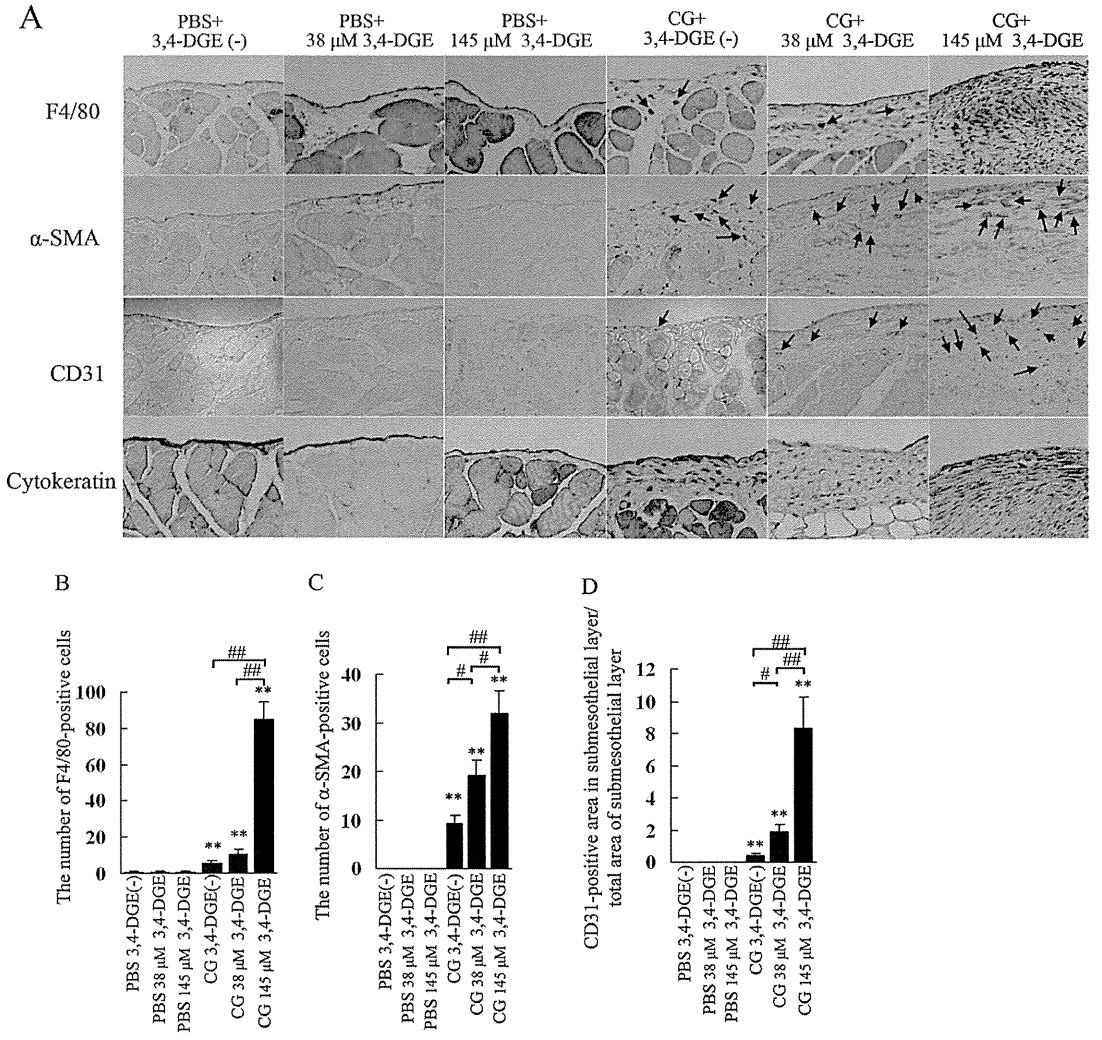


Figure 4 — (A) Immunohistochemical study for F4/80, alpha smooth muscle actin (α-SMA), CD31, and cytokeratin in the peritoneum. Mice receiving chlorhexidine gluconate (CG) plus 38 μmol/L or 145 μmol/L 3,4-dideoxyglucosone-3-ene (3,4-DGE) showed increased F4/80-positive cells (arrows) and α-SMA positive cells (arrows). Vessels positive for CD31 were increased in mice treated with CG plus 38 μmol/L or 145 μmol/L 3,4-DGE. Cytokeratin staining showed that mesothelial cells were detached in mice treated with CG plus 38 μmol/L or 145 μmol/L 3,4-DGE. (B) Number of F4/80-positive cells in the submesothelial area. (C) Number of α-SMA–positive cells in the submesothelial area. (D) Ratio of the area in the submesothelial layer positive for CD31 to the total area of the submesothelial layer. Number of mice: phosphate buffered saline (PBS) without 3,4-DGE [PBS+3,4-DGE(−), $n = 7$]; PBS+38 μmol/L 3,4-DGE ($n = 5$); PBS+145 μmol/L 3,4-DGE ($n = 5$); CG+3,4-DGE(−) ($n = 5$); CG+38 μmol/L 3,4-DGE ($n = 6$); CG+145 μmol/L 3,4-DGE ($n = 5$). All values: mean ± standard error of the mean. * $p < 0.05$; ** $p < 0.01$ versus mice treated with PBS and the same dose of 3,4-DGE; # $p < 0.05$; ## $p < 0.01$.

mice, because apoptosis was not detected in the 38 μmol/L 3,4-DGE group, a result that is not consistent with a previous *in vitro* study that showed induction of mesothelial cell apoptosis even in the presence of 38 μmol/L 3,4-DGE.

We speculated that the 3,4-DGE injected into the peritoneal cavity was rapidly eliminated, especially in injured perineum. The residual concentration of 3,4-DGE in PBS-treated mice was 53% [Figure 6(A)]. In contrast, CG-treated mice showed a much reduced residual level of 3,4-DGE [10%, Figure 6(A)]. Interestingly, in mice given 145 μmol/L 3,4-DGE, the plasma concentration of 3,4-DGE was much higher in the CG-treated group than in the PBS-treated group [Figure 6(B)]. These results indicate that injured peritoneum causes 3,4-DGE to disappear from the peritoneal

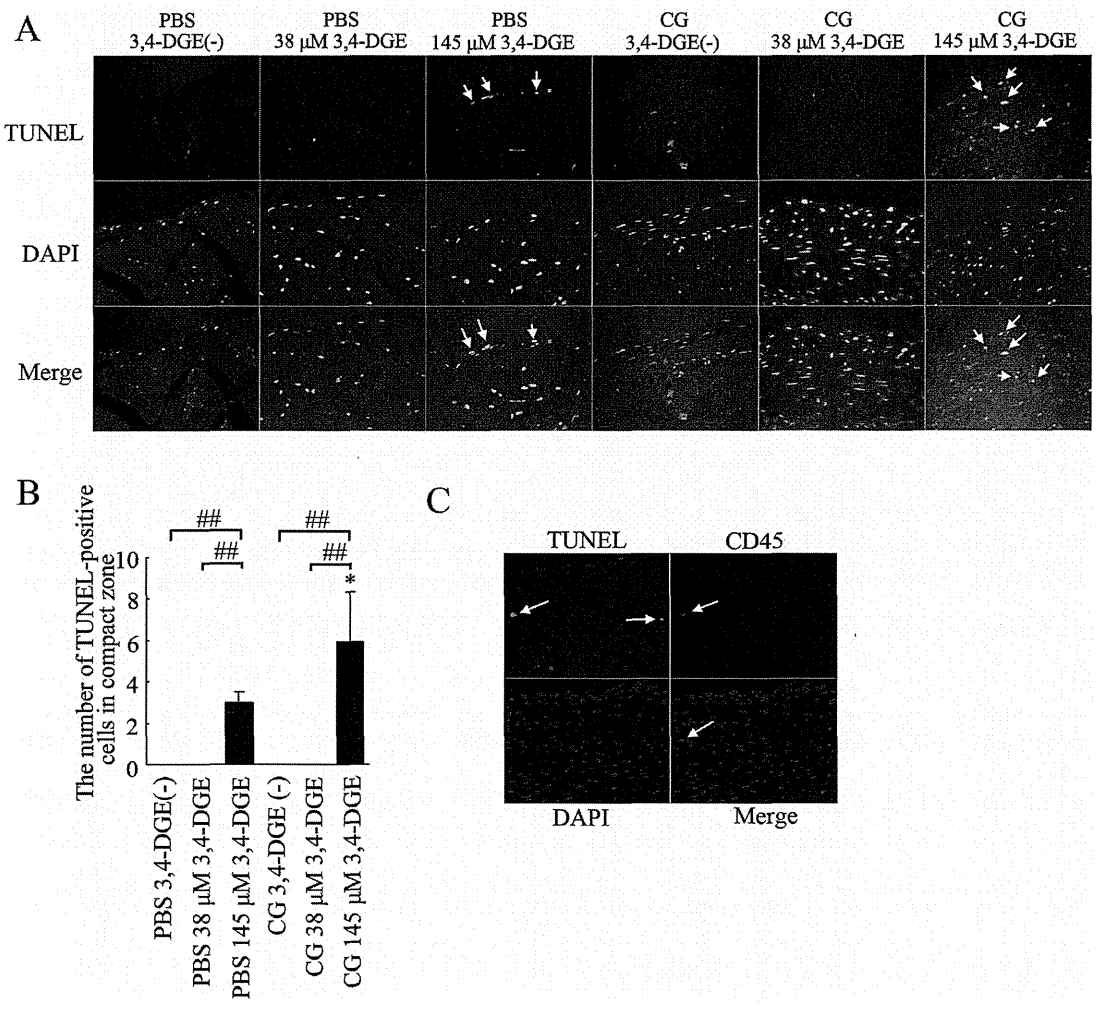
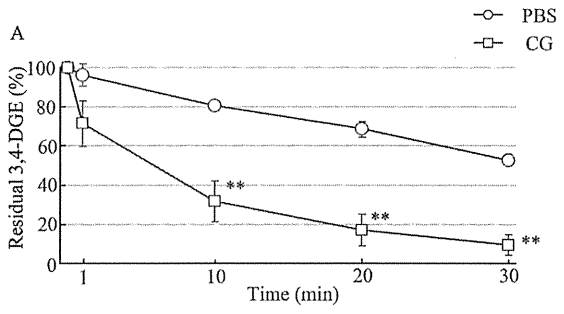


Figure 5 — Apoptotic cells in the peritoneum. (A) TUNEL staining (green), DAPI staining (blue), and merged images. Mice treated with phosphate-buffered saline (PBS) plus 145 μmol/L 3,4-dideoxyglucosone-3-ene (3,4-DGE) showed TUNEL-positive cells (arrows). Mice treated with chlorhexidine gluconate (CG) plus 145 μmol/L 3,4-DGE showed more TUNEL-positive cells than did mice treated with PBS+145 μmol/L 3,4-DGE (arrows). DAPI was used as nuclear staining. Most TUNEL-positive cells were also positive for DAPI. Number of mice: PBS without 3,4-DGE [PBS+3,4-DGE(−), $n = 7$]; PBS+38 μmol/L 3,4-DGE ($n = 5$); PBS+145 μmol/L 3,4-DGE ($n = 5$); CG+3,4-DGE(−) ($n = 5$); CG+38 μmol/L 3,4-DGE ($n = 6$); CG+145 μmol/L 3,4-DGE ($n = 5$). (B) Ratio of TUNEL-positive cells to DAPI-positive cells in the submesothelial area in mice. All values: mean ± standard error of the mean. * $p < 0.05$ versus mice treated with PBS and the same dose of 3,4-DGE; ## $p < 0.01$. (C) Triple staining for TUNEL (green), CD45 (red), and DAPI (blue) in mice ($n = 5$) treated with CG+145 μmol/L 3,4-DGE. Some of TUNEL-positive cells are leukocytes (arrows).

cavity rapidly and that some 3,4-DGE is absorbed into the systemic circulation.

MODIFIED PERITONEAL EQUILIBRATION TEST

Peritoneal equilibration tests were performed to examine the functional role of 3,4-DGE in peritoneal fibrosis. Figure 7(A) shows that the dialysate-to-plasma ratio of creatinine (D/P Cr) in PBS+38 μmol/L 3,4-DGE mice was not different from that in PBS+3,4-DGE(−) mice. In contrast, the D/P Cr was significantly higher in CG+38 μmol/L 3,4-DGE mice than in PBS+38 μmol/L 3,4-DGE or CG+3,4-DGE(−) mice (0.68 vs 0.55 and 0.54 respectively). By analyzing the ratio of net ultrafiltration to body weight, CG-treated mice not receiving 3,4-DGE showed reduced ultrafiltration and CG+38 μmol/L 3,4-DGE mice showed net ultrafiltration failure. The MTAC urea also indicated that, compared with PBS+38 μmol/L 3,4-DGE mice, CG+3,4-DGE mice showed increased urea transport. These results suggest that



B

	PBS	CG
plasma 3,4-DGE (μM)	0.572 ± 0.093	1.073 ± 0.019**

Figure 6 — Elimination rate of 3,4-dideoxyglucosone-3-ene (3,4-DGE) from the peritoneal cavity and appearance rate of 3,4-DGE in plasma. Mice were treated with phosphate buffered saline (PBS) or chlorhexidine gluconate (CG) 3 times in 1 week, and then 4 mL 3,4-DGE–containing PBS was injected into the peritoneal cavity. Peritoneal fluid was collected at 1, 10, 20, and 30 minutes after injection. (A) Residual concentration of 3,4-DGE in peritoneal fluid. Mice treated with CG showed rapid elimination of 3,4-DGE from the peritoneal cavity (PBS-treated: $n = 8$; CG-treated: $n = 7$). (B) Plasma level of 3,4-DGE in PBS- or CG-treated mice at 30 minutes after injection of 145 μmol/L 3,4-DGE. Compared with PBS-treated mice, those treated with CG showed high plasma levels of 3,4-DGE (PBS-treated: $n = 2$; CG-treated: $n = 3$). ** $p < 0.01$ versus PBS-treated mice.

3,4-DGE is associated with high peritoneal transport in peritoneal fibrosis.

DISCUSSION

Although previous reports showed that GDP-containing conventional PD solutions induce peritoneal fibrosis with enhanced peritoneal permeability even after 5 weeks *in vivo* (21), it is not clear whether 3,4-DGE is the sole cause of peritoneal changes. In the present study, we infused 3,4-DGE dissolved in PBS into the peritoneal cavities of mice to examine the effects of that compound on peritoneal fibrosis. Although there is some controversy about the use of CG to induce an encapsulating peritoneal sclerosis model (22), we used this existing model of CG-induced peritoneal injury to investigate the role of 3,4-DGE. Vlijm *et al.* reported that the condition of chronic renal failure can be a "first hit" in peritoneal damage and that perhaps other stimuli can substitute for CG in damaging mesothelial cells (22). Infusion of 3,4-DGE alone did not induce peritoneal changes in conditions of uninjured peritoneum. The peritoneal damage caused by 3,4-DGE alone may be different from that caused by PD solution because of the difference of glucose concentration. The combination of CG and 3,4-DGE caused thickening of the submesothelial zone, suggesting that 3,4-DGE is an aggravating

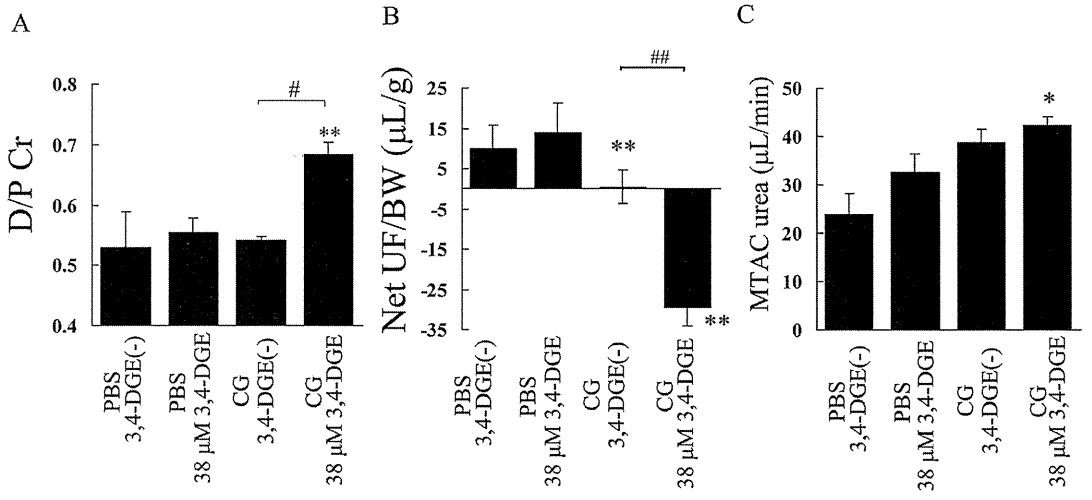


Figure 7 — Modified peritoneal equilibration test. (A) The creatinine (Cr) concentration in 7% glucose dialysate effluent (D) divided by the Cr concentration in plasma (P) in mice at 2 hours. (B) Net ultrafiltration (UF) / body weight (BW). (C) Mass transfer-area coefficient (MTAC). Compared with mice treated with phosphate buffered saline (PBS) plus 38 μmol/L 3,4-dideoxyglucosone-3-ene (3,4-DGE), mice treated with chlorhexidine gluconate (CG) plus 38 μmol/L 3,4-DGE showed high peritoneal transport. Net UF / BW and MTAC urea indicated that CG treatment induced ultrafiltration failure and high urea transport. Number of mice: PBS without 3,4-DGE [PBS+3,4-DGE(–), $n = 3$]; PBS+38 μmol/L 3,4-DGE ($n = 3$); CG+3,4-DGE(–) ($n = 3$); CG+38 μmol/L 3,4-DGE ($n = 5$). All values: mean ± standard error of the mean. * $p < 0.05$; ** $p < 0.01$ versus PBS-treated mice receiving the same dose of 3,4-DGE; # $p < 0.05$; ## $p < 0.01$.

factor in peritoneal fibrosis when peritoneal damage is already present.

The infusion of GDP-containing conventional dialysis solution with lipopolysaccharide induces a high peritoneal transport rate, with mild thickening of the peritoneal membrane (12). In the present study, we used 38 μmol/L or 145 μmol/L 3,4-DGE–containing PBS. The 3,4-DGE alone did not elicit peritoneal fibrosis, probably because half the 3,4-DGE is eliminated from the peritoneal cavity at 30 minutes. In peritoneal mesothelial cells, VEGF-A, which enhances vascular permeability and angiogenesis, has been shown to be increased by MGO and 3,4-DGE (23,24). Blockade of VEGF by anti-VEGF monoclonal antibody prevents hyperglycemia-induced structural and functional peritoneal microvascular alterations in rats, indicating that VEGF plays a role in permeability to small molecules and angiogenesis (25). The present study shows that only CG+145 μmol/L 3,4-DGE mice showed increased levels of VEGF, leading to high peritoneal permeability as indicated by peritoneal equilibration tests. Antiangiogenic reagents TNP-470 and endostatin have been shown to ameliorate peritoneal fibrosis and permeability (26,27). In the present study, increased numbers of CD31-positive vessels were most often noted in CG+145 μmol/L 3,4-DGE mice, which is consistent with increased peritoneal permeability.

Expression of genes associated with extracellular matrix (TGF-β1, CTGF, fibronectin, and COL1A1) was significantly increased in the peritoneum of CG+145 μmol/L 3,4-DGE mice. In cultured human peritoneal mesothelial cells, stimulation with 3,4-DGE has been shown to increase TGF-β secretion (24). Our study indicates that administration of 3,4-DGE without CG did not upregulate TGF-β1 mRNA expression. Taken together, these findings suggest that injured peritoneum expresses increased TGF-β mRNA because of stimulation by 3,4-DGE.

Although the precise mechanism of upregulated TGF-β expression in CG+145 μmol/L 3,4-DGE mice is not clear, we speculate that oxidative stress is one factor affecting TGF-β expression in our model. Reduction of oxidative stress with L-2-oxothiazolidine-4-carboxylic acid (28), a glutathione precursor, or *N*-tert-butyl-α-phenylnitrone (29) lowered the TGF-β level in cultured mesothelial cells, indicating that, in peritoneal mesothelial cells, oxidative stress can increase TGF-β, which is one of the well-known inducers of EMT in mesothelial cells (30,31). Epithelial–mesenchymal transition is characterized by downregulation of E-cadherin and cytoskeletal rearrangement with expression of α-SMA. Our results show that the number of α-SMA–positive cells was increased in the submesothelial zone. Although CG treatment alone or CG plus 38 μmol/L 3,4-DGE increased α-SMA–positive cells, mRNA expression of α-SMA in those groups of mice was not increased. Further investigation is needed into this discrepancy between mRNA and protein levels.

Oxidative stress is a key factor in the development of peritoneal fibrosis. Reactive oxygen species produced after stimulation with high glucose upregulate fibronectin expression through the protein kinase C pathway in human peritoneal mesothelial cells (32). Reactive oxygen species have been shown to play a role in changes of peritoneal membrane structure and function *in vivo*, and antioxidant prevents those changes (33). We showed upregulation of NOX4 and p47phox mRNA expression in CG+145 μmol/L 3,4-DGE mice compared with CG+3,4-DGE(–) mice. Macrophage infiltration was documented in the peritoneum of 38 μmol/L or 145 μmol/L CG+3,4-DGE mice. The infiltrating macrophages aggravate peritoneal fibrosis by secreting profibrogenic cytokines (34). Peritoneal macrophage infiltration is an independent predictor of baseline peritoneal permeability in PD patients (35). At 38 μmol/L, 3,4-DGE can influence macrophage infiltration and vessel formation.

Conventional PD solutions and GDPs induce apoptosis in mesothelial cells. High concentrations of 3-deoxyglucosone or MGO induce mesothelial cell apoptosis (36,37). Administration of 3,4-DGE within the concentration range seen in conventional PD solutions induces apoptosis in cultured mesothelial cells through caspase- and Bax-dependent pathways (10,38), suggesting that 3,4-DGE mediates, at least in part, the cytotoxicity of conventional PD solutions. The precise mechanism and role of apoptosis in the peritoneum *in vivo* needs further investigation. We showed that 145 μmol/L 3,4-DGE in combination with CG induced cell apoptosis in the thickened peritoneal compact zone *in vivo*. As previously reported (39), some of the apoptotic cells were leukocytes. The inability of 38 μmol/L 3,4-DGE to induce apoptosis might be a result of rapid elimination of 3,4-DGE from peritoneal cavity. Peritoneum injured by CG can accelerate that process, elevating plasma levels of 3,4-DGE.

CONCLUSIONS

Our study shows that, when mild peritoneal damage is already present, 3,4-DGE enhances peritoneal injury by augmenting macrophage infiltration and extracellular matrix deposition. These findings help to elucidate the effect of 3,4-DGE in peritoneal fibrosis *in vivo*.

ACKNOWLEDGMENTS

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DISCLOSURES

R. Yamada, S. Namoto, T. Yamamoto, N. Seki, N. Souma, and T. Yamaguchi are employees of JMS Co. Ltd.

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Direct Immunochemiluminescent Assay for proBNP and Total BNP in Human Plasma proBNP and Total BNP Levels in Normal and Heart Failure

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Abstract

Background: Recent studies have shown that in addition to brain (or B-type) natriuretic peptide (BNP) and the N-terminal proBNP fragment, levels of intact proBNP are also increased in heart failure. Moreover, present BNP immunoassays also measure proBNP, as the anti-BNP antibody cross-reacts with proBNP. It is important to know the exact levels of proBNP in heart failure, because elevation of the low-activity proBNP may be associated with the development of heart failure.

Methodology/Principal Findings: We therefore established a two-step immunochemiluminescent assay for total BNP (BNP+proBNP) and proBNP using monoclonal antibodies and glycosylated proBNP as a standard. The assay enables measurement of plasma total BNP and proBNP within only 7 h, without prior extraction of the plasma. The detection limit was 0.4 pmol/L for a 50-μl plasma sample. Within-run CVs ranged from 5.2%–8.0% in proBNP assay and from 7.0%–8.4% in total BNP assay, and between-run CVs ranged from 5.3–7.4% in proBNP assay and from 2.9%–9.5% in total BNP assay, respectively. The dilution curves for plasma samples showed good linearity (correlation coefficients = 0.998–1.00), and analytical recovery was 90–101%. The mean total BNP and proBNP in plasma from 116 healthy subjects were 1.4±1.2 pM and 1.0±0.7 pM, respectively, and were 80±129 pM and 42±70 pM in 32 heart failure patients. Plasma proBNP levels significantly correlate with age in normal subjects.

Conclusions/Significance: Our immunochemiluminescent assay is sufficiently rapid and precise for routine determination of total BNP and proBNP in human plasma.

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Competing Interests: Hiroyuki Okamoto, Masahiro Nakamura, Naoko Ogawa, Kazukiyo Horii and Kiyoshi Nagata are employed by Shionogi& Co., Ltd. Shionogi Company previously developed the BNP kit and they may develop a new assay kit like a proBNP in the future. There are no further patents, products in development or marketed products to declare. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

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Introduction

Brain (also known as B-type) natriuretic peptide (BNP) has been used as a biomarker of heart failure for more than a decade [1]. Indeed, guidelines for the treatment of heart failure recommend measurement BNP before making a diagnosis [2,3]. During the process by which BNP is secreted from cardiac myocytes, its 108-amino acid precursor, proBNP, is cleaved to form the 32-amino acid peptide BNP and the 76-amino acid peptide N-terminal proBNP fragment (NT-proBNP) [4]. Recent studies have shown that in addition to BNP and the NT-proBNP, levels of uncleaved proBNP are also considerably increased in plasma of patients with heart failure [5,6,7]. This is noteworthy in part because the immunoassay system currently being used to measure BNP levels also detects proBNP, as the anti-BNP antibody cross-reacts with proBNP. Consequently, the present assay system actually measures not the active BNP level, but the total BNP (BNP+proBNP) level [8].

It is important to know the proBNP level and/or proBNP/total BNP ratio in heart failure, because proBNP has much less ability to induce cGMP production (about 13–17%) than BNP, and higher levels of the low-activity proBNP may be associated with the development of heart failure [7]. Consistent with that idea, we recently used the combination of gel-filtration and a fluorescent immunoenzyme assay with BNP extracted from plasma to show

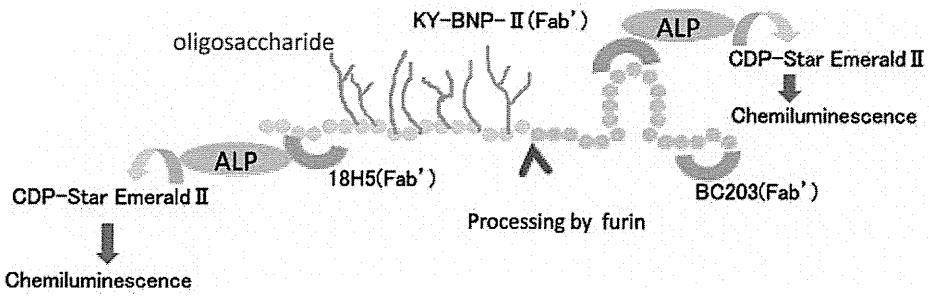

Figure 1. Schematic diagram of the total BNP and proBNP assay systems. BC203(Fab') is a common capture antibody in both systems. KY-BNP-II(Fab') is the detection antibody for the total BNP assay, and 18H5(Fab') is the detection antibody for the proBNP assay. ALP: Alkaline phosphatase; CDP-Star EmeraldII (Chemiluminescent Substrate): Disodium 2-chloro-5-(4-methoxy-spiro{1,2-dioxetane-3,2′-(5′-chloro)-tricyclo [3,3,1,13,7]decan}-4-yl)-1-phenyl phosphate.
doi:10.1371/journal.pone.0053233.g001

that although proBNP/total BNP ratios vary widely in heart failure, they are higher in cases of ventricular overload than in atrial overload [6]. Unfortunately, the method used in that study requires a great deal of time and effort, and extraction of the peptide from plasma may cause underestimation of the proBNP levels due to its high adsorptive property [9].

To overcome those shortcomings, we developed a sensitive method to more quickly and easily measure levels of proBNP and total BNP. Our idea was to make a sandwich immunoassay using a common capture antibody recognizing the C-terminal region of both BNP and proBNP and detection antibodies that recognize different epitopes: the N-terminal region of proBNP and the ring structure of BNP (Figure 1). Using this approach, we were able to develop a sensitive immunochemiluminescent assay for proBNP and total BNP in plasma. Here, we report on the assay's performance and its use to compare plasma levels of total BNP and proBNP in healthy subjects and patients with heart failure. In addition, we measured NT-proBNP and compared it with total BNP and proBNP.

Materials and Methods

All patients provided written informed consent for all blood sample analyses, and the protocol was approved by the Ethical Committee of Kyoto University Graduate School of Medicine. Sample analyses were also conducted in accordance with the policies and procedures of the Institutional Review Board for the use of human subjects in research at the Diagnostics Division of Shionogi & Co., Ltd.

Peptides and Reagents

Glycosylated proBNP and recombinant proBNP were purchased from Hytest Ltd. (Turk, Finland). The protein content was determined by amino acid analysis. BNP was from Peptide Institute, Inc. (Osaka, Japan). EZ-Link-sulfo-NHS-biotinylation kits were from Pierce (Rockford, IL). Sulfo-HMCS (N-(8-maleimidocapryloxy) sulfosuccinimide) was from Dojindo (Kumamoto, Japan). CDP/E (Disodium 2-chloro-5-(4-methoxy-spiro{1,2-dioxetane-3,2′-(5′-chloro)-tricyclo [3,3,1,13,7]decan}-4-yl)-1-phenyl phosphate) was from Applied Biosystems (Foster City, CA).

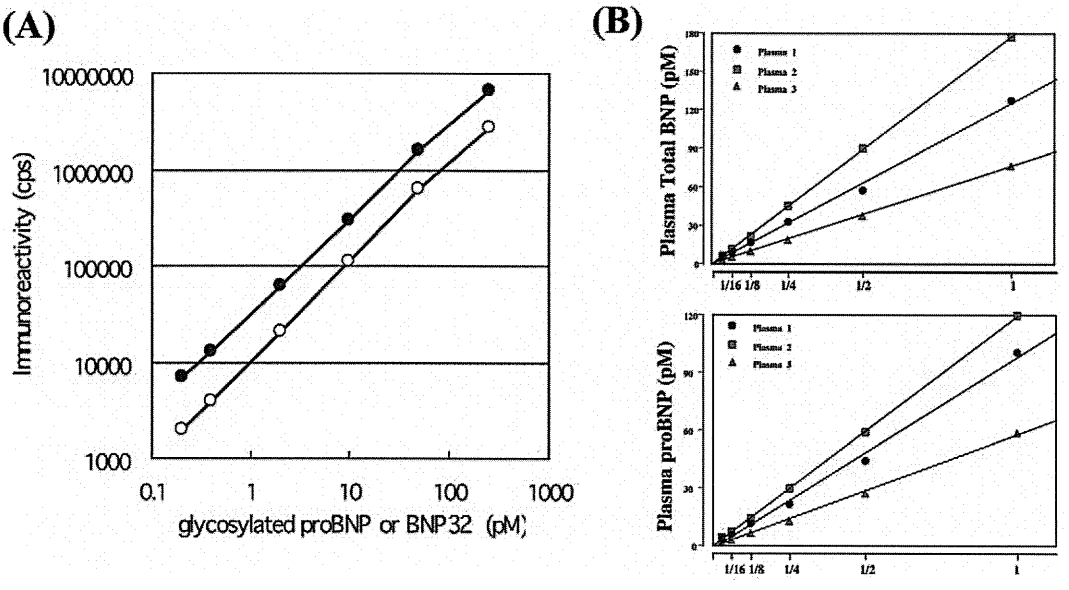

Figure 2. Standard curves for the proBNP (open circle) and total BNP (closed circle) assays (A). Plasma dilution curves (B). Three plasma samples collected from three heart failure patients were serially diluted with buffer.
doi:10.1371/journal.pone.0053233.g002

Table 1. Recovery of standard glycosylated proBNP and BNP added to human plasma.

Added peptide concentration, pmol/L	Recovery, %	Recovery, %
	proBNP assay system	total BNP assay system
2.0	90	85
25.0	101	97
100.0	95	95

doi:10.1371/journal.pone.0053233.t001

Antibodies

The monoclonal antibodies BC203 (IgG1, k) and KY-BNP-II (IgG1, k) were developed by Shionogi & Co., Ltd [10]. BC203 and KY-BNP-II recognize the C-terminal region and the ring region of BNP, respectively. The monoclonal antibody 18H5 was purchased from Hytest Ltd. 18H5 recognizes a region (a.a. 13–20) of proBNP. In the proBNP assay, the combination of BC203 (capture) and 18H5 (detection) was used because 18H5 is not affected by glycosylation [11]. In the total BNP assay, the combination of BC203 (capture) and KY-BNP-II (detection) was used because KY-BNP-II recognizes nearly all bioactive BNPs (Figure 1).

Preparation of BC203 coated immunoassay plates

BC203, which was the capture antibody in both assays, was biotinylated using an EZ-Link-sulfo-NHS-biotinylation kit according to the manufacturer's instructions. The biotinylated BC203 (0.2 mg/well in 100 mL PBS) was added to streptavidin-coated plates and incubated for 18 h at 4°C. After washing with a saline containing 0.01 g/dL Tween 20 and 0.05 g/dL sodium azide (Wash Buffer), the BC203 coated immunoassay plates were dried in a desiccator.

Preparation of 18H5 (Fab')-ALP and KY-BNP-II (Fab')-ALP

The 18H5 and KY-BNP-II mAbs (IgG) were digested with pepsin (IgG/pepsin = 1/0.05) for 4 h at 37°C in 100 mM citrate buffer (pH 4.0) containing 100 mM NaCl. Thereafter, Fab' solution was prepared by reduction with 10 mM 2-mercaptoethylamine in 0.1 M phosphate buffer (pH 6.0) containing 5 mM EDTA using the standard method [12]. Alkaline phosphatase from calf intestine (ALP; 2.0 mg or 14.2 nmol; Kikkoman, Chiba, Japan) in 0.475 mL 0.1 M Tris-HCl buffer (pH 7.0) containing 1 mM MgCl2 and 0.1 mM ZnCl2 was mixed with 31 mg (71 nmol) of Sulfo-HMCS in 0.05 mL of water for 1.5 h on ice, after which the HMCS-activated ALP was purified on a PD-10 column (GE Healthcare, Chalfont St. Giles, UK). Aliquots of HMCS-activated ALP solution (0.96 mg in 0.192 mL) were each added to 0.441 mg of the Fab' in 0.15 mL of 0.1 M phosphate buffer (pH 6.0) containing 5 mM EDTA and mixed for 16 h at 4°C. Unlabeled Fab' antibody was removed using a TSKgel 3000SWxl column. The purified 18H5 (Fab')-ALP and KY-BNP-II (Fab')-ALP were then diluted with a StabilZyme AP (BioFX Lab.) and stored at 4°C until use.

Sandwich 2-step Chemiluminescent Enzyme Immunoassay

After the BC203 coated immunoassay plates were washed with a wash buffer, 50 mL of test sample or calibrator and 50 mL of Assay Buffer (0.05 M Tris-HCl buffer (pH 7.4), 1 g/dL BSA, 0.01 g/dL Tween80, 1 mM MgCl2, 0.1 mM ZnCl2, 1000K IU/mL Aprotinin, 0.1 mg/mL mouse gamma globulin, 0.9 g/dL NaCl) were added to the wells. The plates were then incubated for 3 h at 25°C. After washing with wash buffer, 100 mL of detection antibodies (18H5 (Fab')-ALP, 100 ng/ml; KY-BNP-II (Fab')-ALP, 416 ng/ml) were added to the wells. The plates were then incubated for 1 h at 25°C, followed by washing with wash buffer and addition of substrate (CDP/E) solution. The chemiluminescence from each well was then measured using a plate reader (Wallac 1420 Arvo sx, Perkin Elmer, Inc., MA).

Study Patients

We collected blood samples from heart failure patients (18 men and 14 women; age range, 34–84 years, mean age, 65±11 years) hospitalized at Kyoto University Hospital. The primary causes of the heart failure were ischemic heart disease (n = 8), cardiomyopathy (n = 8), valvular heart disease (n = 7), pulmonary hypertension (n = 7) and others (n = 2), which were diagnosed from the medical history, physical examination and chest radiographic, electrocar-

Table 2. Effects of dilution on recovery rates with the proBNP and total BNP assay systems.

Dilution magnitude	proBNP assay system		total BNP assay system	
	Measured, pmol/L	Recovery, %	Measured, pmol/L	Recovery, %
1	94	-	95	-
2	105	112	101	107
5	96	102	104	109
10	92	98	92	97
20	97	103	93	98
50	99	105	97	103
100	87	92	95	100

doi:10.1371/journal.pone.0053233.t002

Table 3. Intra- and Inter-assay precision of the proBNP assay systems.

	Added proBNP concentration pmol/L	Measured concentration pmol/L		CV	Bias
		Mean	S.D.	%	%
Intra-assay (n = 5)	2.0	2.0	0.2	8.0	2.0
	25	25	1.3	5.2	0.0
	100	101	5.5	5.4	1.0
Inter-assay (n = 15)	2.0	1.9	0.1	5.3	−5.8
	25	23	1.7	7.4	−8.0
	100	96	6.1	6.4	−4.0

doi:10.1371/journal.pone.0053233.t003

diographic, echocardiographic and/or cardiac catheterization findings. Patients with symptomatic heart failure were under medication, including angiotensin-converting-enzyme inhibitors/angiotensin-receptor blockers, digitalis and diuretics. The New York Heart Association (NYHA) functional classes were class I–II (n = 19) and class III–IV (n = 13). Healthy subjects (61 men and 54 women; age range, 30–78 years, mean age, 50±10 years) were selected based on their normal physical, laboratory, chest radiographic, electrocardiographic and echocardiographic findings, and their BNP levels.

Plasma samples

Blood samples were drawn into plastic syringes and quickly transferred to chilled tubes containing EDTA (1.5 mg/mL, blood) and aprotinin (500 U/mL blood) and centrifuged at 1600× g for 20 min at 4°C. The obtained plasma samples were stored at −80°C until assayed.

Assay of plasma NT-proBNP levels

Plasma levels of NT-proBNP were measured using Elecsys proBNP II assay system (Roche Diagnostics, Basel, Switzerland).

Gel filtration chromatography

Plasma samples were extracted using Sep-Pak C18 cartridges (Waters, Milford, MA, USA) as previously described [6]. The eluate was lyophilized and dissolved in 30% acetonitrile containing 0.1% TFA. The resultant solution (300 ml) was separated by gel filtration HPLC on a Superdex 75 10/300 GL columns (10×300 mm×2, GE Healthcare) in the same buffer at a flow rate of 0.4 mL/min. The column effluent was fractionated every minute into polypropylene tubes containing bovine serum albumin (100 mg) and each fraction was analyzed using the total BNP and proBNP assay systems. Because recent studies have shown that glycosylated proBNP with a MW of about 30 K circulates in the plasma [7], we examined the gel filtration positions at which commercial recombinant proBNP and glycosylated proBNP, and synthetic BNP were eluted to determine which is the major molecular form of BNP in human plasma.

Deglycosylation enzyme treatment

We further analyzed the immunoreactive proBNP levels to determine whether immunoreactive proBNP in plasma is glycosylated. Eluate lyophilized after extraction on a Sep-Pak C18 column was dissolved in phosphate buffer and incubated with or without a cocktail of deglycosylation enzymes for 24 h at 37°C, as previously described [13]. The enzyme cocktail included O-glycosidase (Roche Diagnostic) and neuraminidase (Roche Diagnostics) at final concentrations of 4.25 and 42.5 mU/mL, respectively. These two enzymes were essential for the deglycosylation, and the enzyme concentrations and incubation period were selected based on the results of preliminary and previously reported studies [11,13,14]. We then lyophilized the sample again and dissolved it in 30% acetonitrile containing 0.1% TFA, after which it was subjected to gel-filtration HPLC as described above.

Statistical Analysis

All values are expressed as means ± SD. The statistical significance of differences between 2 groups was evaluated using Fisher's exact test or unpaired Student's t test, as appropriate. Variables were compared among three groups using one-way analysis of variance followed by Bonferroni's multiple comparison

Table 4. Intra- and Inter-assay precision of the total BNP systems.

	Added BNP concentration pmol/L	Measured concentration pmol/L		CV	Bias
		Mean	S.D.	%	%
Intra-assay (n = 5)	2.0	2.3	0.2	7.0	15.0
	25	25	2.1	8.4	1.0
	100	99	7.1	7.2	−0.7
Inter-assay (n = 15)	2.0	2.1	0.2	9.5	5.0
	25	24	1.7	2.9	−4.0
	100	100	1.9	1.9	0.0

doi:10.1371/journal.pone.0053233.t004

Table 5. Cross-reactivity between proBNP and BNP.

Added peptide concentration, pmol/L	Added peptide concentration, pmol/L	Measured peptide concentration, pmol/L	Measured peptide concentration, pmol/L
proBNP	BNP	proBNP assay	total BNP assay
50	50	58	114
100	10	113	119
10	100	8	113

doi:10.1371/journal.pone.0053233.t005

test. Correlation coefficients were calculated using linear regression analysis. Values of $P<0.05$ were considered significant.

Results

Standard curve, recovery and precision

Figure 2 shows typical standard curves for the proBNP and total BNP assay systems. The lower detection limits were 0.04 pmol/L (proBNP) and 0.02 pmol/L (total BNP). At these levels the mean value (n = 8 each) of the chemiluminescence intensity (cps) was more than twice that at 0 pmol/L ($P<0.05$). The working range (coefficient of variation (CV)<15%) of both assays was 0.2–250 pmol/L in total BNP and 0.4–250 pmol/L in proBNP, respectively.

Table 1 shows the recovery of standard proBNP and BNP, which was estimated from the levels of glycosylated proBNP or BNP added to clinically available plasma (endogenous total BNP = 0.3 pmol/L and proBNP = 0.2 pmol/L). In the proBNP assay system, using glycosylated proBNP as a standard, the recovery ranged from 90–101%. In the total BNP assay system, using BNP as the standard the recovery ranged from 85–97%. The effect of diluting plasma samples containing 100 pmol/L glycosylated proBNP or BNP is shown in Table 2. At every dilution level, the recovery rate was good. We also investigated the effects of dilution on plasma levels of total BNP and proBNP in three heart failure patients. As shown in Figure 2B, the calculated total BNP and proBNP values varied linearly with dilution (correlation coefficients = 0.998–1.00).

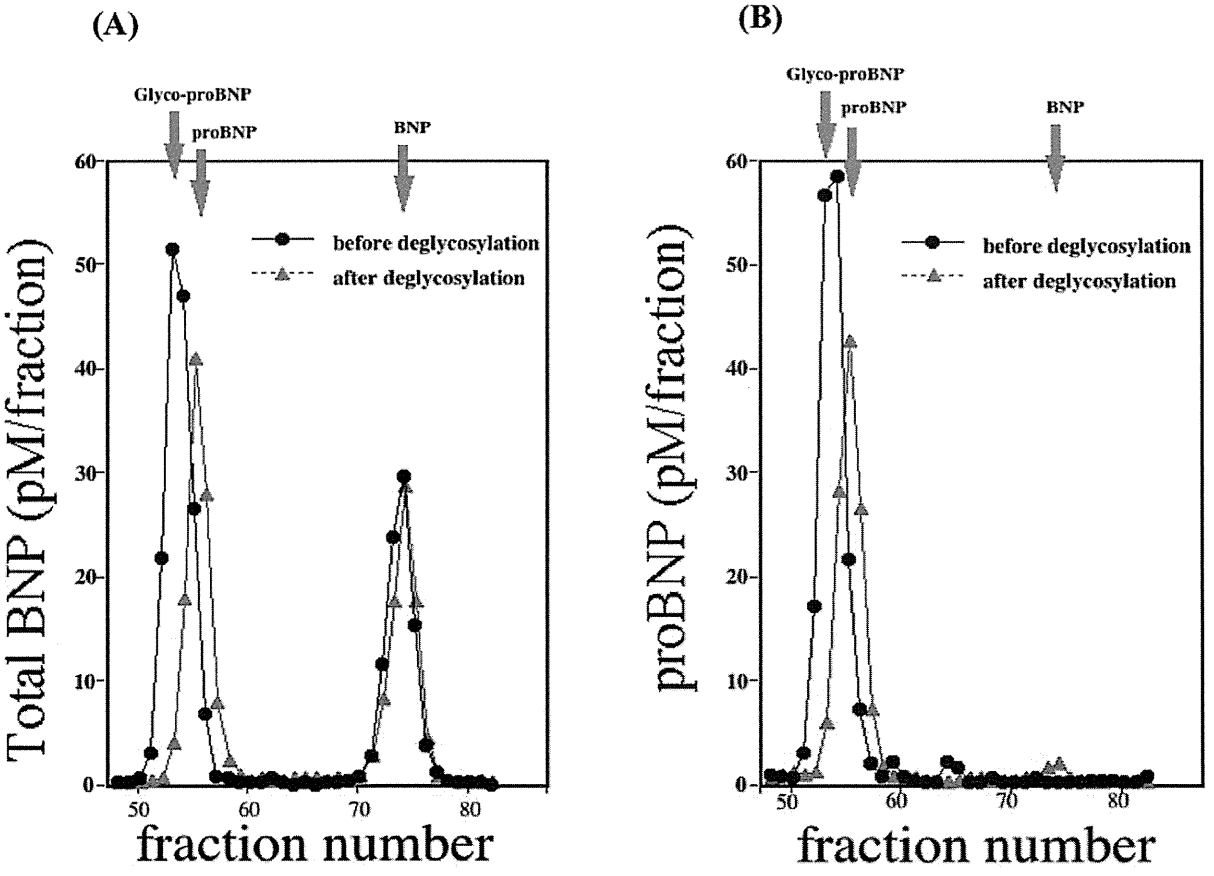

Figure 3. Gel filtration analysis of total BNP (A) and proBNP (B) in plasma from a heart failure patient. Fractions were assayed using the total BNP (A) and proBNP (B) systems. The elution points for glycosylated proBNP, proBNP and BNP are indicated by red arrows. Black and red lines respectively show gel filtration analyses of total BNP (A) and proBNP (B) in the same plasma sample before and after deglycosylation.
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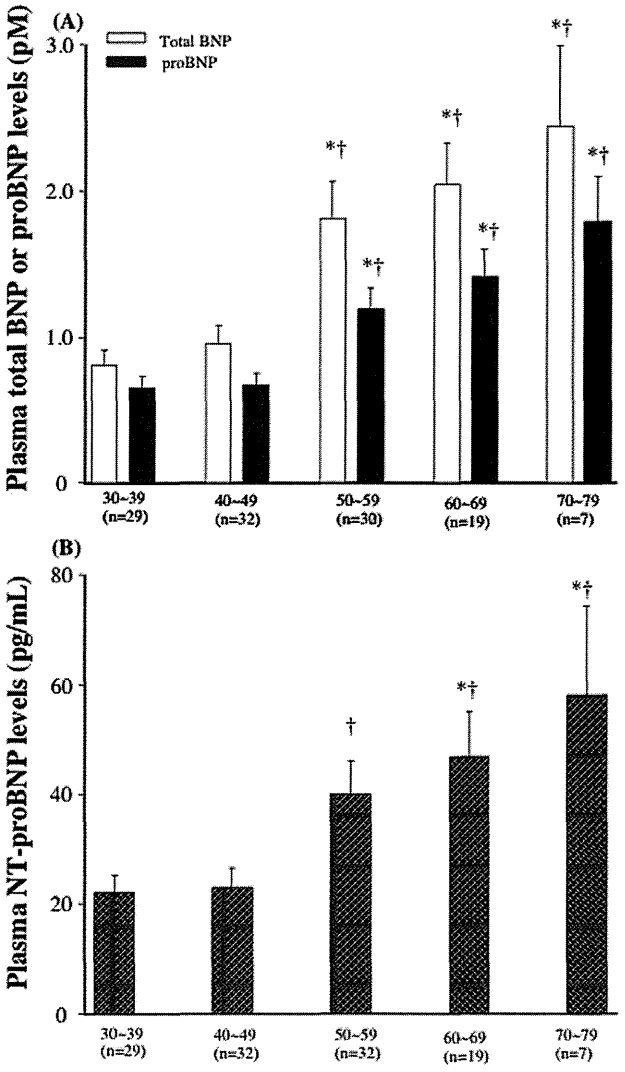

Figure 4. Plasma Levels of total BNP, proBNP, and NT-proBNP in different age groups. Bar graph showing the total BNP, proBNP (A) and NT-proBNP levels (B). Values are means ± SE., *P<0.05 vs total BNP, proBNP, and NT-proBNP in 30~39, †P<0.05 vs total BNP, proBNP, and NT-proBNP in 40~49.
doi:10.1371/journal.pone.0053233.g004

When we then assessed the intra- and inter-assay precision using plasma spiked with glycosylated proBNP or BNP, we found that the intra-assay CV ranged from 5.2%–8.0% in proBNP assay and from 7.0%–8.4% in total BNP assay, while inter-assay CV ranged from 5.3–7.4% in proBNP assay and from 1.9%–9.5% in total BNP assay, respectively (Table 3, 4).

Specificity and sensitivity

We next examined the cross-reactivity between proBNP and BNP. As shown in Table 5, the presence of BNP did not affect the values measured with the proBNP assay system. Moreover, the values measured with the total BNP assay system were the sum of the BNP and proBNP even at different compositions of these two peptides. Thus, the total BNP assay recognized both BNP and proBNP with the same efficiency and sensitivity. Likewise, the proBNP and total BNP assay systems recognized proBNP with the same efficiency and sensitivity.

Gel-filtration chromatography before and after deglycosylation procedure

Figure 3-A shows two immunoreactive BNP peaks detected using the total BNP assay with HPLC fractions. The first peak appeared in fractions 52–55 and the second peak in fractions 72–75. With the same sample, one immunoreactive BNP peak was detected by the proBNP assay (Figure 3-B); the position of that peak was completely consistent with the proBNP peak obtained with the total BNP assay. When subjected to gel filtration HPLC, recombinant proBNP, glycosylated proBNP and BNP were eluted mainly in fractions 53, 56 and 74, respectively. Treating the same plasma sample with an enzyme cocktail catalyzing deglycosylation shifted the first peak to fraction 54–56, which is consistent with the proBNP peak. From these results, we conclude that total BNP assay evaluates the sum of the glycosylated proBNP plus BNP, while proBNP assay detects glycosylated proBNP. The proBNP was not detected in a significant level with either assay system.

Plasma concentrations of proBNP, total BNP, and NT-proBNP in healthy subjects and heart failure patients

Plasma total BNP, proBNP and NT-proBNP levels in different age groups were shown in Figure 4-A, B. Plasma total BNP, proBNP and NT-proBNP levels appeared to increase according to the age. The older age groups (more than 50) had higher total BNP, proBNP and NT-proBNP levels than younger age groups (less than 50); however, there were no statistical differences in NT-proBNP between 30~39 and 50~59. In addition, there were significant positive relationships between plasma total BNP ($r=0.467$, $p<0.001$), proBNP ($r=0.491$, $p<0.001$) and NT-proBNP ($r=0.376$, $p<0.001$) levels and age (Figure 5-A, B, C).

The mean total BNP and proBNP in plasma from 116 healthy subjects were 1.4 ± 1.2 pM and 1.0 ± 0.7 pM, respectively (Figure 6-A). Female had higher total BNP than male (total BNP: 1.7 ± 1.3 vs 1.1 ± 1.1, $P<0.05$; proBNP: 1.1 ± 0.8 vs 0.8 ± 0.6 pM, $P=0.11$) (Figure 6-C). proBNP/total BNP ratio was lower in female than that in male. NT-proBNP was also higher in female than those in male (Figure 6-E). The total BNP and proBNP levels were markedly elevated in heart failure patients, and the magnitude of the increase reflected the severity of the patients' condition as observed in NT-proBNP (Figure 6-A, B).

Discussion

Plasma levels of the cardiac hormone BNP increase in proportion to the severity of heart failure. Indeed, plasma BNP levels are used as a biomarker of heart failure, and the guidelines in many countries recommend that BNP be used as a diagnostic indicator of acute and chronic heart failure [1–3]. The stimuli that increase cardiac BNP production include pressure overload, volume overload and ischemia, as well as various cytokines and neurohumoral factors [15]. In response to these stimuli, BNP mRNA expression is rapidly upregulated. Following translation of the protein, the signal peptide is removed to produce proBNP, which is then cleaved into BNP and the NT-proBNP fragment during secretion [15]. It is noteworthy that BNP and proBNP could not be distinguished from one another in earlier BNP assay systems because the anti-BNP antibodies cross-reacted with proBNP. We therefore endeavored to develop a new assay system that would enable separate measurement of BNP and proBNP. Recent studies have shown that levels of uncleaved proBNP are increased in heart failure to a greater degree than BNP [5–7,16]. Using a combination of gel filtration and an immunoenzyme fluorescent assay for BNP, we previously found that proBNP levels are increased in heart failure and that the proBNP/total BNP

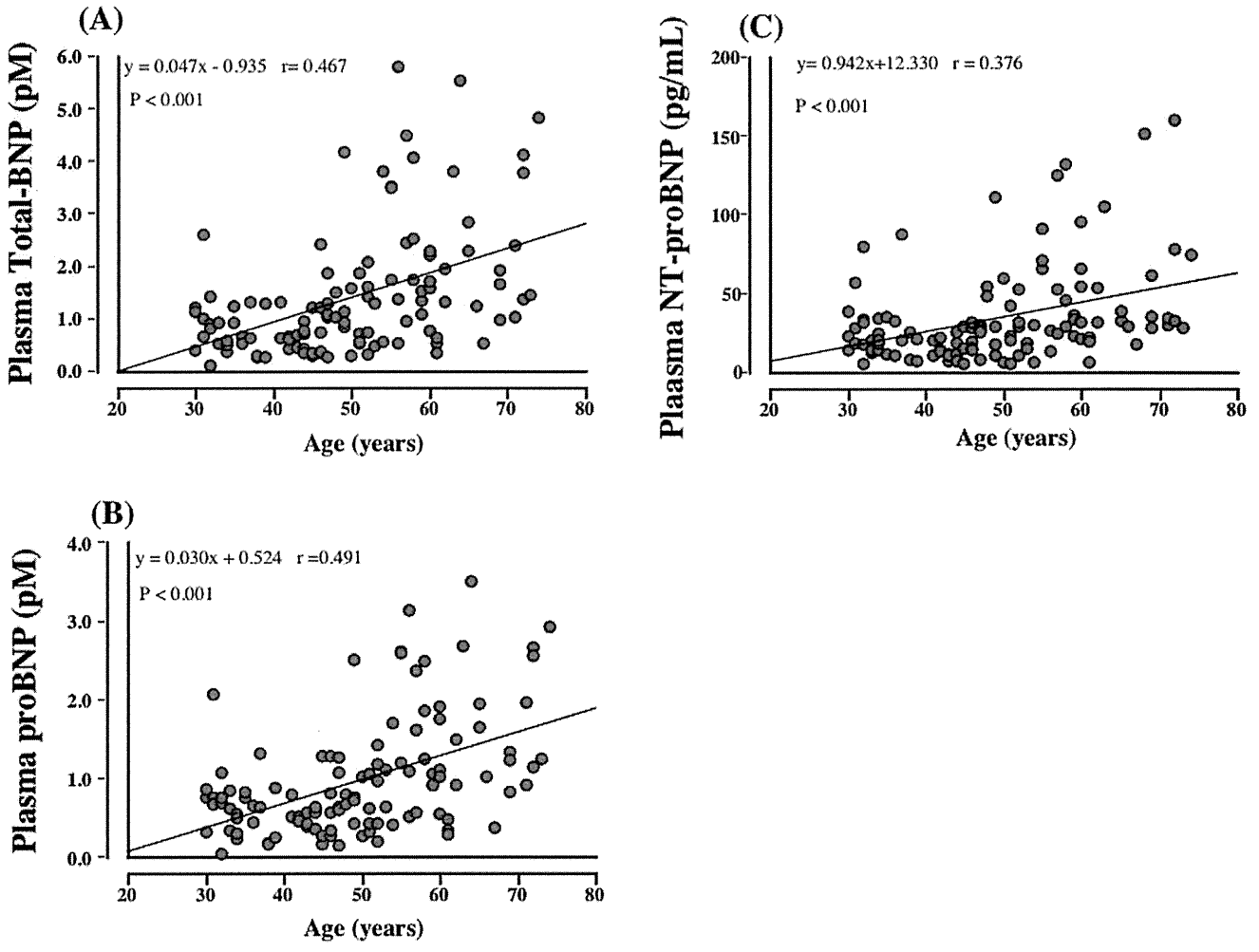

Figure 5. The relationships between total BNP (A), proBNP (B), and NT-proBNP (C) and age.
doi:10.1371/journal.pone.0053233.g005

ratios are higher in heart failure patients with ventricular overload than those with atrial overload [6]. Although this protocol provides useful information, the methodology is time-consuming and impractical for routine assays in clinical laboratories. In addition, recovery of proBNP may be diminished by both extraction and the gel filtration steps [9,16]. To overcome these problems, we developed new direct immunochemiluminescent assays for proBNP and total BNP.

We used two monoclonal antibodies, BC203 and 18H5, to assay proBNP. BC203 recognizes an epitope in the C-terminal of proBNP, while 18H5 recognizes an epitope in the N-terminal. Recent studies showed that proBNP has seven sites suitable for *O*-linked oligosaccharide attachment (Ser36, Thr37, Thr44, Thr48, Thr53, Ser58 and Thr71) within the N-terminal portion of the peptide [14]. Because the *O*-linked oligosaccharide attachments almost completely inhibit the binding of the antibody to the peptide [17], we selected 18H5, which recognizes the N-terminal of proBNP (a.a. 13–20) in a region not subject to glycosylation (Figure 1). To assay total BNP, we used the monoclonal antibodies BC203 and KY-BNP-II, as previously reported [10]. In both assays, BC203 served as the capture antibody. Importantly, because the affinity of 18H5 for the N-terminal portion is similar to the affinity of KY-BNP-II for the ring structure, we are able to calculate the proBNP/total BNP ratio. In addition, our new assays are less time-consuming and more sensitive and accurate than earlier ones, and the lower detection limits for total BNP (0.02 pmol/L) and proBNP (0.04 pmol/L) enabled us to measure plasma proBNP levels in nearly all the healthy subjects tested.

We used gel-filtration on two tandemly connected Superdex 75 columns to determine the molecular mass of plasma proBNP. As shown in Figure 3-A,B, a single peak of proBNP was obtained in both the total BNP and proBNP assay systems. The elution points are consistent with that of glycosylated proBNP, but not deglycosylated proBNP, and deglycosylation treatment significantly shifted the peak rightward (Figure 3-A,B) to an elution point consistent with proBNP. The peak immunoreactivity of proBNP after deglycosylation was slightly smaller than before treatment, suggesting the recovery rate of proBNP after gel-filtration is lower than that of glycosylated proBNP, which is consistent with proBNP being more adsorptive than glycosylated proBNP. Our findings are also consistent with previous Western blot analyses showing that plasma levels of glycosylated proBNP are elevated and no substantial level of proBNP is detected in severe heart failure [7]. Taken together, these results suggest that the major molecular form of proBNP in the plasma of patients with heart failure is the glycosylated form.

ProBNP is also the important molecular form of BNP in the plasma of healthy subjects. When we previously used gel-filtration

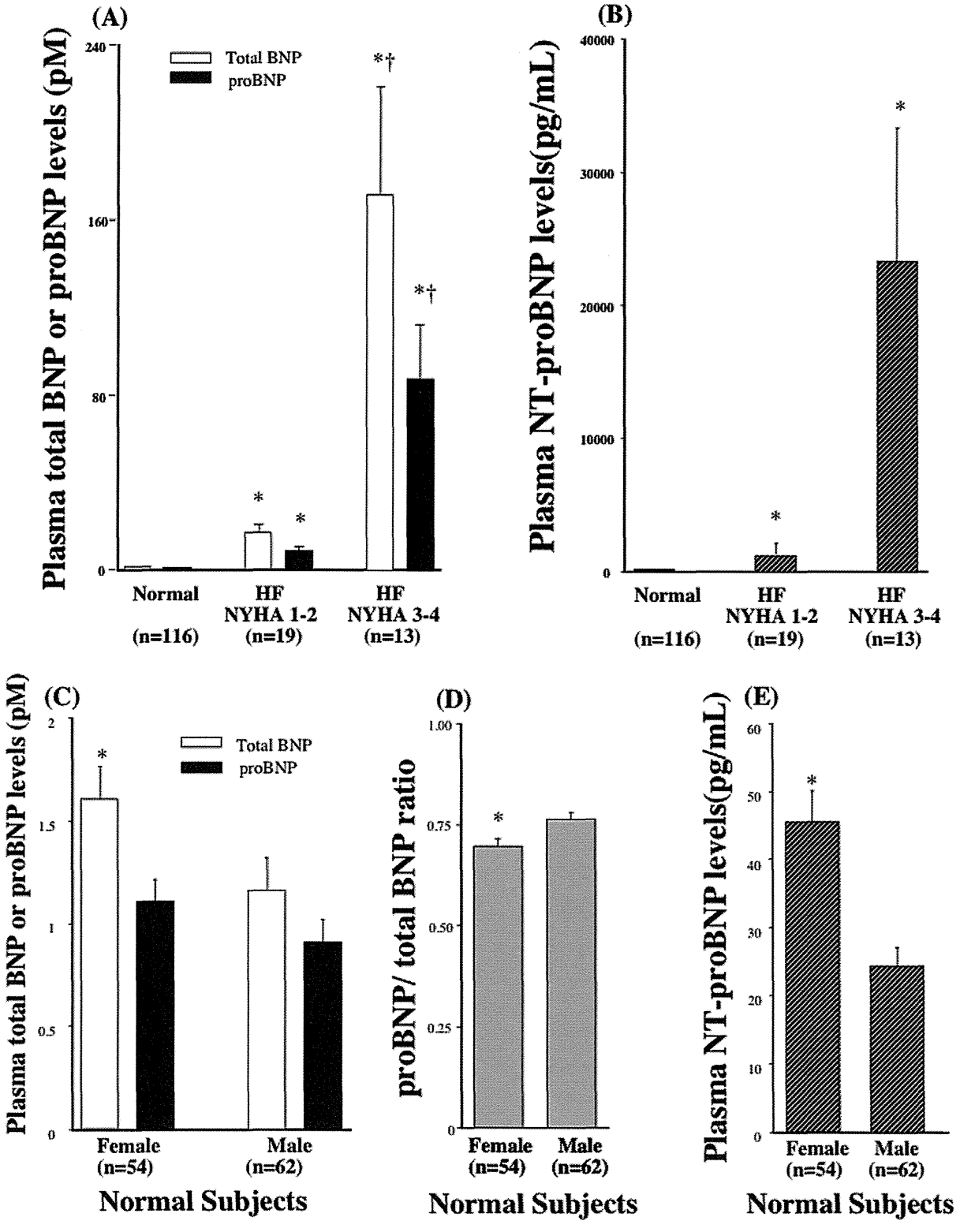

Figure 6. Plasma Levels of proBNP, total BNP, and NT-proBNP in normal and heart failure. Bar graph showing the total BNP, proBNP (A) and NT-proBNP (B) levels in healthy subjects and heart failure patients with NYHA classes 1–2 and 3–4. *P<0.05 vs total BNP and proBNP in normal, †P<0.05 vs total BNP and proBNP in HF NYHA 1–2. Bar graph showing the total BNP, proBNP (C), proBNP/total BNP ratio (D) and NT-proBNP (E) levels in male and female in healthy subjects. Values are means ± SE. *P<0.05 vs male.
doi:10.1371/journal.pone.0053233.g006

and a fluorescent immunoenzyme assay to measure BNP and proBNP, we found that levels of BNP were slightly higher than those of proBNP in both healthy subjects and heart failure patients. The exact reason for the discrepancy in proBNP levels between the earlier study and the present one is unclear; however, the lower recovery caused by the need for extraction from plasma on a Sep-Pak C18 cartridge may have contributed to the lower proBNP levels in the earlier study [9,16]. Recent studies have shown that proBNP has much less ability to induce cGMP production in vascular smooth muscle and endothelial cells than BNP [7,18]. This suggests that increases in the levels of the low-activity proBNP in heart failure may contribute to the so-called "BNP paradox" [19]. That is, administration of exogenous recombinant human BNP to heart failure patients has a substantial clinical and hemodynamic impact, despite the presence of high levels of immunoreactive BNP in their plasma, as measured with commercially used BNP assays.

In the current study, we showed that total BNP and NT-proBNP increased with aging, which are consistent with the previous studies. In addition, the current study first showed that plasma proBNP level increased with aging. However, there were no statistical differences in NT-proBNP between 30~39 and 50~59, whereas there were significant differences in total and proBNP between 30~39 and 50~59, suggesting that total and proBNP are more sensitive than NT-proBNP. In addition, total and proBNP seemed to be well correlated with age ($r = 0.467$. 0.491, each) than NT-proBNP ($r = 0.376$). Thus, total BNP and proBNP may be better marker in discriminating the effect of age than NT-proBNP. Increased myocardial mass and/or reduction of renal clearance of natriuretic peptides with aging may be one of the possible reason for increased BNP and NT-BNP with aging; however, exact mechanism for it still remains unknown and further study is necessary to investigate the relationships between proBNP and aging.

We also analyzed the effects of gender on proBNP, total BNP, proBNP/total BNP ratio and NT-proBNP. Interestingly, in female higher total BNP and NT-proBNP and lower proBNP/total BNP ratio without changing of proBNP was observed. Calculated BNP (total BNP - proBNP) was also increased. This finding may be explained that increased proBNP production and higher processing rate. Further study is necessary to elucidate the mechanism of increased total BNP and NT-proBNP and lower proBNP/total BNP ratio without changing of proBNP in female.

In summary, we have developed rapid and precise immunochemiluminescent assay systems for routine determination of total BNP and proBNP levels in human plasma. Using these assay systems we showed that in addition to BNP, considerable amount of proBNP circulates in both healthy subjects and heart failure patients. The precise relation of the proBNP/total BNP ratio to heart failure remains unknown, as does whether the heart mainly secretes proBNP. Likewise, the effects of age, sex and renal function on proBNP levels remain unknown. We anticipate our new assays for the proBNP and total BNP will be helpful for addressing each of those issues.

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Author Contributions

Gave useful comments and discussion: K. Kamgawa K. Nakao. Conceived and designed the experiments: TN HO NM KH. Performed the experiments: MN NO K. Nagata. Analyzed the data: YN HK CY K. Nakao. Contributed reagents/materials/analysis tools: TM YK K. Koichiro IM. Wrote the paper: TN HO.

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Complexity of molecular forms of B-type natriuretic peptide in heart failure

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INTRODUCTION

In 1988, a Japanese group isolated B-type (or brain) natriuretic peptide (BNP) from porcine brain extracts by monitoring its relaxant effects on chick rectum.[1] Since then studies in humans and rodents demonstrated that BNP is a cardiac hormone mainly expressed in the heart, where its concentration is considerably higher than in brain. BNP possesses a 17-amino acid ring structure containing two cysteine residues, which is essential for its biological activity. Mechanical stress, ischaemia, cytokines and neurohumoral factors, including angiotensin II, stimulate expression of BNP (figure 1),[2] and levels of myocardial BNP mRNA and circulating BNP and N-terminal proBNP (NT-proBNP) are markedly increased in patients with congestive heart failure.[2] BNP is therefore considered to function as an emergency defence against ventricular overload in disease states.

MOLECULAR COMPLEXITY OF IMMUNOREACTIVE BNP IN HUMAN PLASMA

It is thought that human ProBNP is most likely cleaved by furin to BNP and NT-proBNP when it is secreted.[2] Once in the plasma, dipeptidyl peptidase IV removes the two N-terminal amino acids (Ser-Pro) of BNP to generate BNP[3–32], [3] the levels of which are increased in patients with heart failure.[4] Various BNP assay kits (eg, Shionogi, Biosite) similarly detect BNP[3–32] and BNP (figure 2).[5] In addition, other aminopeptidases may further digest the N-terminal region of BNP and/or BNP[3–32], and current assay systems likely also cross-react with these forms (figure 2). Consequently, the actual molecular forms of BNP circulating in plasma remain uncertain.

An early study failed to detect native BNP in plasma from New York Heart Association (NYHA) class-IV patients using solid phase extraction combined with liquid chromatography, immunodetection and mass spectrometry, despite BNP levels having been predetermined to exceed 1000 pg/ml. This suggests that native BNP may be altered in such patients.[6] Later attempts to quantify active BNP in the plasma of patients with heart failure using various extraction and detection methods, including mass spectrometry, detected BNP [3–32] in all patients along with other forms, including BNP[4–32], BNP[5–32], BNP[5–31], BNP[1–25] and BNP[1–26].[7] Incomplete protease inhibition during and after blood collection may partially explain why some investigators detected no BNP in the plasma of heart failure patients. However, when the sum of all the BNP breakdown products was measured in patients using mass spectrometry, it was verified to be only a small fraction of the total immunoreactive BNP determined using the Biosite assay,[7] which may be explained by antibody cross-reactivity with proBNP and related molecules (figure 2). A more recent study using mass spectrometry also confirmed the very low levels of biologically active BNP in the plasma of heart failure patients.[8] Moreover, clinical measurements of BNP made using an immunoassay correlated poorly with BNP levels measured using mass spectrometry, though they correlated well with BNP degradation fragments such as BNP[3–32], BNP[4–32] and BNP[5–32] (figure 2). These results suggest that clinically measured BNP includes numerous fragments that would be expected to have little compensatory biological activity. Whether molecular forms of BNP are altered in healthy individuals as well as in heart failure patients remains unknown. This is because measuring the subfractions of BNP metabolites is difficult in healthy individuals due to the low levels of BNP present.

These findings highlight the need for more specific clinical immunoassays to address the question of atypical proBNP processing and to accurately measure bioactive BNP concentrations. Mass spectrometry is thought to be a suitable technology for such studies.

PRESENCE OF proBNP IN BLOODSTREAM ASSOCIATED WITH HEART FAILURE

The clinical utility of BNP and NT-proBNP as biochemical markers of heart failure was established with BNP's original discovery as a cardiac hormone.[2] However, considerable uncertainty still surrounds the molecular forms of BNP. Earlier studies showing the presence of proBNP in human blood did not garner much attention, but recent studies have shown levels of proBNP to be higher than those of BNP in the plasma of heart failure patients.[9] In addition, one recent study further showed that both proBNP and BNP circulate in the plasma of heart failure patients, and that proBNP/BNP ratios vary widely depending on the heart failure status.[10]

All BNP assays, regardless of the source (eg, Shionogi, Biosite), cross-react with proBNP to some degree because the two antibodies used in the assays recognise epitopes common to BNP and proBNP (figure 2).[5] Whether the BNP values obtained with these assays indicate BNP, proBNP or their combination (BNP +proBNP) remains unknown, and the measured increases in plasma BNP levels seen in heart failure may reflect increases in proBNP as well as BNP. Similarly, all NT-BNP assays (regardless of source) cross-react with proBNP, but react little with glycosylated proBNP, as the attached *O*-saccharide almost completely inhibits antibody binding to the peptide.[11]

In vitro studies have shown that proBNP is much less able to induce guanosine 3′, 5′-cyclic monophosphate (cGMP) production in vascular smooth muscle and endothelial cells than BNP.[12] Plasma cGMP levels are increased in proportion to the severity of mild to moderate heart failure, and correlate with plasma BNP levels. However, the increases in cGMP are attenuated in patients with severe heart failure and a poor prognosis.[2] The observed increase in the levels of less

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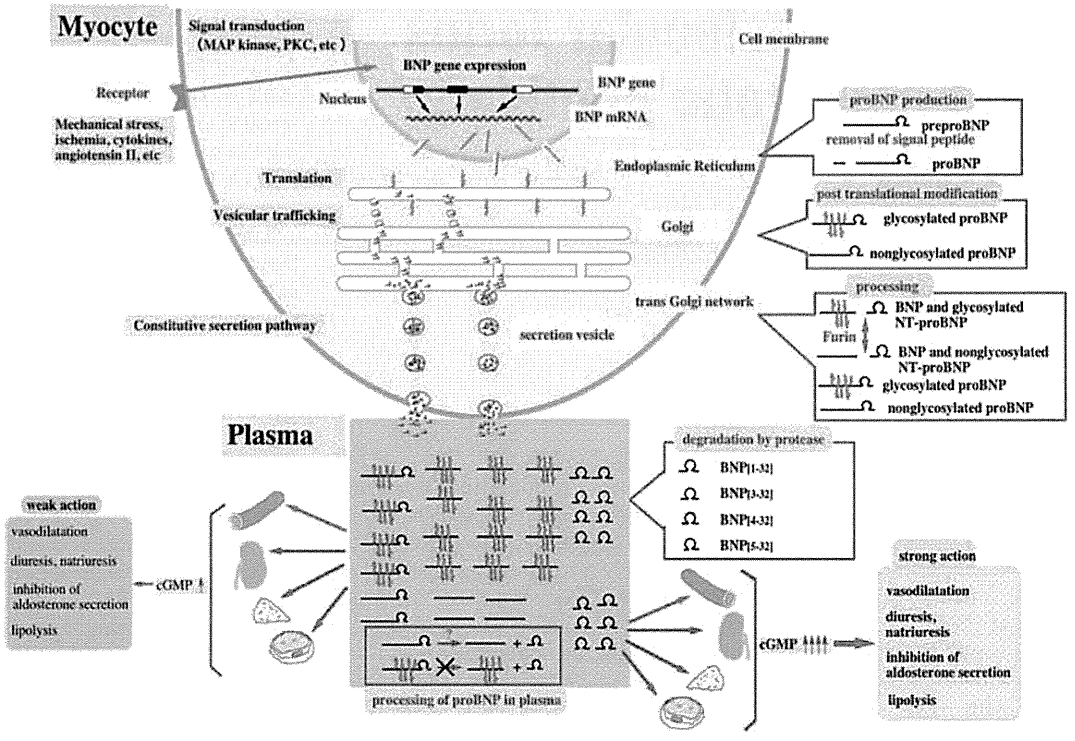

Figure 1 Schematic representation of the stimulus, signal transduction, gene, mRNA, translation, glycosylation, processing and secretion of B-type natriuretic peptide (BNP) in myocytes, and the plasma molecular forms of BNP. Mechanical stress, ischaemia, cytokines and neurohumoral factors (eg, angiotensin II and endothelin-1) stimulate gene expression of BNP via signal transduction mediated by protein kinase C and mitogen-activated protein (MAP) kinase. BNP mRNA is translated in the endoplasmic reticulum, after which preproBNP is converted to proBNP by a signal peptidase. ProBNP is post-translationally *O*-glycosylated within the Golgi apparatus and cleaved to BNP and NT-proBNP in equimolar fashion by furin within the trans-Golgi network. They are then transferred to secretion vesicles and secreted into the circulation via a so-called constitutive secretion pathway. ProBNP is often heavily *O*-glycosylated in the N-terminal region, and furin cannot easily cleave *O*-glycosylated proBNP when Thr71 is *O*-glycosylated. In the plasma, BNP is degraded to BNP [3-32] by dipeptidyl peptidase IV, after which BNP [3–32] is further degraded to BNP [4–32], BNP [5–32] and other metabolites by aminopeptidases. In addition, non-glycosylated proBNP may be processed into BNP and NT-proBNP by an unidentified mechanism, whereas glycosylated proBNP is not processed into BNP and NT-proBNP. BNP stimulates cGMP production via natriuretic peptide receptor-A in various tissues, including the vasculature, kidney, adrenal gland and adipose tissue, and induces vasodilation, diuresis, natriuresis, inhibition of aldosterone secretion and lipolysis. By contrast, glycosylated and non-glycosylated proBNP have little ability to stimulate cGMP production. Glycosylated and non-glycosylated NT-proBNP do not bind receptors and accumulate in the plasma in heart failure. This figure is only reproduced in colour in the online version.

hormonally active proBNP in severe heart failure may explain this phenomenon.[13] Indeed, one recent study showed that the proBNP/BNP ratio is increased in decompensated heart failure, and that medical therapy reduces plasma BNP and the patients' symptoms in concert with a reduction in the proBNP/BNP ratio in some cases.[10] Elucidation of the mechanism associated with the increased proBNP/BNP ratio should help to clarify the pathogenesis of heart failure and/or pave the way towards novel therapies.

PROBNP AND NT-proBNP ARE *O*-GLYCOSYLATED IN SEVERE HEART FAILURE

Not only do the levels of proBNP increase in heart failure, the degree to which proBNP is *O*-glycosylated also increases in proportion to heart failure severity.[13] Thus understanding the clinical relevance of proBNP glycosylation is a matter of obvious importance. Pressure and volume overload, ischaemia and other conditions stimulate BNP gene transcription.[2] BNP mRNA is translated in the endoplasmic reticulum to produce preproBNP. Subsequent removal of the signal peptide yields proBNP, which can be post-translationally glycosylated to varying degrees at several sites in its N-terminal region (Ser36, Thr37, Thr44, Thr48, Thr53, Ser58 and Thr71) while the protein is within the Golgi apparatus.[14] The *O*-glycosylated proBNP is transported to the trans-Golgi network, where it is cleaved to BNP and NT-proBNP by furin.[7] Both BNP and NT-proBNP are thought to be secreted via the constitutive secretion pathway without storage in secretory granules (figure 1).

Plasma levels of glycosylated proBNP, but not proBNP, are increased in patients with severe heart failure.[12] Why glycosylated proBNP is secreted without processing under conditions of severe heart failure is not fully understood at present. One recent study showed that *O*-glycosylation at Thr71 in a region close to the cleavage site impairs proBNP processing by furin in HEK293 cells.[15] But since the effect of *O*-glycosylation on furin-catalysed processing has only been evaluated in vitro, the roles of other possible processing enzymes remain unclear. In addition, whether these events occur in cardiac myocytes in the atria and/or ventricles also remains unknown. Further studies using cardiac myocytes will be required to clarify the precise mechanism of proBNP processing.

NT-proBNP is also *O*-glycosylated in heart failure.[11] An assay for NT-proBNP from Roche Diagnostics (Elecsys I) utilises polyclonal antibodies directed against epitopes proBNP[1–21] and proBNP[39–50], and monoclonal antibodies against epitopes proBNP[27–31] and proBNP[42–46]

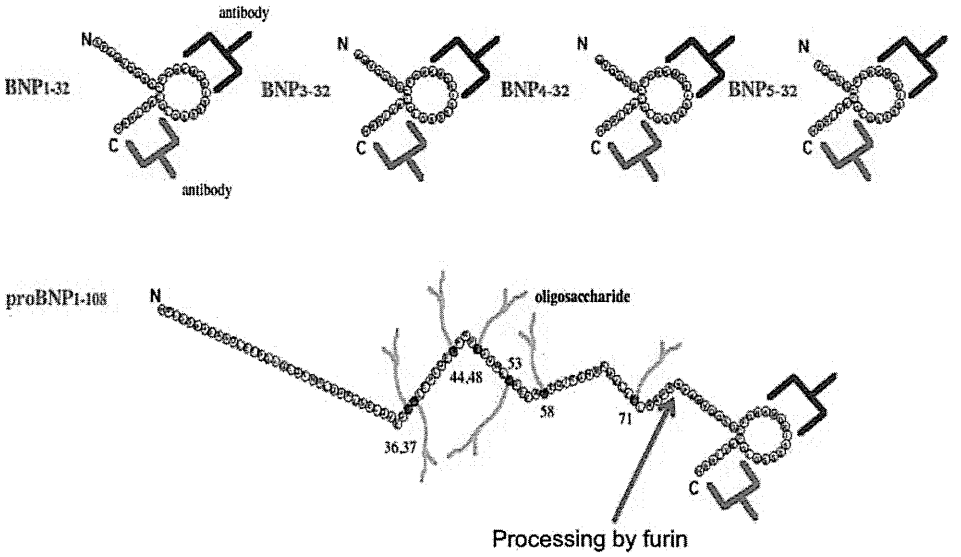

Figure 2 Schematic diagram illustrating the principle underlying the currently used B-type natriuretic peptide (BNP) assay systems. BNP is sandwiched by two antibodies. One is the capture antibody (red), and the other is the detection antibody (blue). This assay system measures BNP independently of the length of the N-terminal extension from the ring structure. Consequently, it cross-reacts with BNP[3–32], BNP[4–32], BNP[5–32] and proBNP[1–108]. This figure is only reproduced in colour in the online version.

have recently been introduced as Elecsys II. In both of these assays, glycosylation of the middle portion of NT-proBNP (Thr44, Thr48) could affect antibody binding (directed against proBNP[39–50] or proBNP[42–46]), potentially leading to an underestimation of the concentration of circulating NT-proBNP.[11] For example, measured levels of plasma NT-proBNP in heart failure increase fourfold to fivefold after enzymatic deglycosylation, as compared with glycosylated NT-proBNP.[11] On the other hand, numerous clinical studies have shown that the current NT-proBNP and BNP assays are equally valid for the diagnosis of heart failure. Therefore, an answer to whether new NT-proBNP assays using antibodies directed against non-glycosylated epitopes will result in better clinical applicability awaits further evaluation. Further studies will also be needed to identify the enzymes involved in the processing of non-glycosylated proBNP in the circulation and to determine how much non-glycosylated proBNP is actually present in human plasma.

CONCLUSION

Here we have briefly summarised the current understanding of the molecular forms of BNP. Several interesting issues remain to be addressed. It is therefore essential to unambiguously identify the molecular forms of BNP present in cardiac tissue and plasma. Also of importance is the relationship between the molecular forms of BNP in plasma and a patient's clinical condition. These studies may contribute not only to more accurate diagnosis of heart failure, but also to the clarification of the pathogenesis of heart failure and the development of new treatments.

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The moyamoya disease susceptibility variant RNF213 R4810K (rs112735431) induces genomic instability by mitotic abnormality

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ABSTRACT

Moyamoya disease (MMD) is a cerebrovascular disease characterized by occlusive lesions in the Circle of Willis. The RNF213 R4810K polymorphism increases susceptibility to MMD. In the present study, we characterized phenotypes caused by overexpression of RNF213 wild type and R4810K variant in the cell cycle to investigate the mechanism of proliferation inhibition. Overexpression of RNF213 R4810K in HeLa cells inhibited cell proliferation and extended the time of mitosis 4-fold. Ablation of spindle checkpoint by depletion of mitotic arrest deficiency 2 (MAD2) did not shorten the time of mitosis. Mitotic morphology in HeLa cells revealed that MAD2 colocalized with RNF213 R4810K. Immunoprecipitation revealed an RNF213/MAD2 complex: R4810K formed a complex with MAD2 more readily than RNF213 wild-type. Desynchronized localization of MAD2 was observed more frequently during mitosis in fibroblasts from patients ($n = 3$, $61.0 \pm 8.2\%$) compared with wild-type subjects ($n = 6$, $13.1 \pm 7.7\%$; $p < 0.01$). Aneuploidy was observed more frequently in fibroblasts ($p < 0.01$) and induced pluripotent stem cells (iPSCs) ($p < 0.03$) from patients than from wild-type subjects. Vascular endothelial cells differentiated from iPSCs (iPSECs) of patients and an unaffected carrier had a longer time from prometaphase to metaphase than those from controls ($p < 0.05$). iPSECs from the patients and unaffected carrier had significantly increased mitotic failure rates compared with controls ($p < 0.05$). Thus, RNF213 R4810K induced mitotic abnormalities and increased risk of genomic instability.

1. Introduction

Moyamoya disease (MMD: MIM 607151) is characterized by occlusive lesions at the terminal portion of internal carotid arteries in the Circle of Willis [1,2]. It is now recognized as one of the major causes of stroke in adults and children worldwide [3–6]. RNF213 has been recognized as the susceptibility gene for MMD, and the p. R4810K polymorphism (rs112735431 or ss179362673: G > A; herein referred to as RNF213 R4810K) as a founder variant commonly found in East Asian (Japanese, Korean and Chinese) MMD patients [7].

We recently found that vascular endothelial cells developed from induced pluripotent stem cells (iPSECs) of patients with MMD, carrying RNF213 R4810K, had reduced angiogenic activity [8]. This was partially mediated by the down-regulation of *Securin* [8]. In addition to *Securin*, various mitosis-associated genes were down regulated in iPSECs from patients [8]. Furthermore, the overexpression of RNF213 R4810K inhibited the proliferation of human umbilical vein endothelial cells (HUVECs) [8]. The primary aim of our study was to characterize the phenotypes associated with RNF213 R4810K in the cell cycle.

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