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Glycosylation of NT-proBNP Molecules and NT-proBNP Immunoassays

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INTRODUCTION

Measurements of NT-ptoBNP (N-terminal part of the precursor of brain natriuretic peptide) in patients' blood are successfully used for heart failure (HF) diagnosis, assessment of left ventricular function and prognosis after myocardial infarction. However, though this analyte is used in clinical practice for a long time, biochemical and immunochemical properties of NT-proBNP molecule haven't been studied precisely. In recent studies Schellenberger et al. (2006) demonstrated, that proBNP in human blood is modified by glycosylation. Our studies of the biochemical properties of endogenous NT-proBNP molecule revealed that NT-proBNP in patients' blood is also modified by glycosylation. The aim of the present study was to determine the influence of glycosylation on NT-proBNP measurements in human blood by immunochemical methods.

HyTest

Materials and Methods

Monoclonal antibodies specific to human NT-proBNP were from HyTest (Finland).

Antigen: Recombinant human NT-proBNP (expressed in *E. coli*) and NT-proBNP peptides were also from HyTest.

Plasma samples from heart failure patients were pooled and stored at –70ºC until used.

Sandwich immunofluorescent assay (IFA). Capture antibodies (2 µg per well, 0.1 mL) were incubated in the wells of 96-well plates for 30 min at room temperature with gentle shaking. After washing, tested sample (0.05 mL) and detection antibodies (200 ng per well, 0.05 mL) labeled with Eu^{3+}-chelate - were added to the wells. The plates were incubated for 30 min at room temperature with gentle shaking and washed. After addition of the solution enhancing fluorescent signal, the mixture was incubated for 3 min at room temperature with gentle shaking. The fluorescence was measured on a Victor 1420 multilabel counter (Wallac-Perkin Elmer, Finland).

Endogenous NT-proBNP extraction from pooled plasma of heart failure patients was performed using affinity chromatography on a column containing immobilized monoclonal antibodies specific to different parts of NT-proBNP molecule.

Deglycosylation of NT-proBNP: Extracted NT-proBNP was incubated with endo-alpha-N-acetylgalactosaminidase (o-glycosidase) and alpha-3,6,8,9-neuraminidase (sialidase) in 75 mmol/l sodium phosphate buffer, pH 5.0 at 37ºC for 1 hour.

Results and Discussion

In our study we used twenty monoclonal antibodies (MAbs) (HyTest, Finland) specific to different parts of NT-proBNP molecule with epitopes covering the whole NT-proBNP sequence. 3 MAbs were specific to region 1-12, 2 MAbs - to region 5-12, 2 MAbs – to region 13-20, 3 MAbs – to region 13-24, 1 MAb – to region 28-45, 2 MAbs – to region 31-39, 1 MAb – to region 34-39, 3 MAbs – to region 46-56, 1 MAb – to region 63-71, 1 MAb – to region 67-73 and 1 MAb- to region 67-76 (Fig. 1). All antibodies were tested as capture with two forms of NT-proBNP - *recombinant*, expressed in *E. coli*, and *endogenous*, extracted by affinity chromatography from pooled plasma samples of HF patients. MAb 13G12 (epitope 13-20) and MAb 24E11 (epitope 67-76) were used as detection (labeled with stable Eu^{3+}-chelate).

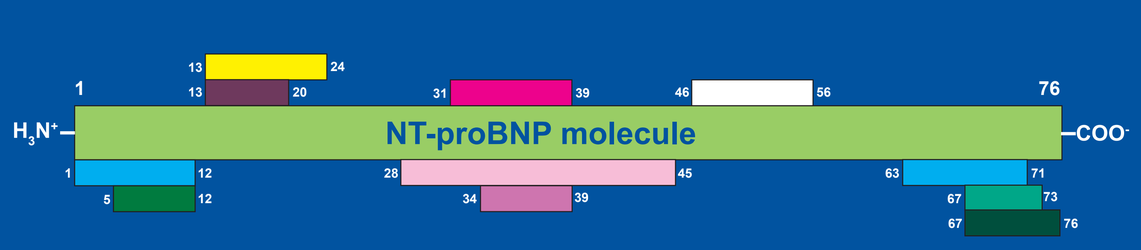

Figure 1: Epitope map of NT-proBNP-specific antibodies used in the study.

Two-site MAbs combinations were composed in the following way: capture MAbs with epitopes located in the region of NT-proBNP 1-24 were tested in pairs with detection MAb 24E11 (epitope 67-76); capture MAbs with epitopes located in the region of NT-proBNP 28-76 were tested in pairs with detection MAb 13G12 (epitope 13-20) (Fig. 2). All two-site MAbs combinations, utilizing antibodies with remote epitopes, were able to recognize *recombinant* antigen. But only few combinations gave response with *endogenous* protein. So, in the case when at least one MAb was specific to the region 1-12 or 28-60, two-site combination scarcely detected endogenous protein. Treatment of *endogenous* NT-proBNP with glycosydases, specific to O-type glycosylation, resulted in the significant growth of the signal in the case of MAbs, specific to the region 28-60 (Fig. 3). We concluded, that 28-60 fragment of endogenous NT-proBNP is heavily glycosylated and because of glycosylation MAbs specific to this region can't recognize endogenous protein.

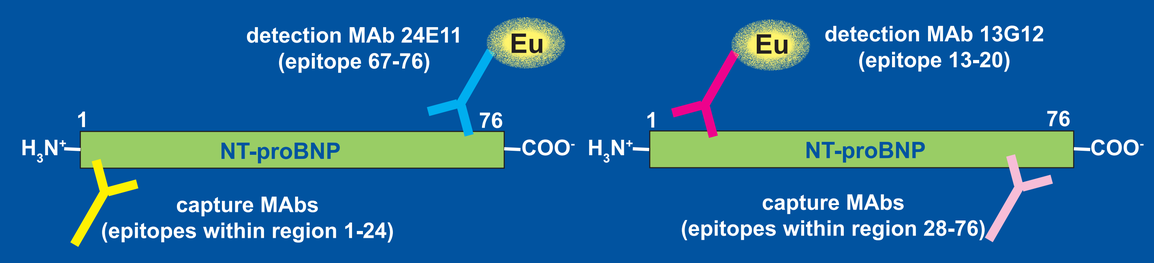

Figure 2: Schematic sketch of two-site MAbs combinations used in the study.

In the case while both MAbs utilized in two-site combination were specific to the region 5-24 or 63-76 there was almost no difference in the recognition of native and deglycosylated endogenous NT-proBNPs. Most likely these two regions are not glycosylated and antibodies specific to these fragments of NT-proBNP molecule are the best candidates for the development of the precise NT-proBNP immunoassay. Deglycosylation did not improve the recognition of the endogenous NT-proBNP by MAbs specific to the region 1-12. We assumed that this region (the very N-terminal part of it) of endogenous antigen is unstable and could be partially cleaved by proteases. This suggestion is proved by our stability studies of endogenous antigen (data not shown).

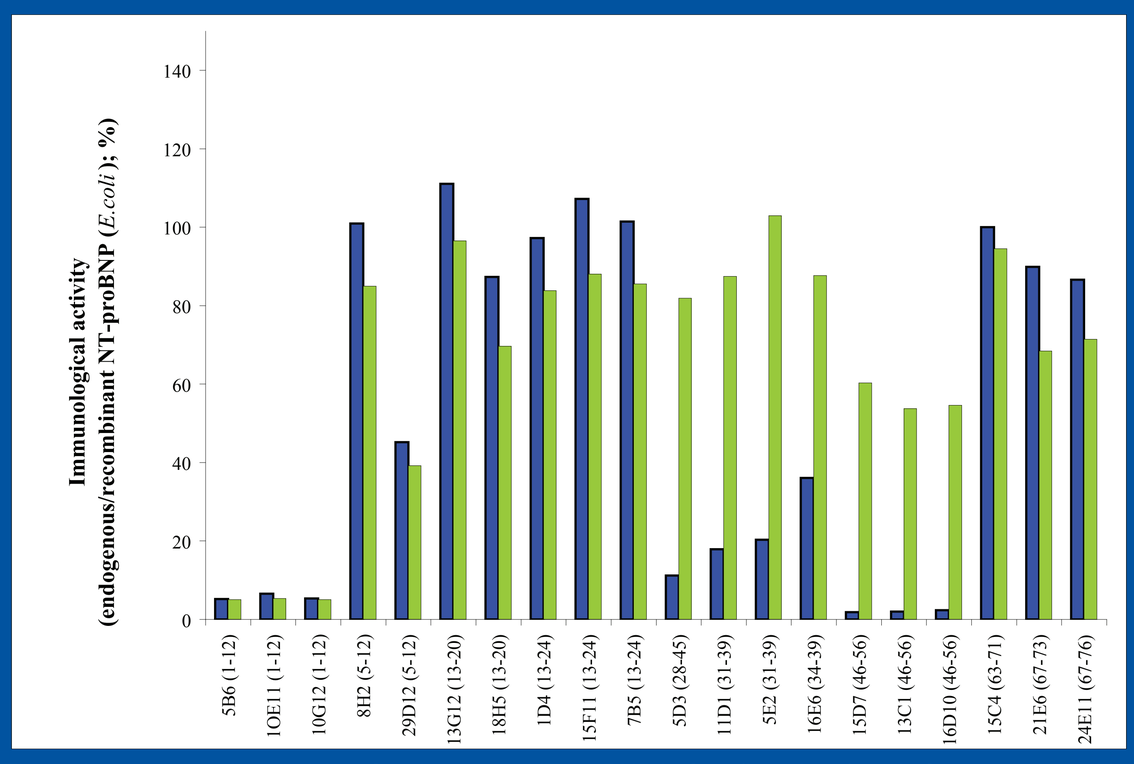

Figure 3: Immunological activity of endogenous NT-proBNP before (blue bars) and after treatment with o-glycosidase and sialidase (green bars). The results are presented as ratio of immunological activity of endogenous NT-proBNP to immunological activity of recombinant NT-proBNP (expressed in *E. coli,* not glycosylated) used in the same concentration as endogenous antigen and measured by different MAbs pairs. Names and epitope specificity (in brackets) of capture MAbs are represented below the X-axis.

Conclusions

We have shown that glycosylation of the mid fragment of *endogenous* NT-proBNP molecule greatly influences the recognition of the antigen by the antibodies specific to this region. We suggest here that this fact should be considered while selecting antibodies to be utilized in new generation of precise NT-proBNP immunoassays.