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Expression of Atrial and Brain Natriuretic Peptides and Their Genes in Hearts of Patients With Cardiac Amyloidosis

GENZOU TAKEMURA, MD,*† YOSHIKI TAKATSU, MD,* KIYOSHI DOYAMA, MD,‡ HIROSHI ITOH, MD,‡ YOSHIHIKO SAITO, MD,‡ MASATOSHI KOSHIJI, MD,† FUMITAKA ANDO, MD,* TAKAKO FUJIWARA, MD,§ KAZUWA NAKAO, MD,‡ HISAYOSHI FUJIWARA, MD†

Hyogo, Gifu and Kyoto, Japan

Objectives. **We investigated the expression of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) and their genes in the hearts of patients with cardiac amyloidosis and those with isolated atrial amyloidosis.**

Background. **The expression of ANP and BNP is augmented in the ventricles of failing or hypertrophied hearts, or both. The expression of ANP and BNP in the ventricles of hearts with cardiac amyloidosis, which is hemodynamically similar to restrictive cardiomyopathy, is not yet known. ANP is the precursor protein of isolated atrial amyloid.**

Methods. **We analyzed the immunohistocytochemical localizations of ANP and BNP as well as the expression of their mRNAs by in situ hybridization in the myocardium and measured the plasma levels of ANP and BNP in patients with cardiac amyloidosis.**

Results. **Four of the five right and all six left ventricular endomyocardial biopsy specimens obtained from six patients with cardiac amyloidosis were immunohistochemically positive for both ANP and BNP; none of the biopsy specimens from eight normal subjects were positive for ANP or BNP. All four of the right atria obtained at operation showed positive immunoreactions for both peptides. Electron microscopy identified specific secretory granules in ventricular myoctyes of the patients with cardiac amyloidosis, but not in ventricular myocytes from the normal control subjects. Double immunocytochemical analysis revealed the co-localization of ANP and BNP in the same granules and that isolated atrial amyloid fibrils were immunoreactive for ANP and BNP, whereas ventricular amyloid fibrils were negative for both peptides. Both ANP mRNA and BNP mRNA were expressed in the ventricles of the patients with cardiac amyloidosis but not in the normal ventricles. The autopsy study of four patients with cardiac amyloidosis revealed an almost transmural distribution of ANP and BNP, with predominance in the endocardial side. Plasma BNP levels in the patients were markedly elevated ([mean ± SD] 1,165.1 ± 561.2 pg/ml) compared with those in the control subjects (8.9 ± 6.0 pg/ml, $p < 0.05$).**

Conclusions. **Expression of ANP and BNP and their genes was augmented in the ventricular myocytes of the patients with cardiac amyloidosis. Both regional mechanical stress by amyloid deposits and hemodynamic stress by diastolic dysfunction may be responsible for the expression of the peptides in patients with cardiac amyloidosis.**

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Atrial natriuretic peptide (ANP) is a cardiac hormone secreted mainly from the atria and is involved in fluid, electrolyte and vascular homeostasis (1,2). ANP is synthesized not only in the atria but also in the ventricles of normal animals (3,4), and ventricular ANP synthesis is increased in various pathologic conditions, causing hemodynamic overload (5–9). Brain natriuretic peptide (BNP) has a striking similarity to ANP with regard to both its amino acid sequence and pharmacologic spectrum (10–12). Patients with congestive heart failure have extremely high plasma levels of BNP, and the secretion of this peptide from the ventricles is greatly augmented in these patients (12,13). These studies suggest a pathophysiologic role for ANP and BNP in cardiovascular disease as endogeneous vasodilator and diuretic agents, and the finding that ANP or BNP administration produces clinical improvement in patients with congestive heart failure supports this hypothesis (14,15).

Dilated cardiomyopathy is one of the myocardial diseases that cause heart failure, and thus augmented ANP and BNP in ventricles afflicted with this disease have been well studied (7–9,13). We found (16,17) that in the ventricles of hearts with hypertrophic cardiomyopathy, the expression of ANP and BNP was augmented despite the absence of heart failure. ANP or BNP, or both, was found to be overexpressed in the myocytes surrounding myocardial lesions, such as myocardial infarction

From the *Department of Internal Medicine and Department of Cardiovascular Surgery, Hyogo Prefectural Amagasaki Hospital, Hyogo; †Second Department of Internal Medicine, Gifu University School of Medicine, Gifu; ‡Third Department and Second Department of Internal Medicine, Kyoto University School of Medicine and §Kyoto Women's University, Kyoto, Japan.

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Address for correspondence: Dr. Hisayoshi Fujiwara, Second Department of Internal Medicine, Gifu University School of Medicine, 40 Tsukasa-Machi, Gifu 500, Japan. E-mail: gifuim-gif@umin.u-tokyo.ac.jp.

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Abbreviations and Acronyms

ANP = atrial natriuretic peptide
BNP = brain natriuretic peptide
ELISA = enzyme-linked immunosorbent assay
ET-1 = endothelin-1
LVEDP = left ventricular end-diastolic pressure
LVMI = left ventricular mass index
PBS = phosphate-buffered saline
PRA = plasma renin activity
SSC = standard saline citrate

and myocarditis (18–20). These findings suggest that focal as well as hemodynamic stress is responsible for the ventricular augmentation of these peptides. Cardiac amyloidosis presents a hemodynamically restrictive pattern and is sometimes categorized as restrictive cardiomyopathy (21). In general, hearts with cardiac amyloidosis preserve systolic function until the terminal stage, but diastolic function is damaged, mainly as a result of the mechanical disturbance of myocyte movement by the diffuse deposition of amyloid. A previous study (22) reported immunohistochemical ANP expression in the ventricles of patients with restrictive cardiomyopathies, mainly idiopathic restrictive cardiomyopathy. However, ANP and BNP have not yet been studied in detail in cardiac amyloidosis.

Another interesting relation between amyloidosis and natriuretic peptides is that in isolated atrial amyloidosis, amyloid fibrils themselves show antigenicity against both ANP and BNP, and thus ANP is regarded as the precursor protein of isolated atrial amyloid (23–25). Amyloid in cardiac amyloidosis is indeed derived from monoclonal immunoglobulin light chain (26), but it is of interest as to whether it also contains the immunogenicity of natriuretic peptides, in light of the above-mentioned isolated atrial amyloidosis case.

In the present study, we investigated the expression and tissue localization of ANP and BNP and their genes in the myocardium of patients with cardiac amyloidosis by means of immunohistochemical analysis at both the light and electron microscopic levels and in situ hybridization histochemical analysis. We also measured the plasma levels of both peptides in these patients by means of radioimmunoassay.

Methods

Tissue specimens and experimental procedure. Myocardial tissues (right atrial appendage or ventricle) were obtained at biopsy, operation or autopsy from patients with cardiac amyloidosis, patients with isolated atrial amyloidosis and control subjects. The tissue specimens were used for histologic, electron microscopic and immunohistochemical studies at both the light and electron microscopic levels and for in situ hybridization histochemical analysis to detect ANP and BNP and their genes. Blood sampling was also performed in some patients to measure the plasma concentrations of ANP and BNP (by radioimmunoassay) and other humoral factors.

Endomyocardial biopsy. Six patients who met the clinical and histologic criteria for the diagnosis of restrictive cardiomyopathy on the basis of cardiac involvement of AL amyloidosis (systemic amyloidosis with light chain deposition) were enrolled in the study (five men, one woman; 46 to 71 years old, mean [±SD] 57 ± 8). Clinical findings included the presence of monoclonal protein in serum or urine, the involvement of other organs with amyloidosis, the absence of underlying diseases other than myeloma and clinical syndromes, such as nephrotic and carpal tunnel syndrome. The classification of restrictive heart disease was based on the presence of classical restrictive hemodynamic features at catheterization or by Doppler echocardiographic examination. The histologic diagnosis was confirmed at biopsy by the presence of amyloid deposits identified by a positive staining reaction with alkaline Congo red with dichronic birefringence that was not attenuated after treatment with $KMnO_4$ (27). No patient had coronary artery disease, myocardial infarction, valvular heart disease or any heart disease other than cardiac amyloidosis.

Control endomyocardial biopsy specimens were taken from eight patients with clinically suspected cardiac disease because of chest pain, syncope, slight electrocardiographic changes or arrhythmia. Noninvasive and invasive examinations, including biopsy, revealed no organic heart disease in these patients.

Two-dimensional echocardiographic examination was performed in all 14 patients. The left ventricular mass index was calculated by the method of Feneley et al. (28). All patients underwent biventricular cardiac catheterization, biplane left ventriculography and selective coronary angiography by standard techniques. During the procedure, endomyocardial biopsy samples were taken from both the right and left ventricles in five of the six patients with cardiac amyloidosis and all eight control patients and from only the right ventricle in one patient with cardiac amyloidosis. Two to five pieces of tissue were obtained from each site. The largest specimens were immediately immersed in 10% buffered formalin solution, and other pieces were fixed with 2.5% glutaraldehyde solution. Some specimens from two patients with cardiac amyloidosis and three control patients were fixed with 4% cold paraformaldehyde solution for 4 h at 4°C. After being soaked in graded sucrose, the specimens were embedded in cryomold by dry ice-acetone. Table 1 summarizes the clinical data of the patients and histologic findings in the endomyocardial biopsy specimens.

Right atrial appendages resected at operation. Right atrial appendages were obtained from four patients who underwent aortocoronary bypass surgery. These specimens were treated in the same way as the endomyocardial biopsy specimens.

Autopsy hearts. Four autopsy hearts from patients with cardiac amyloidosis (other than the previously described patients) were studied. All four patients had died of heart failure accompanied by prominent congestion of the lungs, liver and kidneys. The hearts showed extensive deposits of amyloid and fibrosis. Four hearts from age- and gender-matched subjects with neither amyloidosis nor other cardiac disease served as control hearts. The control patients died of noncardiac causes

Table 1. Data of Patients Who Underwent Biopsy or Operation

Specimen and Pt Group	Age (yr)/ Gender	HR (bpm)	CI (liters/min per m^2)	EDVI, ESVI (ml/m^2)	LVEF (%)	LVSP, LVEDP (mm Hg)	RVSP, RVEDP (mm Hg)	IVST, LVPWT (mm)	LVMI (ml/m^2)	Size of Myocyte: RVB, LVB (μm)	Serum Creatinine, BUN (mg/dl)	Plasma ANP, BNP (pg/ml)*	PRA (ng/ml per h), Aldosterone (pg/ml)*	Plasma ET-1 (pg/ml)*
Ventricle														
Amyloidosis														
Pt A1	52/M	80	3.90	73, 24	67	85, 15	40, 10	12, 13	180	20.0, 26.0	1.0, 16.0	—, —	—, —	—
Pt A2	46/M	88	3.30	53, 36	31	128, 26	24, 5	12, 14	182	17.9, 21.9	1.1, 12.0	—, —	—, —	—
Pt A3	59/M	80	2.06	60, 28	53	120, 20	40, 15	14, 13	191	17.2, 17.0	1.0, 18.0	47.0, 640.5	0.4, 10.0	5.39
Pt A4	58/M	105	3.94	76, 18	76	106, 10	34, 7	12, 12	172	14.4, 17.9	0.6, 10.0	—, —	—, —	—
Pt A5	53/M	76	3.42	98, 52	47	129, 24	43, 7	12, 11	182	22.4, 22.2	1.3, 11.0	211.5, 1,097.8	0.4, 26.0	4.72
Pt A6	71/F	130	2.47	46, 18	61	124, 17	34, 10	15, 14	224	—, 16.3	0.7, 13.0	911.6, 1,756.9	2.8, 160.0	1.47
Mean ± SD	57 ± 8 (5/1)	93 ± 21	3.18 ± 0.77	68 ± 19, 32 ± 18	56 ± 16	113 ± 15,† 19 ± 6‡	36 ± 7, 9 ± 4	13 ± 1,‡ 13 ± 2‡	188 ± 18‡	18.4 ± 3.0,‡ 20.2 ± 3.9†	1.0 ± 0.3, 13 ± 3	390.0 ± 459.1, 1,165.1 ± 561.2†	1.2 ± 1.4, 65 ± 82	3.86 ± 2.10†
Control (n = 8)														
Mean ± SD	57 ± 13 (6/2)	80 ± 20	3.03 ± 1.16	69 ± 10, 17 ± 5	75 ± 8	141 ± 14, 9 ± 3	32 ± 5, 6 ± 2	11 ± 1, 11 ± 1	113 ± 23	13.4 ± 1.3, 15.8 ± 1.2	1.1 ± 0.3, 17 ± 5	13.4 ± 9.0, 8.9 ± 6.0	1.2 ± 1.2, 61 ± 19	1.57 ± 0.74
RAA														
ACBG (n = 4)														
Mean ± SD	62 ± 15 (3/1)	73 ± 24	3.17 ± 1.12	85 ± 33, 37 ± 43	66 ± 31	147 ± 26, 9 ± 3	33 ± 8, 8 ± 4	10 ± 1, 10 ± 0	115 ± 13	—, —	1.0 ± 0.1, 15 ± 3	—, —	—, —	—

*Plasma levels of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), plasma renin activity (PRA), aldosterone and endothelin (ET)-1 were measured in three patients with cardiac amyloidosis and six control subjects. †$p < 0.05$, ‡$p < 0.01$, significant difference versus other groups. ACBG = aortocoronary bypass graft; bpm = beats/minute; BUN = blood urea nitrogen; CI = cardiac index; EDVI and ESVI = left ventricular end-diastolic and end-systolic volume index, respectively; F = female; HR = heart rate; IVST and LVPWT = interventricular septal and left ventricular posterior wall thickness by two-dimensional echocardiography, respectively; LVB = left ventricular biopsy; LVEF = left ventricular ejection fraction; LVMI = echocardiographic left ventricular mass index; LVSP and LVEDP = left ventricular systolic and end-diastolic pressure, respectively; M = male; Pt = patient; RAA = right atrial appendage; RVB = right ventricular biopsy; RVSP and RVEDP = right ventricular systolic and end-diastolic pressure, respectively.

Table 2. Autopsy Data

Group	Age (yr)/ Gender	Heart Wt (g)	IVS Thickness (mm)	LVFW Thickness (mm)	Staining Pattern					
					ANP			BNP		
					End	Mid	Epi	End	Mid	Epi
Amyloidosis (n = 4)										
Pt B1	64/F	490	20	20	+, d	+, d	+, f	+, f	—	—
Pt B2	73/F	460	15	15	+, d	—	—	+, f	—	—
Pt B3	78/M	410	14	12	+, d	+, d	+, f	+, d	+, d	+, f
Pt B4	72/M	605	20	18	+, d	+, d	+, f	+, d	+, d	+, f
Mean	72	491	17	16						
±SD	±6	±83*	±3†	±4						
	(2/2)									
Control (n = 4)					All	All	All	All	All	All
Mean	72	286	12	12	—	—	—	—	—	—
±SD	±5	±23	±1	±1						
	(2/2)									

*$p < 0.01$, †$p < 0.05$, significant difference versus control group. d = diffuse; End, Mid and Epi = subendocardial, middle and subepicardial one-third layer of transversely sectioned ventricular walls, respectively; f = focal; IVS = interventricular septum; LVFW = left ventricular free wall; Wt = weight; + = positive; other abbreviations as in Table 1.

(cancer in two patients, a traffic accident in one and cerebrovascular disease in one). No autopsy heart had any congenital anomalies, significant stenosis of the major coronary arteries or valvular disease. All subjects were autopsied within 12 h of death. The autopsy data are summarized in Table 2.

After fixation with 10% buffered formalin, the right atrial free wall and appendage were resected. The ventricles were cut into serial transverse slices from the base to the apex, and a slice from the midpoint of the heart was cut into six to eight blocks. The slices were embedded in paraffin and sectioned at a thickness of 4 μm.

Histologic evaluation. Light microscopic observations of the sections were made after hematoxylin-eosin staining and measurement of myocyte size (mean diameter 30 to 50 myocytes/specimen). The paraffin sections were also stained with alkaline Congo red or with Daylon and examined under polarized light.

Transmission electron microscopy. Biopsied specimens of the ventricles from three patients with amyloidosis and from three control subjects and three atrial tissues obtained at operation were fixed in 2.5% glutaraldehyde at 4°C, followed by postfixation with 1% osmium tetroxide. The specimens were then dehydrated and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and viewed under an electron microscope (model H-600, Hitachi, Tokyo, Japan).

Immunohistochemical analysis. Monoclonal antibodies against human BNP-32 and alpha-human ANP were prepared as previously described (12,29).

Serial 4-μm paraffin sections were alternately immunostained with the antibodies to ANP and BNP to compare their distribution. Immunostaining was done according to the indirect immunoperoxidase method reported previously, with some modifications (9,13,16–20). In the biopsy and surgical samples, the largest two specimens from each side of the heart were used, and sections from three different depths in the blocks were subjected to immunostaining. In the autopsy cases, whole ventricular transverse sections were used. Antigen retrieval was performed by soaking the deparaffinized sections in hot water (95°C) for 10 min. Intrinsic peroxidase activity was blocked by incubation with 0.3% hydrogen peroxide for 20 min, and nonspecific binding was blocked with normal goat serum. As the primary antibody, the monoclonal antibody was added at the dilution of 1:1,000 to the sections, followed by incubation for 48 h at 4°C. Next, peroxidase-conjugated $F(ab')_2$ fragment of the secondary antibody (goat anti-mouse IgG[H+L], Jackson Immunoresearch Laboratories) was added for 45 min at room temperature. The sections were subsequently stained with 45 mg of 3,3′-diaminobenzidine tetrahydrochloride (Sigma) and 0.05% hydrogen peroxide in 100 ml of 0.05 mol/liter Tris buffer for 3 min at room temperature. Between each step, the sections were washed four times (for 10 min each) with 0.01 mol/liter phosphate-buffered saline (PBS). Finally, counterstaining with hematoxylin was carried out.

The specificity of the immunohistochemical reaction was tested by preabsorption of the primary antibody with the corresponding peptide antigen (1 nmol peptide/ml of diluted antigen) and substitution of the primary antibody with nonimmune mouse serum. In addition, to exclude the possibility of cross-reaction between ANP and BNP, the primary antibody was preabsorbed with heterogeneous antigen (1 nmol/ml).

Immunocytochemical analysis at electron microscopic level. Small pieces of the biopsy specimens from two patients with cardiac amyloidosis and from three control subjects and two surgical specimens of right atrial appendages were fixed in 2.5% phosphate-buffered glutaraldehyde without postfixation by osmium tetroxide. The specimens were washed, dehydrated and embedded in Epon medium. Ultrathin sections of 80 nm were cut and mounted on bare 300-mesh nickel grids. For

double staining for ANP and BNP, sequential immunogold staining of the two faces of each ultrathin section was performed as previously described, with slight modifications (13,30). In the first staining sequence, only one side of the section was exposed to the solutions. The grids were floated, tissue sections face down, on drops of PBS containing 3% normal goat serum for 30 min before incubation with anti-BNP antibody (diluted 1:500) for 20 h at 4°C. After being washed in PBS, the sections were placed on drops of 10-nm gold-labeled goat anti-mouse IgG (diluted 1:20, Amersham, UK) for 1 h and then washed in PBS, rinsed in distilled water and dried. The grids were then turned over, and the other side of the sections was labeled according to the same procedure using anti-ANP antibody (diluted 1:250) for 20 h at 4°C and 5-nm gold-labeled anti-mouse IgG (diluted 1:20, Amersham) for 1 h at room temperature. After completion of the incubation procedures, the grids were rinsed in PBS and distilled water and counterstained with uranyl acetate and lead citrate before electron microscopic examination.

RNA probes and in situ hybridization histochemistry. The 581-base pair *Sac*I–*Pst*I fragment of human ANP cDNA (31) was subcloned into pBluescript SK^- (Stratagene). This vector was linearized with *Sac*I or *Pst*I. The *Sac*I overhanging ends of the linearized DNA templates were modified to blunt ends with a Blunt-end kit (Takara Shuzo, Otsu, Japan). Digoxigenin-labeled riboprobes were transcribed using a DIG RNA labeling kit (Boehringer Mannheim Biochemica, Mannheim, Germany). Antisense ANP and sense ANP riboprobes were transcribed with T7 or T3 RNA polymerase, respectively.

The cDNA of human BNP prepared by reverse transcriptase-polymerase chain reaction (32) was subcloned into the vector pCRII using a T-A cloning kit (Invitrogen). The sequence of BNP cDNA subcloned into the plasmid was determined by the dideoxy chain termination method. This vector was linearized with *Eco*R or *Hin*dIII. Antisense and sense BNP riboprobes were transcribed with SP6 and T7 RNA polymerase, respectively.

The specificity of the riboprobes was also confirmed by Northern blotting using a DIG nucleic acid detection kit (Boehringer).

Six-micrometer thick cryosections were mounted on glass slides coated with silane. The hydrated sections were rinsed in PBS and then placed subsequentially in 2 μg/ml of proteinase K in PBS for 20 min at 37°C and postfixed in 4% paraformaldehyde for 10 min. They were then placed in 0.2 N HCl for 10 min. The slides were rinsed in PBS between each of these procedures, which were carried out at room temperature, unless otherwise stated. They were hybridized overnight at 50°C in hybridization buffer (Hybrisol I containing 50% formamide [Oncor]) at a probe concentration of 200 ng/ml. The slides were then washed in 2 × standard saline citrate (SSC) with 50% formamide at 50°C for 15 min twice and incubated with RNase A (Boehringer) for 30 min at 37°C. Finally, the slides were washed in 2 × SSC and in 0.2 × SSC for 15 min each at 50°C. For immunodetection, the DIG nucleic acid detection kit (Boehringer) was used.

To ensure the specificity of the mRNA signals, in situ hybridization with sense probes and RNase digestion of the sections before hybridization were performed as the negative controls.

Plasma sampling. Of the subjects who underwent the endomyocardial biopsy, plasma samples were obtained from three patients with cardiac amyloidosis and six control subjects within 1 week before the biopsy procedure. Blood was withdrawn from the antecubital vein while the subject was in a recumbent position in the morning, after medications had been discontinued the night before. The samples were immediately transferred to chilled glass tubes containing Na_2EDTA (1 mg/ml) and aprotinin (1 million IU/ml) and centrifuged at 4°C. Plasma was frozen immediately and stored at −70°C until assay.

Measurement of plasma ANP and BNP. Measurement of plasma levels of ANP and BNP was performed by radioimmunoassay, as previously reported (8,12).

Measurement of plasma renin activity, aldosterone and endothelin-1. Plasma renin activity (PRA) and plasma aldosterone concentrations were measured with commercially available kits, renin radioimmunoassay beads (Dainabot, Tokyo, Japan) and an aldosterone radioimmunoassay kit II (Dainabot), respectively.

The plasma endothelin-1 (ET-1) concentration was measured with an enzyme-linked immunosorbent assay (ELISA) kit (Wako Pure Chemicals, Osaka, Japan) according to the manufacturer's instructions. This ELISA is a two-step sandwich method using a monoclonal antibody that recognizes the N-terminal of ET-1 and a peroxidase-conjugated polyclonal antibody that recognizes the C-terminal of ET-1. In this system, cross-reactivity with ET-3, or big ET-1, is $< 0.4\%$.

Statistical analysis. The clinicopathologic values are expressed as the mean value ± SD. Statistical analyses were performed using the Fisher exact test, Student *t* test or one-way analysis of variance, followed by the Newman-Keul multiple comparison test when appropriate. A p value < 0.05 was considered significant.

Results

Hemodynamic and histopathologic characterization of patients. Table 1 summarizes the hemodynamic and angiographic data of the patients who underwent biopsy or operation. Left ventricular end-diastolic pressure (LVEDP) was significantly elevated in patients with cardiac amyloidosis compared with control group values. Right ventricular pressure records showed a dip and plateau pattern in all patients with cardiac amyloidosis but not in the control subjects. However, left ventricular ejection fraction and volume indexes were not different between the two groups. Hemodynamic variables showed no significant differences between patients who underwent aortocoronary bypass surgery and control subjects.

In both the right and left ventricular biopsy specimens, the myocyte size was larger in the cardiac amyloidosis group (right: 18.4 ± 3.0 μm, left: 20.2 ± 3.8 μm) than in the control group

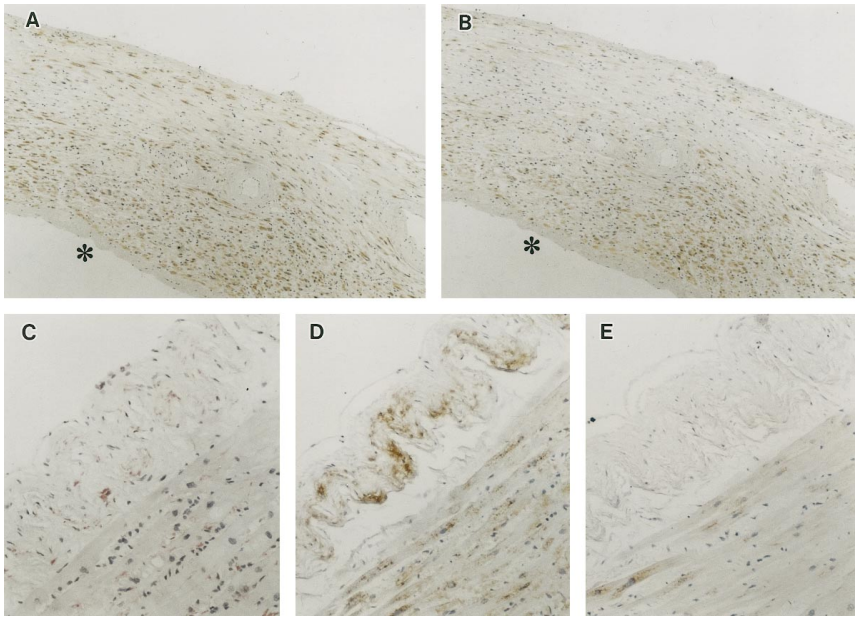


Figure 1. Light micrographs showing transmural gradient of immunohistochemical expression of ANP (**A**) and BNP (**B**) in a right atrial appendage. Note that ANP is distributed in atrial myocytes transmurally, but BNP is predominantly localized in the endocardial side (**asterisks**). The right atrial appendage with isolated atrial amyloidosis stained with Congo red (**C**) and immunohistochemically with anti-ANP (**D**) and anti-BNP antibody (**E**) is shown. Small amyloid deposits and ANP immunoreactions are seen in the subendocardial connective tissue. **A and B:** ×40; **C to E:** ×200, reduced by 35%.

(right: 13.4 ± 1.3 μm, left: 15.8 ± 1.2 μm). Specimens from the cardiac amyloidosis group showed amyloid deposition in the vascular structures and interstitium among myocytes or beneath the endocardium. A small amount of amyloid deposit was noted in two of the four right atrial tissue specimens obtained at operation (isolated atrial amyloid).

Immunohistochemical expressions of ANP and BNP. Control sections incubated with nonimmune mouse serum as the primary antibody, or with the primary antibody preabsorbed with homologous antigen, showed no specific immunoreaction. Moreover, staining with the antibody to human BNP-32 was not reduced by preabsorption with alpha-human ANP, or vice versa.

In the right atrial appendages, almost all atrial myocytes showed immunoreactivity for ANP, whereas BNP was predominantly localized in the myocytes of the subendocardial side and was rare on the epicardial side (Fig. 1). Although these peptides were generally not found in the endocardium, epicardium or connective tissue, their immunoreactions were noted on small deposits of atrial amyloid (Fig. 1).

None of the biopsied ventricles, neither the right nor left side, of the control group showed immunoreactivity for ANP or BNP. In contrast, four of five right and all six left ventricular biopsy specimens from the cardiac amyloidosis group showed immunoreactivity for both ANP and BNP (Fig. 2). In specimens from both sides, the incidence was significantly higher in the cardiac amyloidosis group than in the control group. The endocardium, connective tissue and vasculature were not

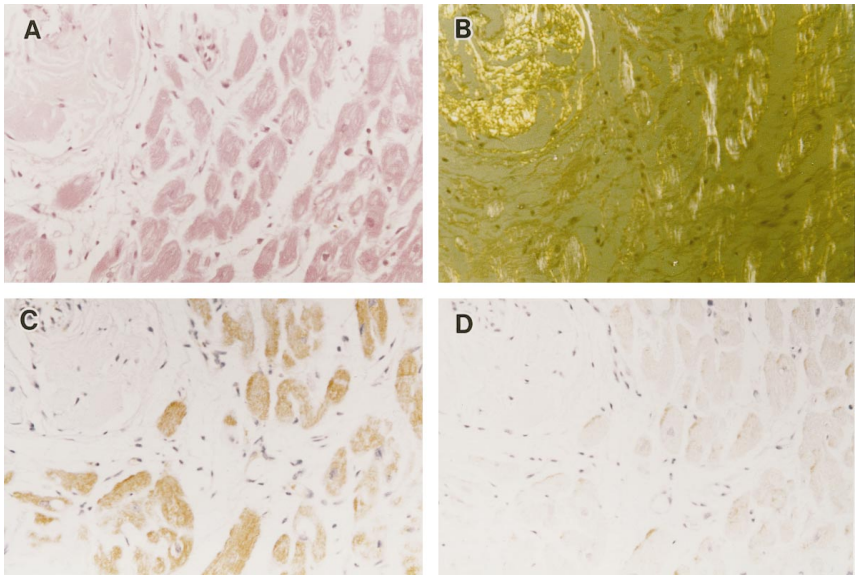


Figure 2. Light micrographs of left ventricular endomyocardial biopsy specimen from a patient with cardiac amyloidosis **A,** Hematoxylin-eosin stain. **B,** Congo red stain with dichronic birefringence. **C and D,** Immunohistochemical expression of ANP (**C**) and BNP (**D**). Vascular and interstitial amyloid deposits are seen. ANP and BNP immunoreactions are present in the myocytes but not in the amyloid deposits. ×200, reduced by 35%.

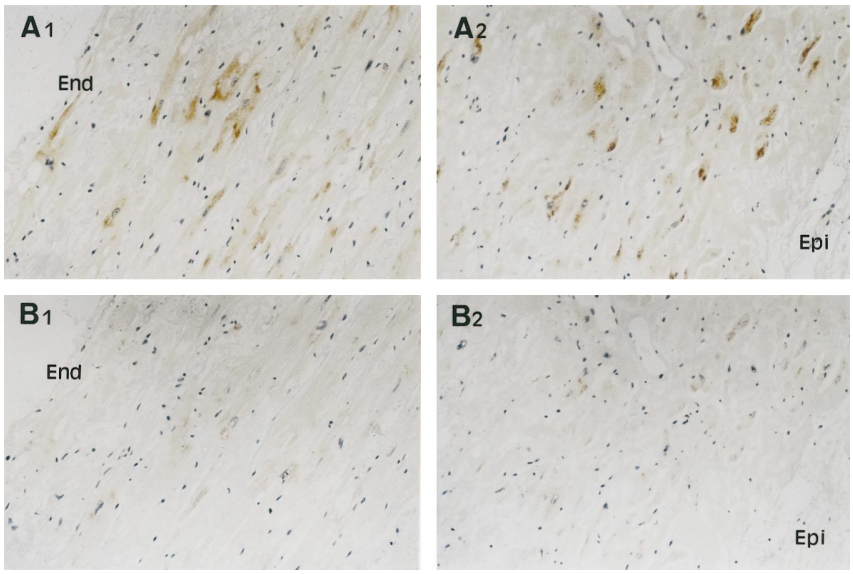


Figure 3. Light micrographs of left ventricle from an autopsied heart of a patient with cardiac amyloidosis showing immunohistochemical expression of ANP (**A1 and A2**) and BNP (**B1 and B2**) in the endocardial (End **[A1 and B1]**) and epicardial side (Epi **[A2 and B2]**). In this patient, ANP and BNP expression is transmural. ×100, reduced by 35%.

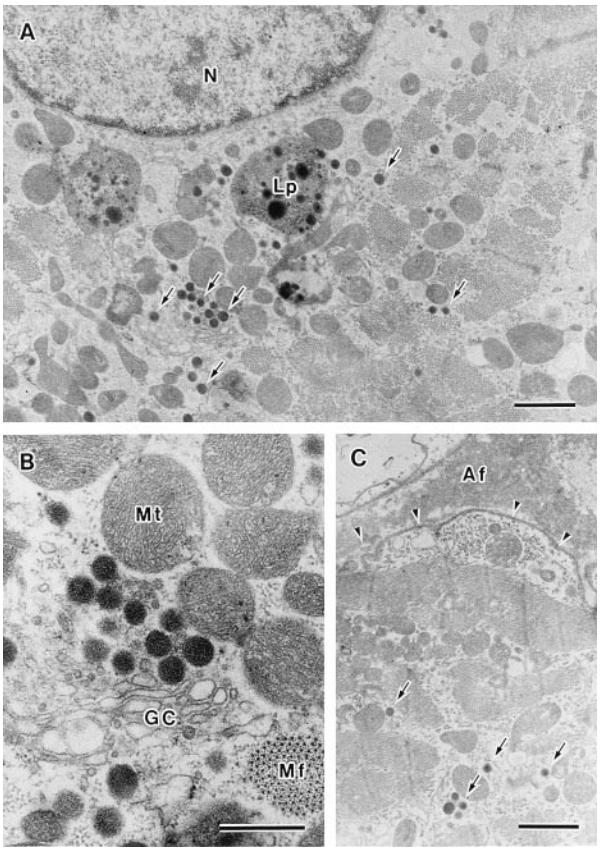
immunoreactive to ANP or BNP. Unlike the atrial amyloid, the ventricular AL amyloid in cardiac amyloidosis did not show ANP or BNP immunoreactivity (Fig. 2).

ANP or BNP immunoreactive products were not seen in the ventricles of any autopsy control heart. However, ANP and BNP immunoreactions were seen in the all ventricles with cardiac amyloidosis (Table 2). The immunoreactions were exclusively noted in myocytes but not in the endocardium, epicardium or interstitium, including amyloid fibers. The ANP-positive myocytes were distributed transmurally in three of the four cases, and BNP-positive myocytes were distributed transmurally in two of the four cases; not only in the subendocardium but also in the subepicardium (Fig. 3). Both the density of the positive myocytes and the intensity of staining were more predominant on the endocardial side. The myocytes surrounding amyloid deposits tended to show more intense staining. Our observations of the serial sections showed that the distribution of ANP- and BNP-positive myocytes was similar.

Electron microscopy. In the atrial myocytes, many secretory granules were gathered around the nuclei but were also found frequently in the peripheral cytoplasm (Fig. 4). A deposit of amyloid fibrils was found in one atrial specimen used for electron microscopy. Secretory granules were not noted in any ventricular myocytes of the biopsy specimens obtained from the normal control subjects. In contrast, myocytes containing secretory granules similar to the atrial myocytes were found in all biopsy specimens from patients with cardiac amyloidosis (Fig. 4). All cardiac amyloidosis specimens contained amyloid fibrils to some degree in the interstitium.

Immunocytochemistry at the electron microscopic level. The control studies using nonimmune mouse serum as the primary antibody and preabsorption tests for double immunocytochemical analysis at the electron microscopic level showed

Figure 4. Electron micrographs of left ventricular endomyocardial biopsy specimen from a patient with cardiac amyloidosis showing specific granules in myocytes. **A,** Electron-dense specific granules **(arrows)** are seen in the perinuclear region. N = nucleus; Lp = lipofuscin. ×7,000, reduced by 35%. **B,** Higher magnification of panel **A.** Golgi complex (GC) is seen near the specific granules. Mt = mitochondria; Mf = myofibrils. ×20,000, reduced by 35%. **C,** Perimyocyte amyloid fibrils (Af) and a myocyte containing specific granules **(arrows)**. The plasma membrane of the myocyte is indicated by **arrowheads.** ×6,000, reduced by 35%. **Bars** = 1 μm **(A and C)** and 0.5 μm **(B).**



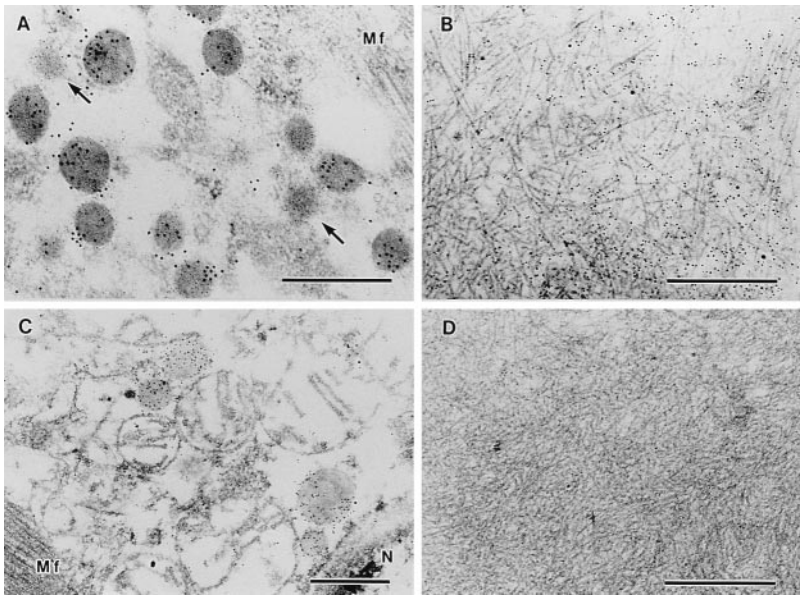


Figure 5. Double immunocytochemical studies for ANP and BNP in a right atrial tissue specimen obtained at operation **(A and B)** and ventricular tissue from a patient with cardiac amyloidosis **(C and D).** Smaller gold particles (5 nm) indicate ANP, and bigger particles (10 nm) indicate BNP. **A, Arrows** indicate granules containing ANP alone (type 1 granules); granules without arrows contain both ANP and BNP (type 2 granules). ×30,000, reduced by 35%. **B,** Amyloid fibrils of isolated atrial amyloidosis on which both smaller and larger gold particles are concentrated. ×7,000, reduced by 35%. **C,** Ventricular granules show immunoreactions for both ANP and BNP (type 2 granules). The labeling intensities of BNP are more prominent in ventricular granules than within atrial granules. ×30,000, reduced by 35%. **D,** Amyloid fibrils, in a ventricle from a patient with cardiac amyloidosis, are labeled with neither the smaller nor the larger gold particles. ×7,000, reduced by 35%. **Bars** = 0.5 μm.

no cross-reactions between the first and second staining sequences.

Gold particles were concentrated on the myocyte secretory granules. In the atrial myocytes, two types of secretory granules presenting different immunostaining patterns were noted as previously reported (13,30): monohormonal granules labeled only with small gold particles, indicating ANP (type 1 granules); and multihormonal granules containing both small and large gold particles, respectively, indicating ANP and BNP (type 2 granules) (Fig. 5). The amyloid fibrils in an atrial specimen were positive for both ANP and BNP (Fig. 5). Gold particles did not gather on any organelles other than myocyte granules and amyloid fibrils in the right atrial appendages.

In the ventricular myocytes of the normal control subjects, we found neither secretory granules nor accumulations of gold particles. However, in the ventricular myocytes of the cardiac amyloidosis specimens, the gold particles then were concentrated on secretory granules were type 2, showing an intense immunoreaction for both ANP and BNP. Unlike the isolated atrial amyloid, the ventricular amyloid fibrils of the cardiac amyloidosis were not positive for ANP or BNP immunoreaction (Fig. 5).

In situ hybridization. The signals of digoxigenin-labeled riboprobes hybridized with mRNA of ANP and BNP were seen as dark precipitates in the cytoplasm of atrial myocytes (Fig. 6). Hybridization signals were observed exclusively in the myocytes and were not seen in any connective tissue or vasculature. The signals for ANP were stronger than those for BNP in all atrial specimens. Neither the sense ANP or sense BNP riboprobe yielded any hybridization (Fig. 6).

No hybridization signal was noted in any ventricular biopsy specimen from the control subjects. However, the signals for both ANP mRNA and BNP mRNA were noted in the ventricular biopsy specimens from two patients with cardiac amyloidosis: in both the right and left ventricular specimens from one patient and in the left ventricular specimen from the other (Fig. 6). The signal intensities of ANP mRNA and BNP mRNA showed no marked difference, in contrast to those in

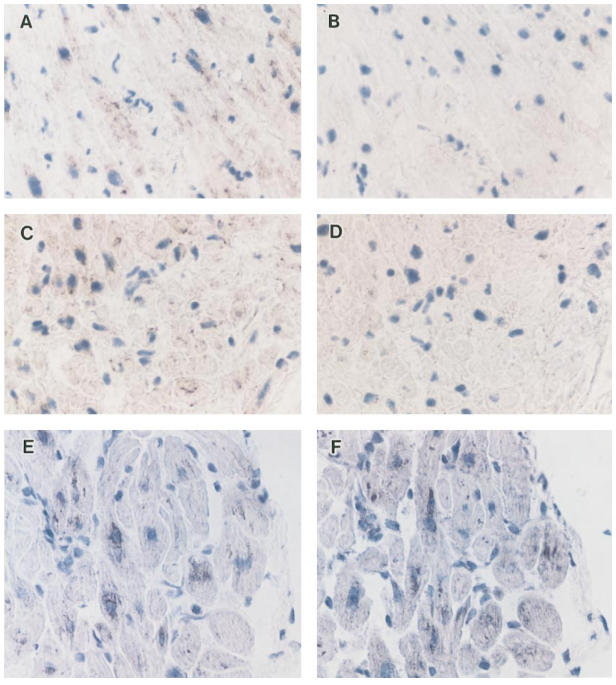


Figure 6. In situ hybridization immunohistochemical studies for ANP mRNA and BNP mRNA in a right atrial tissue specimen obtained at operation **(A to D)** and a left ventricular endomyocardial biopsy tissue specimen from a patient with cardiac amyloidosis **(E and F)**. ×200, reduced by 35%. **A and B,** Serial sections treated with, respectively, antisense and sense probes for ANP mRNA. **C and D,** Serial sections treated with antisense and sense probes for BNP mRNA, respectively. **E and F,** Serial sections treated with the antisense probe for ANP mRNA and BNP mRNA, respectively.

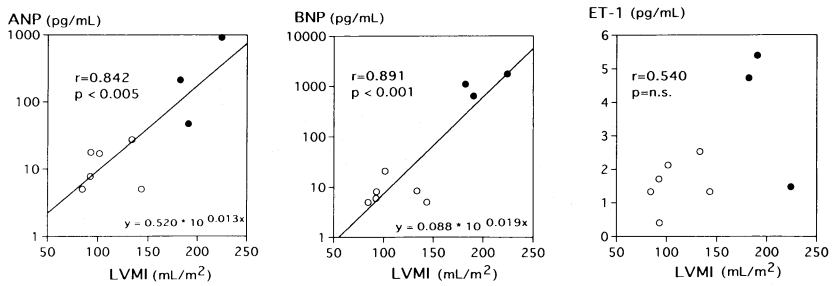


Figure 7. Plots of correlations between the plasma concentrations of ANP, BNP and ET-1 and LVMI. **Solid circles** = patients with cardiac amyloidosis; **open circles** = normal control subjects.

the atria. Neither sense ANP or sense BNP riboprobe yielded any hybridization.

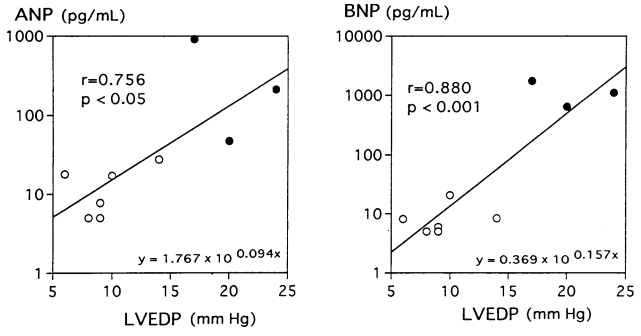
Plasma levels of ANP, BNP and other humoral factors. The plasma levels of both ANP and BNP were significantly higher in patients with cardiac amyloidosis than in control subjects, but the elevation of plasma BNP level was more marked (Table 1). In one patient with cardiac amyloidosis in particular, the plasma BNP level was markedly elevated (640.5 pg/ml) despite a slight elevation of the plasma ANP level (47.0 pg/ml). The mean ratio of BNP to ANP in the plasma was 6.9 ± 6.0 in the cardiac amyloidosis group and 0.8 ± 0.4 in the control group.

Patients with cardiac amyloidosis showed levels of PRA and aldosterone similar to those in the control subjects, whereas patients had a more than twofold higher level of ET-1 than did the control subjects (3.86 ± 2.10 vs. 1.57 ± 0.74 pg/ml); the difference was significant ($p < 0.05$) despite the small number of samples (Table 1).

To explore the relation between the humoral factors and cardiac hypertrophy, we examined the correlations between ANP, BNP, ET-1 and left ventricular mass index (LVMI). Both BNP and ANP levels showed a significant linear correlation with LVMI: $r = 0.886$, $p < 0.001$ for BNP; $r = 0.730$, $p < 0.05$ for ANP. When the plots were fit exponentially, the correlation coefficients were higher: $r = 0.891$, $p < 0.001$ for BNP; $r = 0.842$, $p < 0.005$ for ANP (Fig. 7). ET-1 was not significantly correlated with LVMI ($r = 0.540$, p = NS for linear fit; $r = 0.512$, p = NS for exponential fit) (Fig. 7).

We also checked the correlations between ANP, BNP and LVEDP to further explore the relation between the natriuretic peptides and diastolic dysfunction. Both BNP and ANP levels showed a significant correlation with LVEDP when the plots were exponentially fit, with the higher correlation coefficient between BNP and LVEDP: $r = 0.880$, $p < 0.001$ for BNP; $r = 0.756$, $p < 0.05$ for ANP (Fig. 8).

Figure 8. Plots of correlations between the plasma concentrations of ANP and BNP and LVEDP. Symbols as in Figure 7.



Discussion

Overexpression of natriuretic peptides in ventricles with cardiac amyloidosis. The present study revealed the diffuse expression of natriuretic peptides in the autopsied ventricles of patients with end-stage cardiac amyloidosis who died of heart failure. In these specimens, the expression was almost transmural but was more prominent in the endocardial than in the epicardial side. Ventricular wall stress is greater on the endocardial side than on the epicardial side (33,34). Thus, the result suggests that the "hemodynamic overload" hypothesis for the ventricular expression of these peptides (8,9,12,13) remains true in patients with cardiac amyloidosis. However, the present study also showed a high incidence of augmented ANP and BNP expression in the ventricular endomyocardial biopsy specimens of patients with cardiac amyloidosis who had relatively well preserved systolic function but poor diastolic function. These findings suggest that hemodynamic stress, particularly during the diastolic phase due to diastolic dysfunction, is important for the overexpression of ventricular natriuretic peptides in cardiac amyloidosis. In addition, the myocytes neighboring amyloid deposits tended to show more intense staining. Amyloid deposits may produce regional mechanical stress by restricting myocyte motion and interfering with their cooperative movement. Previous studies (9,13,16–18,20) pointed out a close association of ventricular expression of these peptides with a histologic abnormality, such as fibrosis in hearts with dilated and hypertrophic cardiomyopathy, myocardial infarction and myocarditis. Thus, regional stress related to amyloid deposits may be another contributor to overexpression of the peptides in cardiac amyloidosis. The transmural pattern of the natriuretic peptides in cardiac amyloidosis is considered to agree with diffuse and transmural distribution of amyloid deposits in the interstitium. Therefore, both hemodynamic stress accompanied by ventricular diastolic dysfunction and regional mechanical stress restricting myocardial movement by amyloid deposits may be important triggers for the expression of these peptides in patients with cardiac amyloidosis.

Subcellular distribution of natriuretic peptides in cardiac amyloidosis and isolated atrial amyloidosis. We identified ultrastructural specific secretory granules in the ventricular myocytes of patients with cardiac amyloidosis. This finding is compatible with previous reports on the occurrence of specific granules in the stressed ventricular myocytes of cardiomyopathic hamsters (35), spontaneously hypertensive rats (36) and patients with dilated cardiomyopathy (13). In the present study, electron microscopic immunocytochemical analysis revealed that similar to dilated cardiomyopathy (13), all granules detected in the ventricular myocytes of cardiac amyloidosis showed an immunoreaction for both ANP and BNP, in contrast to atrial myocytes, where two types of granules were found: one with ANP immunoreactivity only; the other with both ANP and BNP immunoreactivity. The different distribution of the granule types between the ventricular and atrial myocytes may reflect a more active production of BNP in the ventricles. It has been reported (3,12,32) that both natriuretic peptides are secreted from ventricles through a constitutive pathway. Immunoreactivity for these peptides may thus also exist in the cytoplasm or in organelles other than granules. However, they are not likely to be in a concentrated form outside the granules and therefore would not be identified by electron microscopic immunocytochemical analysis. The presence of granules immunoreactive for both natriuretic peptides in the ventricular myocytes of patients with cardiac amyloidosis suggests that these peptides are, at least in part, synthesized simultaneously and secreted together by the same granules.

In the isolated atrial amyloid, we detected immunogenicity of ANP and BNP at the light or electron microscopic level, or both. We observed these immunogenicities in the atria of nonfailing hearts, whereas Pucci et al. (24) observed them in the atria of failing hearts. This finding supports the hypothesis that ANP and BNP or their precursor peptides are constituents of amyloid fibrils of isolated atrial amyloidosis (23,24). In contrast, neither ANP nor BNP immunogenicity was detected in the ventricular amyloid fibrils of the cardiac amyloidosis specimens by light and electron microscopic immunohistocytochemical analysis in the present work. This is not a surprising result considering that amyloid fibrils of AL amyloidosis are induced from the light chains of monoclonal immunoglobulins (26), but our finding confirmed that ANP and BNP are not additional components of the amyloid fibrils in cardiac amyloidosis despite the fact that the ventricular myocytes of patients with cardiac amyloidosis contain both peptides. This phenomenon may reflect a more active and rapid turnover of these peptides in the ventricle than in the atrium.

Relation between plasma BNP levels and diastolic dysfunction. Plasma BNP levels in patients with cardiac amyloidosis were surprisingly high such that they showed significant difference from those values in the control subjects (n = 6) despite the small sample number (n = 3). Renal function was not impaired in our patients with cardiac amyloidosis (Table 1). Therefore, the increase in circulating ANP and BNP in these patients does not seem to be due to decreased clearance. Mean left ventricular ejection fraction (systolic function) was not greatly decreased in these three patients (54 ± 7%), but LVEDP (diastolic function) was greatly increased (20 ± 4 mm Hg), reminiscent of the high plasma BNP levels in patients with hypertrophic cardiomyopathy (17,37,38). According to our previous studies (17,38), plasma BNP levels in hypertrophic cardiomyopathy were higher in patients with than in those without a left ventricular outflow tract obstruction. Ventricular BNP expression also positively correlated with histologic changes and an LVEDP pressure elevation (17). Because BNP is secreted mainly from the ventricles (32), ventricular BNP may contribute much to plasma BNP levels in disease states. Cardiac amyloidosis resembles hypertrophic cardiomyopathy in presenting diastolic dysfunction. Thus, both in hypertrophic cardiomyopathy (the obstructive type in particular) and cardiac amyloidosis, the extreme increase in plasma BNP may be due to a ventricular overexpression of BNP that is triggered by the combined effects of hemodynamic stress, mainly at diastole, and regional mechanical stress induced by histologic abnormalities. We therefore postulate that the expression of BNP is highly sensitive to ventricular diastolic dysfunction and regional mechanical stress and that BNP expression is one of the specific compensatory mechanisms in such disease states. BNP has been reported (39) to be released in increased amounts throughout the ventricular myocardium but principally from the infarct zone in patients with an old myocardial infarction, presumably in response to increased regional wall stress. In addition, a recent study (40) showed beneficial hemodynamic and neurohormonal effects of a BNP infusion during exercise in patients with isolated diastolic heart failure. These findings support our speculation.

The question arises as to whether the activation of ANP and BNP as observed is unique to cardiac amyloidosis among restrictive cardiomyopathies. We observed the overexpressions of ANP and BNP in the endomyocardial biopsy specimens from a patient with idiopathic restrictive cardiomyopathy and from a patient with endocardial fibroelastosis (unpublished data). Moreover, the present study revealed good correlations between the plasma levels of the peptides and LVEDP (Fig. 8). Taking these findings together with the report of Edwards et al. (22), the overexpression of the ventricular natriuretic peptides as observed may be extended to restrictive cardiomyopathies with an etiology other than cardiac amyloidosis.

Relation between natriuretic peptides and other variables. The present patients with cardiac amyloidosis showed levels of PRA and aldosterone similar to those in the control subjects. However, ET-1 levels were more than twofold higher in the patients than in the control subjects. Physiologic effects of ET-1 (e.g., an increase in systemic vascular resistance or a decrease in cardiac output [41]), were not observed in our patients with cardiac amyloidosis; rather, this group was relatively hypotensive (Table 1). It is therefore conceivable that ANP and BNP may counteract the hemodynamic effects of ET-1 in the patients with cardiac amyloidosis.

In the present study, both BNP and ANP showed a significantly positive correlation with LVMI. BNP was more closely correlated with LVMI than was ANP. ET-1 also had a

tendency toward a positive correlation with LVMI that was not significant. Thus, BNP may be the best marker among these humoral factors for cardiac hypertrophy. However, it is not clear at present whether these differences in the findings among ANP, BNP and ET-1 are significant or are attributable to the low number of data analyzed. More extensive studies are necessary for elucidation.

Conclusions. The major findings of the present study are as follows: 1) The expression of ANP and BNP was augmented at both the peptide and mRNA levels in ventricular myocytes of cardiac amyloidosis. These peptides tended to be distributed in the myocytes predominantly on the endocardial side and near amyloid deposits. 2) The ANP and BNP immunogenicities were confined to specific granules of ventricular myocytes but were not noted in the amyloid fibrils of the cardiac amyloidosis specimens, unlike those of isolated atrial amyloidosis, where their antigenicities were confirmed. 3) The plasma concentrations of both ANP and BNP were elevated in the patients with cardiac amyloidosis, and the elevation of BNP was more extreme.

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