarizes the immunohistochemical results. Immunoreactivity to anti-CA-VI antiserum diffused through the cytoplasm of all tested samples. Some vascular endothelial and interstitial cells were shown to be weakly to moderately nonspecific immunopositive reactions. Stratified columnar epithelium in the vestibule of the nose and ductal epithelial cells in the vestibule glands were moderately immunopositive to the anti-CA-VI antiserum, and the vestibule gland and serous acinar cells in the superficial layer of subepithelial connective tissue were weakly to moderately immunopositive. In contrast, serous acinar cells in the deep layer of subepithelial connective tissue were negative to weakly immunoreactive (Figs. 1 and 2). Ciliated mucous epithelium and ductal epithelial cells of the nasal glands in the respiratory region of the nose were moderately immunopositive, whereas goblet cells and serous acinar cells of the nasal glands were immunonegative (Fig. 3). In the olfactory mucosa, olfactory cells have one process to the luminal surface and other side to the basal layer of the epithelium. Some thin spindle shaped olfactory cells with slender dendrite were intensely immunopositive to the anti-CA-VI antiserum, while other epithelial layer cells are moderately immunopositive. Serous acinar and ductal epithelial cells in the olfactory glands were moderately immunopositive to the anti-CA-VI antiserum (Fig. 4). In the lateral nasal gland, serous acinar cells were immunonegative, whereas striated and intercalated ductal epithelial cells were moderately immunopositive (Fig. 5).

We detected CA-VI gene transcripts by RT-PCR in the parotid gland, vestibule of the nose, respiratory region, olfactory region, and lateral nasal gland. The β-actin gene was detected in the same areas as the CA-VI mRNA signals (Figs. 6 and 7).

DISCUSSION

We detailed the immunohistolocalization of a secretory carbonic anhydrase isoenzyme (CA-VI) in the canine nasal cavity for the first time. Immunoreactivity to CA-VI was detected in the respiratory mucous epithelial cells including the vestibule of the nose, the vestibule gland, and in the ductal epithelial cells of all glands of the nasal mucosa. However, serous acinar cells in the nasal and lateral nasal glands were not immunoreactive. The CA-VI mRNA expression in each canine nasal tissue coincided with the immunohistochemical findings. Both immunohistochemistry and RT-PCR have detected CA-VI signals in the murine respiratory region, nasal epithelial cells and serous acinar cells [15], and only RT-PCR has identified CA-VI in humans [35].

The physiological roles of nasal CA-VI might be to provide a greater buffering capacity in the nasal mucosa, to protect the upper respiratory tract against excessive acidity [15, 19] and electrolyte transportation via epithelial cells [5]. Excessive organic acids or endogenous acid generated from CO_2 could be rapidly neutralized by the CA enzymes [1, 30, 32]. The present results also support the suggestion that CA-VI from the mucous epithelia protects the lining of the

Table 1. Immunohistochemical distribution of CA-VI in nasal mucosa

	Mucous epithelial cells Mouse[a]/Dog	Acinar secretory cells Mouse[a]/Dog	Ductal epithelial cells Mouse[a]/Dog
Parotid gland	*	ND/++	ND/+[e]
Vestibule of nose	ND/+	ND/–~+[b]	ND/+
Respiratory region	+/±	–~+[c]/–	+/+
Olfactory region	+/±~++[d]	–/+	–/+
Lateral nasal gland	*	+/–	+/+[e]

a) Data from [16].; b) Immunoreactivity of serous acinar cells in superficial layer, ±~+; in deep layer, –~±; c) Immunoreactivity of nasal septum: anterior gland, + [16]; posterior gland, – [16].; d) Immunoreactivity of olfactory cells, ++; sustentacular cells and basal cells, ±~+; e) Striated and intercalated ducts are moderately immunopositive.

±, weak; +, moderate; ++, intense; –, negative. ND, no data; *, absent.

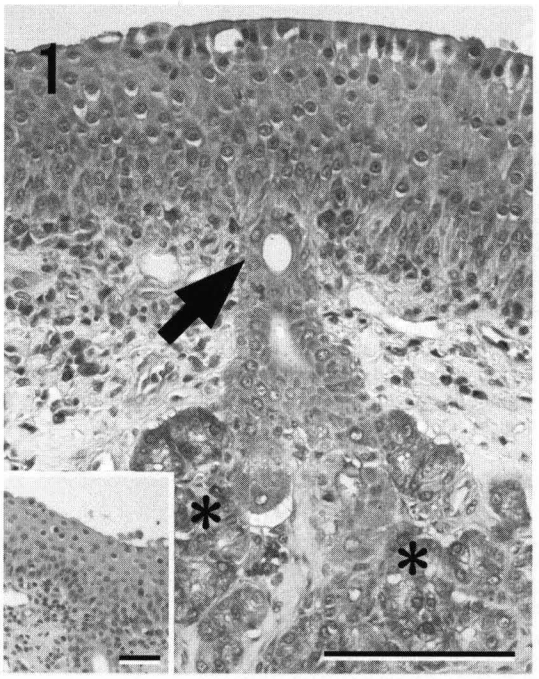

Fig. 1. Immunoreactivity against anti-canine CA-VI antiserum in vestibule region and vestibule gland in superficial layer. Bar, 100 μm. CA-VI is expressed in mucous; stratified columnar epithelial cells, and ductal epithelial cells; simple cuboidal epithelial cells (arrow). Serous acinar cells (*) in superficial layer of subepithelial connective tissue are weakly to moderately immunopositive to anti-CA-VI antiserum. Insert shows negative control.

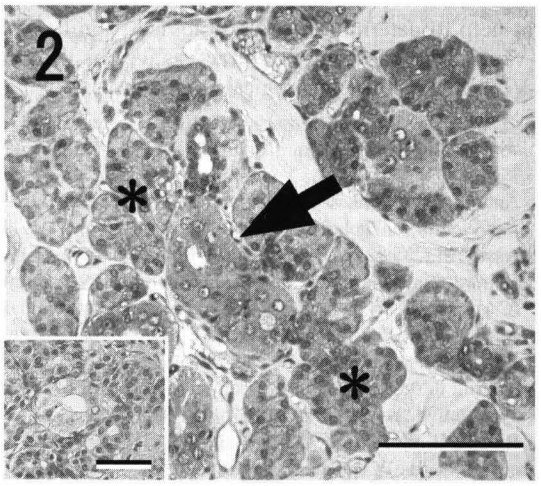

Fig. 2. Immunoreactivity against anti-canine CA-VI antiserum in vestibule gland in deep layer of subepithelial connective tissue. Bar, 100 μm. CA-VI is expressed in ductal epithelial cells; simple cuboidal epithelial cells (arrow). Serous acinar cells (*) in deep layer of subepithelial connective tissue are negative to weakly immunoreactive to anti-CA-VI antiserum. Insert shows negative control.

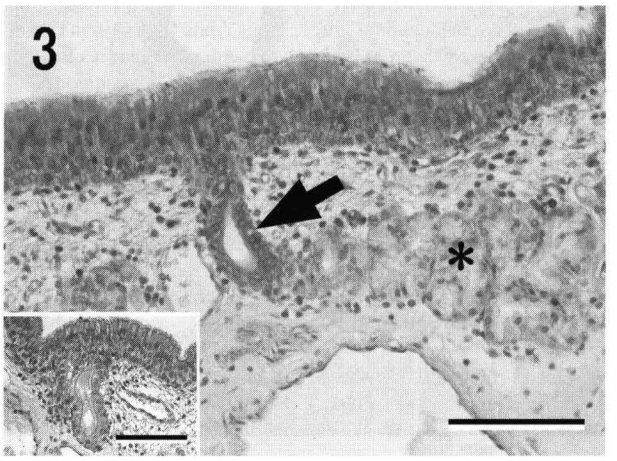

Fig. 3. Immunoreactivity against anti-canine CA-VI antiserum in respiratory region of nasal mucosa. Bar, 100 μm. CA-VI is expressed in mucous and ductal epithelial cells (arrow), but not in serous acinar cells of nasal glands (*). Insert shows negative control.

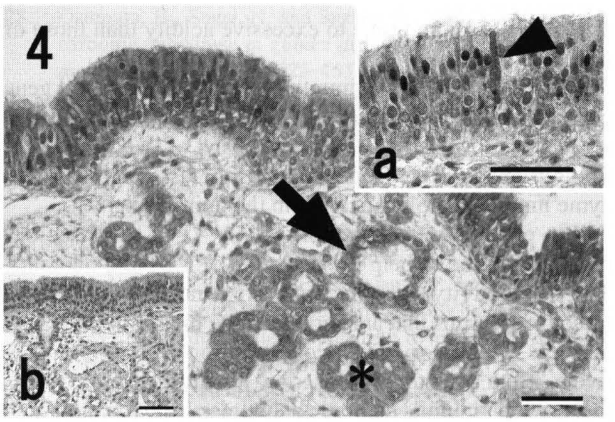

Fig. 4. Immunoreactivity against anti-canine CA-VI antiserum in olfactory region of nasal mucosa. Bar, 100 μm. CA-VI is expressed moderately to intensely in mucous and ductal epithelial cells (arrow) and moderately in serous acinar cells of olfactory glands (*). Some spindle shaped olfactory cells are intensely immunopositive to anti-CA-VI antiserum (arrowhead). Other epithelial layer cells are moderately immunopositive. Figure 4a is a high magnification image in olfactory epithelium. Figure 4b is negative control.

respiratory tract against the excessive acidity of the respiratory milieu [19]. Ogawa *et al.* [22] described that there were two types of CA-VI, differentiated by their subcellular localizations. The first type is found in serous granules, while the second type is found diffusely in the cytoplasm. The function of second type is suggested to catalyse ion secretion and exchange in the cell. Based on these findings, stratified columnar epithelium in the vestibule of the nose may contain second type of CA-VI. Dogs must often imbibe large quantities of water for evaporative cooling through panting [3], so the upper respiratory canal of the

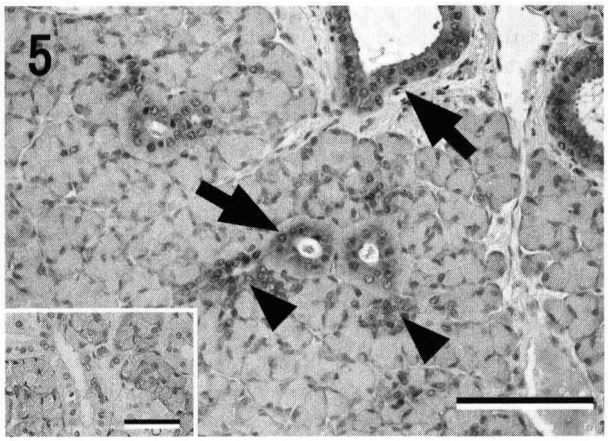

Fig. 5. Immunoreactivity against anti-canine CA-VI antiserum in lateral nasal gland in nasal mucosa. Bar, 100 μm. CA-VI is moderately expressed in striated (arrows) and intercalated (arrowheads) ductal epithelial cells, but not in serous acinar cells. Insert shows negative control.

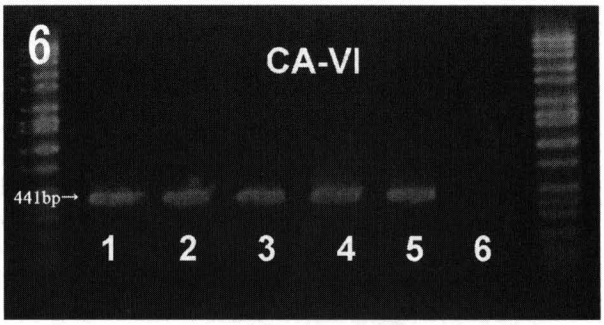

Fig. 6. RT-PCR analysis of CA-VI mRNA in nasal mucosa. Lanes: 1, parotid gland; 2, vestibule region; 3, respiratory region; 4, olfactory region; 5, lateral nasal gland; 6: negative control.

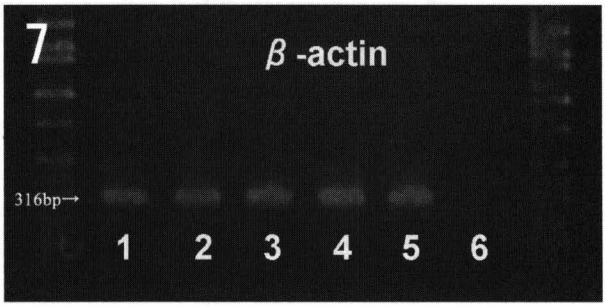

Fig. 7. RT-PCR analysis of β-actin mRNA in nasal mucosa. Lanes: 1, parotid gland; 2, vestibule region; 3, respiratory region; 4, olfactory region; 5, lateral nasal gland; 6: negative control.

dog might be more prone to excessive acidity than those of mice and humans.

We confirmed CA-VI immunohistolocalization and gene expression in the mucous epithelial, ductal epithelial and serous acinar cells of the olfactory gland of the dog. These findings are similar to those of cytosolic CA-I and II isoenzyme immunohistolocalization in the guinea pig [28].

The physiological function of CA-VI in the olfactory region might be to help the detection of CO_2 and odorants. The nasal cavity contains trigeminal nerve endings and olfactory neurons that are stimulated by high (noxious CO_2) and low (respiratory CO_2) concentrations of CO_2, respectively. Both receptor responses are attenuated by acetazolamide, which inhibits carbonic anhydrase [6], indicating that CA is involved in CO_2 sensing by the nasal mucosa. Some spindle shaped olfactory cells are intensely immunopositive to anti-CA-VI antiserum than other epithelial layer cells. These findings might be suggesting that some olfactory cells are associated with CO_2 sensing in the nasal mucosa. In addition, the CA-VI isoenzyme is considered to cover the entire surface of the olfactory cells in the epithelium. This layer serves to maintain mucosal moisture and to furnish the necessary solvents for primary contact with odor stimuli [4]. Frings *et al.* [10] reported that the concentration of electrolytes such as Na, K, Ca in the mucous layer over the olfactory epithelium affected the sensitivity of odor detection. We also detected CA-VI in the canine olfactory epithelial and glands cells. These results suggest that CA-VI is associated with the neutralization of excessive organic acid and endogenous acids synthesized from CO_2, as well as with CO_2 sensing and the sensitivity of odor detection.

In summary, we discovered that the principal sites of normal canine CA-VI secretion are the serous secretory cells and ductal segments in the olfactory gland, the vestibule gland and the nasal mucosa in particular. These findings might help to determine the physiological significance of CA-VI in the upper respiratory tract, as well as the role of CA-VI under various pathological conditions.

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