Immunodetection of Glycosylated NT-proBNP Circulating in Human Blood

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BACKGROUND: Brain natriuretic peptide (BNP) or NT-proBNP (N-terminal fragment of BNP precursor) measurements are recommended as aids in diagnosis and prognosis of patients with heart failure. Recently it has been shown that proBNP is O-glycosylated in human blood. The goal of this study was to map sites on the NT-proBNP molecule that should be recognized by antibodies used in optimal NT-proBNP assays.

METHODS: We analyzed endogenous NT-proBNP by several immunochemical methods using a broad panel of monoclonal antibodies specific to different epitopes of the NT-proBNP molecule.

RESULTS: Treatment of endogenous NT-proBNP by a mixture of glycosidases resulted in significant improvement of the interaction between deglycosylated NT-proBNP and monoclonal antibodies (MAbs) specific to the mid-fragment of the molecule. MAbs specific to the N- and C-terminal parts of NT-proBNP (epitopes 13–24 and 63–76) were able to recognize glycosylated and deglycosylated protein with similar efficiency.

CONCLUSIONS: The central part of endogenous NT-proBNP is glycosylated, making it almost “invisible” for the antibodies specific to the mid-fragment of the molecule. Thus sandwich assays using even one antibody (poly- or monoclonal) specific to the central part of the molecule could underestimate the real concentration of endogenous NT-proBNP. MAbs specific to the N- and C-terminal parts of NT-proBNP (epitopes 13–24 and 63–76) are the best candidates to be used in an assay for optimal NT-proBNP immunodetection.

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Proteomics and Protein Markers

Materials and Methods

REAGENTS
We obtained O-glycosidase (endo-α-N-acetylgalactosaminidase) from QA-Bio; sialidase A (N-acetylneuraminyl galactosidase), β(1-4) galactosidase, and β-N-acetylgalactosaminidase from Prozyme; normal human EDTA-plasma from Innovative Research; Sepha-
rose CL 4B from GE Healthcare; 96-well plates for immunoassays from Costar; and streptavidin-coated plates from Wallac-Perkin Elmer. All other chemicals were from Sigma.

**ANTIBODIES AND RECOMBINANT PROTEINS**

Monoclonal antibodies (MAbs) 5B61–12, 10E111–12, 29D125–12, 3H83–12, 13G123–20, 16F313–20, 18H513–20, 1D413–24, 15F1113–24, 7B513–24, 5D328–45, 11D131–39, 5E231–39, 16E634–39, 15D746–56, 13C146–56, 16D1046–56, 15C463–71, 21E667–73, and 24E1167–76 are specific to different regions of the NT-proBNP molecule and recognize recombinant NT-proBNP and proBNP expressed in *E. coli* that are originally nonglycosylated. MAb 50E1 is specific to the 26–32 region of the BNP molecule, MAb 24C5 to region 11–22. We obtained all MAbs, human recombinant NT-proBNP and proBNP expressed in *E. coli* (nonglycosylated forms) from HyTest.

**PATIENTS**

Plasma samples were from 67 City Hospital, Moscow, Russia. Venous blood was collected into EDTA-containing vacuette tubes (Greiner Bio-One) and centrifuged at 3000 g (15 min, 4 °C). Plasma specimens were stored at −70 °C until use. The diagnosis of HF was based on clinical symptoms (dyspnea, orthopnea, lung rales, leg edema) and confirmed by echocardiography studies and x-ray examination. All studies using human blood samples were in accordance with the current revision of the Helsinki Declaration.

**NT-proBNP-FREE PLASMA**

To remove any traces of NT-proBNP and proBNP from normal human plasma, we loaded it onto an affinity column containing several MAbs specific to different regions of NT-proBNP (18H513–20, 15F1113–24, 15C463–71, 24E1167–76) and BNP (50E126–32 and 24C511–22) molecules. Selected antibodies were coupled with BrCN-activated Sepharose CL 4B according to the protocol suggested by GE Healthcare.

**SANDWICH IMMUNOFLUORESCENCE ASSAYS**

*Sandwich 2-step NT-proBNP immunofluorescence assay (IFA)*. We used MAbs labeled with stable Eu³⁺ chelate as detection antibodies (5). Capture antibodies, 2 μg per well in 100 μL PBS, were incubated in immunoassay plates for 30 min at room temperature with constant shaking. After washing with 0.01 mol/L Tris-HCl (pH 7.8) buffer containing 0.15 mol/L NaCl, 0.25 mL/L Tween 20, and 0.5 g/L NaN₃ (buffer A), 50 μL tested sample or calibrator and 50 μL detection antibodies in assay buffer (0.05 mol/L Tris-HCl buffer, pH 7.7, 9 g/L NaCl, 0.1 mL/L Tween 40, 5 g/L bovine serum albumin, 0.5 g/L NaN₃) was added to the wells. The plates were incubated for 30 min at room temperature with constant shaking. After washing with buffer A, 200 μL enhancement solution (1.75 mol/L NaSCN, 1 mol/L NaCl, 50 mL/L glycerol, 200 mL/L 1-propanol, 0.005 mol/L Na₂CO₃, 0.05 mol/L glycine-NaOH, pH 10.0) per well was added and incubated for 3 min at room temperature with gentle shaking. Fluorescence was measured on a Victor 1420 multilabel counter (Wallac-PerkinElmer). These assays were used in the preliminary testing of 2-site MAb combinations with endogenous NT-proBNP.

*Sandwich 1-step NT-proBNP IFAs (15C463–71 capture-13G1213–20 detection and 11D131–39 capture-13G1213–20 detection).* Capture antibodies were biotinylated (5), whereas detection antibodies were labeled with stable Eu³⁺ chelate. We incubated a mixture of equal quantities (200 ng per well) biotinylated and detection antibodies in 50 μL assay buffer in streptavidin-coated plates with 50 μL tested sample or calibrators for 30 min at room temperature with gentle shaking. After washing with buffer A, we added 200 μL enhancement solution per well and measured fluorescence after 3 min of incubation. Total assay time was about 40 min. For assay validation, recominant NT-proBNP was reconstituted in NT-proBNP-free plasma and used as a calibrator. The same matrix was used for dilution of HF patients’ plasma. The detection limit was defined as a concentration (measured 20 times in a single run) producing a signal 2 SD above the mean for a matrix free of analyte. We assessed within-assay imprecision (CV) by measuring 20 replicates of 3 different concentrations of calibrator (100 ng/L, 1 μg/L, and 10 μg/L) in NT-proBNP-free plasma.

**ProBNP sandwich IFA.** For proBNP quantification, we used a HyTest in-house proBNP assay using BNP-specific MAb 50E126–32 as capture and NT-proBNP specific antibody 16F313–20 as detection (6).

**AFFINITY MATRIX AND NT-proBNP AFFINITY PURIFICATION**

To prepare affinity matrix for NT-proBNP extraction, we coupled several MAbs specific to different NT-proBNP regions (29D125–12, 18H513–20, 15F1113–24, 11D131–39, 16E634–39, 15D746–56, 15C463–71, and 24E1167–76) with BrCN-activated Sepharose CL 4B. For the affinity extraction, 20 mL pooled plasma from 9 HF patients containing 62 μg/L NT-proBNP (determined by reference assay 15C463–71-13G1213–20) was filtered through 0.45-μm membrane and loaded onto the affinity matrix containing approximately 100-fold molar excess of antigen binding sites over the amount of NT-proBNP. After loading, the matrix was washed with 24 volumes of 0.02 mol/L Tris-HCl buffer, pH 7.5,
containing 0.15 mol/L NaCl. NT-proBNP was eluted with 5 volumes of 0.01 mol/L HCl. Eluted proteins were immediately frozen and stored at -70 °C until use. NT-proBNP was also extracted from 52 individual plasma samples (6–7.2 mL) using the same protocol.

**NT-proBNP DEGLYCOSYLATION**

We adjusted the pH of extracted NT-proBNP preparations to 5.4 by addition of 0.5 mol/L Na2HPO4, pH 6.0. Each sample was divided into 2 equal portions and incubated with either an enzyme mixture [3.75 U/L O-glycosidase, 33.5 U/L sialidase A, 112 U/L α-N-acetylhexosaminidase, 6.6 U/L β(1–4)galactosidase] or without enzymes for 1.5 h at 37°C. Samples of endogenous NT-proBNP treated and not treated with glycosidases were reconstituted in assay buffer, immediately frozen, and stored at -70 °C until use.

**STUDIES OF IMMUNOCHEMICAL PROPERTIES OF ENDOGENOUS NT-proBNP BEFORE AND AFTER DEGLYCOSYLATION**

We used 17 2-site MAb combinations to compare immunoreactivity of endogenous NT-proBNP extracted from pooled plasma from 9 HF patients before and after deglycosylation. Capture antibodies in each assay were specific to different epitopes, covering the NT-proBNP molecule from N- to C-terminal ends (Fig. 1). We used detection antibody 24E1167–76 to form pairs with capture MAbs specific to the N-terminal and central portions of NT-proBNP, whereas we used detection MAb 13G1213–20 in the assays with capture antibodies specific to the C-terminus of NT-proBNP. We used recombinant NT-proBNP as calibrator. Samples of endogenous NT-proBNP, treated and not treated with a mixture of glycosidases, were reconstituted in assay buffer up to concentration 18 µg/L (measured by the reference assay 15C463–71-13G1213–20) and analyzed by 17 IFAs.

**MEASUREMENTS OF NT-proBNP FROM INDIVIDUAL SAMPLES BEFORE AND AFTER DEGLYCOSYLATION**

We measured the quantity of NT-proBNP extracted from 52 individual plasma samples before and after deglycosylation using 2 HyTest in-house assays, 15C463–71-13G1213–20, 11D131–39-13G1213–20 and the Roche Elecsys 2010 NT-proBNP assay that uses polyclonal antibodies specific to 2 different NT-proBNP fragments, peptide 1–21 and peptide 39–50 (3). Assays were calibrated using recombinant NT-proBNP.

**NT-proBNP GEL-FILTRATION STUDIES**

A Superdex 75 10/300 GL column (GE Healthcare) was equilibrated with 0.1 mol/L potassium phosphate buffer, pH 7.4, containing 0.7 mol/L NaCl, 0.005 mol/L EDTA. Endogenous NT-proBNP (treated or not treated with glycosidases) or recombinant NT-proBNP was reconstituted in the same buffer containing 5 g/L bovine serum albumin to the final concentration of 300 µg/L. We loaded 150 µL sample containing 45 ng NT-proBNP onto the column with the pump speed at 0.8 mL/min and collected 0.5-mL fractions. The column was calibrated with a set of standard proteins: albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa), ribonuclease A (13 kDa) (all GE Healthcare), and aprotinin (6.5 kDa) (Sigma).

**Results**

**PRELIMINARY HF PLASMA TESTING IN DIFFERENT SANDWICH IFAS**

Preliminary testing of pooled plasma samples from HF patients in 2-site MAb combinations covering epitopes for the whole NT-proBNP sequence revealed that antibody pairs using at least one of the MAbs specific to the very N-terminal region (peptide 1–12) or to the mid-fragment of NT-proBNP (peptide 28–56) recognized endogenous NT-proBNP poorly. In contrast, the same pairs that were not able to recognize endogenous protein detected recombinant NT-proBNP or proBNP with high sensitivity. The results of pooled plasma testing by 2-site MAb combinations were similar (data not shown) to the results of extracted endogenous protein testing by the same antibody combinations (Fig. 2; grey columns).

**ASSAY VALIDATION**

As was demonstrated in the preliminary studies, sandwich immunoassay 15C463–71-13G1213–20 exhibited the same immunoreactivity for both recombinant and endogenous NT-proBNPs and was accepted as a refer-
ence assay. Assay 11D131–39-13G1213–20 was able to recognize recombinant NT-proBNP with high sensitivity but recognized endogenous protein poorly.

Typical calibration curves for assays 15C463–71-13G1213–20 and 11D131–39-13G1213–20 and serial dilutions of human plasma samples are shown in Supplemental Fig. 1, A and B, respectively, in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol54/issue5. Assay 15C463–71-13G1213–20 was linear in the range of 15 to 100 000 ng/L and assay 11D131–39-13G1213–20 in the range 20 to 100 000 ng/L. The detection limits of both assays were 10 ng/L. For assay 15C463–71-13G1213–20, the CV was 5.4% at 100 ng/L and 1.926 ng/L concentrations and 2.8% at 10 ng/L. For assay 11D131–39-13G1213–20, the CV was 4.2% at low calibrator concentrations and 4.1% at 10 ng/L.

**EXTRACTION OF ENDOGENOUS NT-proBNP FROM POOLED PLASMA**

Because MAbs used to prepare affinity matrix were also able to recognize proBNP, proteins extracted from pooled plasma contained about 8% of proBNP (in comparison with NT-proBNP).

**IMMUNOCHEMICAL PROPERTIES OF ENDOGENOUS NT-proBNP BEFORE AND AFTER DEGLYCOSYLATION**

Samples of endogenous NT-proBNP extracted from pooled HF plasma, treated and not treated with the mixture of glycosidases, were quantified by 17 IFAs (Fig. 2). MAbs specific to the very N-terminal fragment of NT-proBNP molecule (amino acid residues 1–12) did not recognize endogenous NT-proBNP either before or after deglycosylation. MAbs 29D125–12 and 3H85–12 demonstrated significantly better recognition of endogenous NT-proBNP. NT-proBNP treatment with glycosidases resulted in a slight increase in signal (1.2-fold) for these 2 MAbs. Antibodies specific to fragments 13–24 and 63–76 were the most effective in recognizing endogenous NT-proBNP and were insensitive to deglycosylation. The signal increase after deglycosylation was on average 1.12-fold (range 0.99–1.25) greater for combinations using these MAbs. The most dramatic changes after deglycosylation were observed with MAb combinations utilizing one antibody specific to the mid-fragment of NT-proBNP (amino acid residues 28–56). As demonstrated in the preliminary experiments, such assays recognize endogenous NT-proBNP poorly. These MAb combinations were
able to recognize only about 7.5% (range 2.4% to 18.4%) of NT-proBNP immunoreactivity presented in the sample compared with the 15C463–71-13G1213–20 reference assay. After treatment with glycosidases, we observed a substantial increase of the signal, from 5.8- to 41-fold (mean value 19) for the assays with MAbs specific to the mid-fragment of the molecule. NT-proBNP concentration measured by these assays after deglycosylation was comparable with that measured by the reference assay.

GEL-FILTRATION STUDIES


Peak NT-proBNP immunoreactivity was detected by assay 15C463–71-13G1213–20 in fraction no. 19, corresponding to proteins with molecular masses of about 24.5 kDa. Deglycosylation of the protein resulted in the shift of the peak of immunoreactivity toward the proteins with smaller molecular masses, 17 kDa, fraction no. 21 (Fig. 3A). Assay 11D131–39-13G1213–20 was able to detect only minor traces of NT-proBNP before deglycosylation, with a smaller peak in fraction no. 20. After enzyme treatment, the signal in peak fractions increased 36-fold, and the maximum signal shifted to the next fraction (no. 21), as in the case of the 15C463–71-13G1213–20 assay (Fig. 3B). In both cases, the positions of the immunoreactivity maximum for the deglycosylated samples did not absolutely coincide with those established for recombinant NT-proBNP.

IMMUNOCHEMICAL DETECTION OF NT-proBNP EXTRACTED FROM INDIVIDUAL PLASMA SAMPLES BEFORE AND AFTER DEGLYCOSYLATION

We compared results of measurements of NT-proBNP extracted from the blood samples of 52 HF patients (before and after deglycosylation) by the Roche and HyTest in-house 11D131–39-13G1213–20 assays with results of HyTest in-house 15C463–61-13G1213–20 reference assay measurements (Fig. 4, A–F). Before deglycosylation of the samples, there was poor agreement between assays using one antibody specific to the central part of the molecule (assay 11D131–39-13G1213–20 or Roche assay) and assay 15C463–71-13G1213–20. NT-proBNP concentrations measured by assay 11D131–39-13G1213–20 were in some cases 40-fold lower than values obtained by assay 15C463–71-13G1213–20.

NT-proBNP concentrations determined in the samples by assay 11D131–39-13G1213–20 and the Roche assays increased after deglycosylation. In some cases, assay 11D131–39-13G1213–20 was able to detect up to 50-fold more NT-proBNP (range 3.1–53, mean 14.3) than before deglycosylation. Increase of the measurable concentrations for the Roche assay varied from 1.9- to 10.7-fold (mean 4.4). At the same time, assay 15C463–71-13G1213–20 was not so sensitive to deglycosylation—changes were not higher than 2.3-fold (mean 1.37). After deglycosylation, correlation between results of measurements by these 3 assays was substantially better.
Fig. 4. Correlation between results of measurements of NT-proBNP extracted from 52 individual HF patients plasma samples.

(Figs. 4B and D), with almost the same amounts of NT-proBNP detected in the same samples.

Discussion
We analyzed the interaction of different monoclonal antibodies with NT-proBNP from the plasma of HF patients. We showed that MABs specific to fragment 28–56, when used in sandwich immunoassay, detected only a minor portion (about 10%) of endogenous NT-proBNP measured by assay 15C463–71-13G1213–20, which was accepted as a reference (since it demonstrated equal response to recombinant and endogenous proteins). Concordant data have been published. Hughes et al. (2) demonstrated that antibodies specific to region 37–49 recognize endogenous NT-proBNP poorly in contrast to antibodies specific to epitope 65–76. Such observations suggested that endogenous NT-proBNP could be modified or complexed with other molecules in comparison with recombinant protein expressed in E. coli. This assumption is confirmed by the fact that endogenous NT-proBNP demonstrates different (higher) mobility in GF studies than recombinant protein (Fig. 3A). Recently, Schellenberger et al. (4) demonstrated that proBNP expressed in CHO cells is glycosylated and has several sites of O-glycosyl addition (Thr36, Ser37, Ser44, Thr48, Ser53, Thr58, Thr59). These investigators also demonstrated that endogenous proBNP was glycosylated (4). These observations led us to verify the hypothesis that endogenous NT-proBNP might also be glycosylated and that glycosylation affects the recognition of the antigen by antibodies.

After endogenous NT-proBNP was treated by a mixture of enzymes removing O-linked oligosaccharides, we observed a substantial increase in the response in the case of the pairs utilizing one of the antibodies specific to the fragment 28–56. These MAB pairs recognized both deglycosylated endogenous and recombinant NT-proBNP with almost the same efficiency. We conclude that glycosylation is the major reason that antibodies specific to the central region of NT-proBNP are unable to recognize endogenous protein. Enzymatic deglycosylation, by removing carbohydrates from the amino acid residues in the central part of endogenous NT-proBNP, makes antigen–antibody interaction possible. Deglycosylation did not improve the recognition of endogenous NT-proBNP by antibodies specific to the very N-terminal region of the molecule (epitope 1–12). This observation is in agreement with data described by Lam et al. (7), who reported that endogenous proBNP is truncated at the N-terminus, lacking 2 amino acid residues. Most likely, antibodies specific to this portion of the molecule were not able to recognize endogenous NT-proBNP because of its proteolytic degradation. MAB pairs with one of the antibodies specific to region 13–24 and another antibody specific to region 61–76 recognized endogenous NT-proBNP almost with the same efficiency before deglycosylation as after enzyme treatment, with only slight signal growth noted in deglycosylated specimen, on average 1.12-fold.

In GF-HPLC studies of endogenous protein, assay 15C463–71-13G1213–20 detected the peak of NT-proBNP immunoreactivity in the fraction corresponding to proteins with molecular masses of about 24.5 kDa. This finding was in agreement with our previous studies (6), as well as with other studies demonstrating that, in GF experiments, endogenous NT-proBNP has substantially higher mobility (higher molecular weight) than its recombinant counterpart. Assay 11D131–39-13G1213–20 recognized only a small amount of the NT-proBNP, with a peak value in the fraction corresponding to proteins of molecular mass of approximately 20 kDa, almost coincident with the peak of deglycosylated protein (Fig. 3B). These observations suggest that endogenous NT-proBNP protein contains a small portion (about 5%) of nonglycosylated or slightly glycosylated protein and that this portion can be detected by antibodies specific to the central part of the molecule. The fact that deglycosylation resulted in the shift of the NT-proBNP immunoreactivity peak, measured by assay 15C463–71-13G1213–20 toward the proteins with significantly lower molecular masses, and that immunoreactivity measured by assay 11D131–39-13G1213–20 in the sample after deglycosylation was 36-fold higher than in the case of untreated NT-proBNP, allows us to conclude that glycosylation is the major reason for the following: a) endogenous protein has apparent molecular mass higher than recombinant protein; and b) MABs specific to the mid-fragment of NT-proBNP are not able to recognize endogenous protein. Observed differences in apparent molecular masses between deglycosylated endogenous and recombinant NT-proBNP could be caused by remaining sugar residues or by other possible posttranslational modifications. Contamination of the NT-proBNP preparation by proBNP cannot explain this observation, owing to insignificant quantity of proBNP in the specimen (about 8% of NT-proBNP quantity).

To investigate how deglycosylation influences measurements of NT-proBNP between individuals, we analyzed NT-proBNP extracted from blood samples of 52 HF patients using 3 assays: 2 HyTest in-house assays, 15C463–71-13G1213–20 and 11D131–39-13G1213–20, described above, and the Roche Elecsys 2010 NT-proBNP assay. The Roche assay uses 2 polyclonal antibodies. The capture antibody recognizes peptide 1–21, whereas the detection antibody is specific to peptide 39–50, comprising possible sites of glycosylation (Ser44 and...
Ser$_{48}$) and known sites of proBNP glycosylation (Ser$_{37}$ and Ser$_{53}$) which are located very close to the epitope recognized by the Roche antibodies. Thus, the detection antibody of the Roche assay is similar to MAb 11D$_{31-39}$, specific to a part of the peptide sequence that includes 2 possible sites of glycosylation: Thr$_{36}$ and Ser$_{37}$ (4). Because the detection antibody of the Roche assay is specific to the fragment of the molecule affected by glycosylation, we assumed that the Roche assay should be also sensitive to glycosylation. As expected, the effect of sample deglycosylation was highly pronounced in the Roche assay (Fig. 4, D and F), but not as much as the 11D$_{31-39}$-13G$_{12-20}$ assay (Fig. 4, B and E). A possible explanation for the smaller changes in concentrations measured by the Roche assay before and after deglycosylation, in comparison with 11D$_{31-39}$-13G$_{12-20}$ assay, is that polyclonal antibodies used in the Roche assay could contain a population of antibodies recognizing small epitopes that do not contain glycosylation sites, and thus are not (or are only slightly) affected by glycosylation. We also observed that in both assays (11D$_{31-39}$-13G$_{12-20}$ and Roche), changes of measurable concentrations after deglycosylation varied across different blood samples (Fig. 4, E and F). In some cases, measured concentrations increased only 2- to 3-fold, in other cases 10-fold, in still others >20-fold. We conclude that NT-proBNP is glycosylated in all analyzed patients and that the level of glycosylation differs between individual patients. We further conclude that NT-proBNP assays using at least one antibody specific to the mid-fragment of the molecule seriously underestimate the real concentration of NT-proBNP in blood samples. The degree of error is unpredictable and depends on the amount of glycosylation of the site recognized by antibodies. The clinical consequence of such antigen underestimation is not clear yet, but could be important. Assay 15C$_{463-71}$-13G$_{12-20}$, with both MAb specific to the terminal parts of the molecule, was not as sensitive to deglycosylation of endogenous protein from individual blood samples. We observed only a small increase in signal after deglycosylation (mean 1.37-fold) (Fig. 4, B and E). Finally, we conclude that antibodies specific to the N- and C-terminal parts of the NT-proBNP molecule (but not to the very N-terminal) represent the best choices for the development of an optimal NT-proBNP assay.

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