NOTE *Anatomy*

Preliminary Immunohistochemical Study of Natriuretic Peptide Receptor Localization in Canine and Feline Heart

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ABSTRACT. We examined the immunohistochemical distributions of natriuretic peptide receptor (NPR)-A, -B and -C that bind with natriuretic peptide hormones A, B and C in four healthy crossbreed young canine and feline cardiac tissues using specific antibodies against human antigens. Cross-immunoreactivities between antigens and antibodies were confirmed using western blot analysis. NPR-A and -C were expressed more strongly in dogs than cats. In both species, these expressions were stronger in the atria than the ventricles, with stronger expression in the left ventricles than the right. NPR-B was largely very weekly or undetected. In canine and feline cardiac tissues, the expressional distribution of NPR-A, -B, and -C closely matched with that of atrial natriuretic peptide, brain natriuretic peptide, and C-type natriuretic peptide as the ligands for corresponding receptors.

KEY WORDS: canine, feline, heart, immunohistochemistry, NPRs.

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Isolation of the natriuretic peptide family began with isolation of atrial natriuretic peptide (ANP) from the human and the rat atrium in 1983 [2], followed by isolation of brain natriuretic peptide (BNP) from the porcine brain in 1988 [4, 6]. C-type natriuretic peptide (CNP) was subsequently isolated from the porcine brain in 1990 [4, 6]. In the heart, ANP is synthesized and secreted in the atria [14], and BNP is firstly reported in the cerebral ventricles and also synthesized in the heart, in response to stretching of the myocardium, and also control of the renin-angiotensin-aldosterone (RAA) system. CNP is secreted in the vessel endothelium and the macrophage of the kidney, the lung and the heart, in response to proliferation control of the fibroblast. It is 20 years since the first biochemical research peptides [4], and immunohistochemical research localization of natriuretic peptides has been reported in the horse [8], rat [2], mouse [10, 14], and other animals [9]. Laboratory tests and clinical researches for the therapeutic uses of human ANP and BNP have also been widely reported [16]. Researches of receptors that bind to ANP, BNP, and CNP has focused on the molecular structure of these receptors and the way in which they bind to diuretic peptides. Results have shown that receptor A (NPR-A) exhibits strong ligand-specific binding in the order of strength ANP > BNP > CNP. Receptor B (NPR-B) binds mainly to CNP, and receptor C (NPR-C) binds to ANP, BNP, or CNP with equal strength [3, 5]. However, there has been few immunohistochemical research into the tissue localizations of these receptors in dogs and cats.

The present study was devised to clarify the localizations of natriuretic peptide receptors (NPRs) in the atria and ventricles of dogs and cats with the ultimate aim of using BNP and ANP in the clinical diagnosis and treatment of heart failure in small animals.

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Samples were two male and two female dogs, and three male and three female cats, all crossbreeds in good health and aged 1.0 to 1.5 years. Animals were euthanized by excess anesthesia using intravenous injection of somnopentyl in accordance with the regulations of the Ethics Committee of Nippon Veterinary and Life Science University. After the perfusion fixation with 10% neutral phosphate-buffered formalin, tissue was sampled from the left and right atria and the left and right ventricles in the conventional methods. After the embedding in the paraffin, histological sections aproximately 3 μm thick were prepared from the samples. Immunohistochemical staining was performed according to the conventional protocols, using specific antibodies to the receptor molecules that bind to the various human NPR-A (H-125) rabbit polyclonal IgG (× 100 dilution), human NPR-B (N-18) goat polyclonal IgG (× 100 dilution), and human NPR-C (N-20) goat polyclonal IgG (× 100 dilution); Santa Cruz Biotechnologies Co., Ltd., CA, U.S.A. Further, to enhance the immunoreactivity of the different tissue sample, microwave treatment using 0.01 M citric acid buffer (pH 4.0) was performed prior to immunohistochemical staining. Following the immunohistochemical staining, the samples were observed with an optical microscope.

The following western blot protocol was performed to confirm the antigen recognition specificity of the antibodies used. Kidneys from a dog and a cat were homogenized in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X100, 0.1% sodium dodecyl sulfate (SDS), 0.1% sodium deoxycholate) containing proteinase inhibitor cocktail (Complete Mini, Roche Diagnostics, Ltd., Mannheim, Germany). We denatured 30 μg of these solubi-

lized materials in SDS under reduced conditions using 7.5% (for NPR-A and NPR-B) or 10% (for NPR-C) SDS-polyacrylamide gel only electrophoresis and transferred them electrophoretically to a polyvinylidene difluoride membrane (Millipore Co., Ltd., Billerica, MA, U.S.A.). The membranes were rinsed in 20 mM Tris HCl, pH 7.4, containing 500 mM NaCl and 0.1% Tween-20 (TBST) and then incubated in 5% blocking reagent (GE Healthcare UK Ltd., Buckinghamshire, England) in TBST overnight at 4°C. The membranes were then incubated with anti-NPR-A, anti-NPR-B, or anti-NPR-C (Santa Cruz Biotechnologies Co., Ltd., CA, U.S.A.) polyclonal antibodies for 1 hr at room temperature. After treatment with sheep anti-rabbit IgG (GE Healthcare UK Ltd., Buckinghamshire, England) or donky anti-goat IgG (Santa Cruz Biotechnologies Co., Ltd., CA, U.S.A.) conjugated with horseradish peroxidase, the membranes were developed with the Amersham Western Blotting System (GE Healthcare UK Ltd., Buckinghamshire, England) according to the manufacturer's protocol. Signals of immunoreactivity were detected by LAS400 according to the manufacturer's protocol.

The results of the western blot analysis are shown in Fig. 1. Bands specific to canine and feline NPR-A and NPR-B were found at 120 kDa, and those specific to canine and feline receptor C at 60 kDa, which indicating that the antibodies used in this study recognized the canine and the feline NPR families. Comparing the dog to the cat tissues, while there were some slight individual differences in the staining, expressions of NPR-A and NPR-C were stronger in dogs than in cats (Figs. 2C, 2D). In both normal dogs and cats, NPR-B, which bind to CNP, showed weak or no expression (data not shown). Staining showed very little age-or sex-related difference in each species.

Table 1 summarizes the expression of canine and feline NPR-A, -B, and -C, and typical histological images are shown in Figs. 2A-2E.

Expressions of NPR-A and -C was stronger in the atria than the ventricles in dogs and cats. Besides, their expressions in the canine ventricles were stronger in the right than the left. NPR-B expression was weak in both the atria and the ventricles, with no difference found between left and right atria or between left and right ventricles in dogs and cats. In addition, each NPR was widely distributed within the cardiac muscular cytoplasm rather than strongly local-

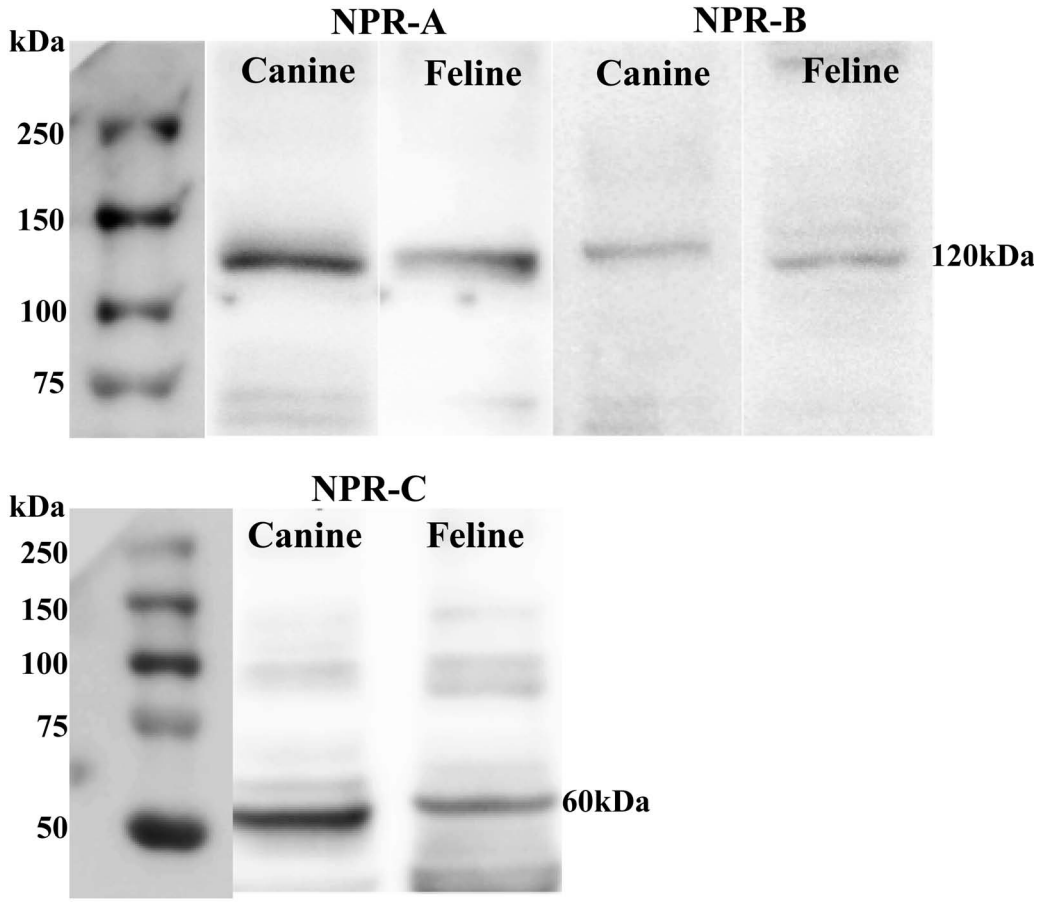

Fig. 1. Western blot analysis. Specific bands of NPR-A, B and C from canine and feline kidney samples are observed at a single band. NPR-A and B are detected at the 120 kDa band, and NPR-C is detected at the 60 kDa band.

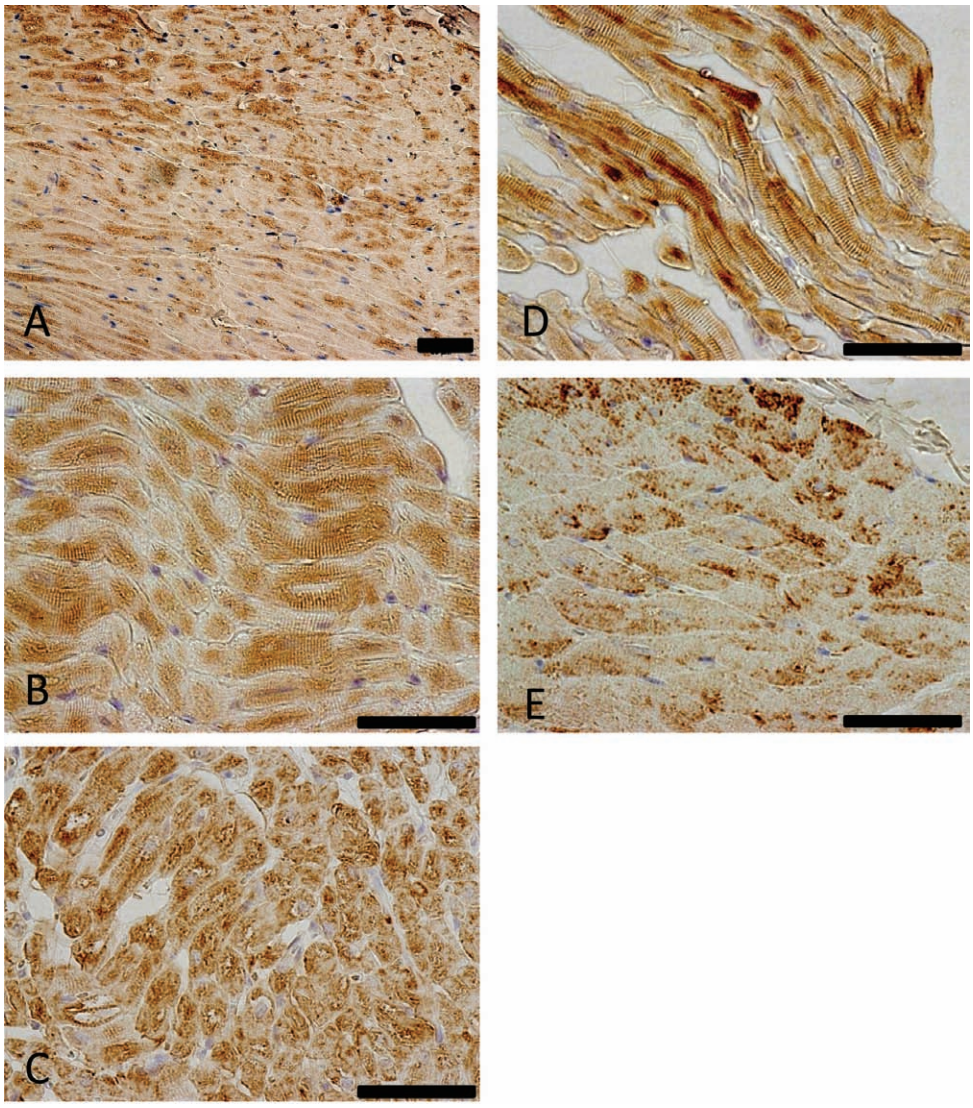

Fig. 2. Results of immunohistological staining. Bar: 50 μm. (A)NPR-A distribution is detected on canine right ventricle tissue. (B)NPR-A distribution is detected on canine left atrium tissue. (C)NPR-C distribution is detected on feline left atrium tissue. (D)NPR-C distribution is detected on canine right atrium tissue. (E)NPR-C distribution is detected on canine right ventricle tissue.

Table 1. Immunohistochemical results for NPR-A, -B and -C

Canine	NPR-A	NPR-B	NPR-C
Left atrium	++	+	+++
Right atrium	++	+	+++
Left ventricle	+	+	++
Right ventricle	++	+	+++
Feline	**NPR-A**	**NPR-B**	**NPR-C**
Left atrium	++	+	++
Right atrium	++	+	++
Left ventricle	+	+	+
Right ventricle	+	+	+

+, Weak positive. ++, Moderate positive. +++, Strong positive.

ized on the cell membrane in dogs and cats. There was no expression of NPRs in the nucleus of myocardial cells.

The highest levels of BNP in the human heart are found in elderly women [13, 14], and the hightest leveles of ANP are also reported in older Mongolian gerbils [7]. However, no differences attributable to age or gender were noted in the present experiment, probably because all the animals used were aged 1.0 to 1.5 years and the experimental population number was small. Nonetheless, expressions of NPR-A and -C were higher in the atria than in the ventricles, and slightly higher in the left atrium than the right. This distribution pattern appears to result from the different secretory pathways of ANP and BNP. Through a regulated pathway in the atria, ANP and BNP, the ligands that bind with NPR-A and NPR-

C, are synthesized in cardiac myocytes of the atria, subsequently stored in granular form in the normal myocardial cells, and when necessary released by secretion from the cell. In the ventricles, however, ANP and BNP are controlled by a constitutive pathway, in which the ligand peptides are synthesized and secreted as necessary by the ventricular cardiac myocytes. Thus, in normally functioning heart, ligand expression is constantly higher in the atria than the ventricles. Regarding the binding of ligands with receptors, there is a constant localized level of the ANP precursor proatrial natriuretic peptide (proANP), which is composed of 126 amino-acid residues. At the time of secretion, proANP is cleaved into the biologically active ANP (28 residues) and N-terminal proANP (Nt-proANP) (98 residues), and an ANP residue binds with the receptor [3]. In a similar fashion, BNP is stored as proBNP (108 residues), which is cleaved into BNP (32 residues) and N-terminal proBNP (Nt-proBNP) (76 residues) when secreted into the blood. In the normal cardiac ventricle, NPR-A and -C might always correspond to the expression levels of the ligands that bind to the receptor. Ultrastructural finding using doble immunocytochemical labeling has shown that ANP and BNP coexist in granular form in cardiac myocytes in the rat [1] and equine [8] atria. It has also been reported that, in humans, the amount of ANP in the normal ventricles is at most 1/30 of that in the atria [16]. We observed that in canine and feline hearts, the ligands ANP and BNP were more strongly expressed in the atria than in the ventricles and their expressions were stronger in the left atrium than the right (data not shown).

NPR-A, -B, and -C are all single-span transmembrane proteins. NPR-A and -B act through the mediation of membrane guanylate cyclase in the cell membrane, with a cyclic guanosine monophosphate (cGMP) as a second messenger [11, 15]. NPR-C, however, lacks the guanylate cyclase domain, so guanylate cyclase exists in both membrane-bound and soluble forms [11, 15]. It is known from X-ray crystal structure analysis [12] of NPR-A that monomolecular ligand ANP is sandwiched between receptor-dimer pair, forming a complex with a 2:1 molecular ratio [11]. It is also known that the same type of binding occurs between other receptors and ligands. A characteristic of this binding of receptor and ligand molecules is that a quantitative correspondence can be shown between the distribution of ligands and that of receptors, and theoretically twice as many receptors as ligands should be expressed in the atrium. This means that as the ligands ANP and BNP are more abundant in the atria than the ventricles during normal heart functioning, and that the expressions of receptors A and C are amplified in response to this ligand distribution, so that there is greater expression of these receptors in the atrium. Moreover, greater expressions of receptors A and C were found in the canine right ventricle than the left one, and as the receptors show the same type of distribution as the ligand here as well, it is clear that there is a relationship between ligand and receptor expression. At the same time, the weak expression of NPR-B in the cardiac tissue appears to be reflection of the scarcity or noexistence in the normal heart of CNP, only receptor may existed in the heart. These results are a clear reflection that the distribution of NPRs family matches the intramyocardial distribution of ligand molecules.

In this preliminary study, there is no distributional differencies between dogs and cats. However, little more strongly signal levels was detected at dogs than cats. Future work is needed to investigate age and gender differences and the expression of NPRs family in the tissue of organs other than the heart.

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