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Investigation of immunochemical and biochemical properties of circulating IGFBP-4 fragments – novel biomarkers for cardiac risk assessment

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Introduction

The dimeric form of the zinc metalloproteinase Pregnancy Associated Plasma Protein-A (dPAPP-A) is overexpressed in vulnerable atherosclerotic plaque and is associated with the plaque destabilization [1]. Insulin-like growth factor-binding protein 4 (IGFBP-4) was described as one of dPAPP-A substrate molecules. It was shown that dPAPP-A is able to specifically cleave IGFBP-4, forming the N- and C-terminal fragments (NT- and CT-IGFBP-4; Fig. 1 and 2). Recent findings [2] show that NT- and CT-IGFBP-4 can be utilized as biomarkers for the prediction of major adverse cardiac events in patients with suspected acute coronary syndrome (ACS). It was suggested that increased NT- and CT-IGFBP-4 levels in patients' blood is associated with increased expression and proteolytic activity of dPAPP-A and thus with atherosclerotic plaque destabilization. Novel NT- and CT-IGFBP-4 immunoassays utilizing monoclonal antibodies specific to proteolytic neo-epitopes originated by PAPP-A dependent cleavage of IGFBP-4 were described (Fig. 2) [2]. However the level of truncation or degradation of NT- and CT-IGFBP-4 released into the circulation has not been investigated yet. The degraded or truncated forms of circulating IGFBP-4 fragments do not contain the native neo-epitopes. By this reason NT- and CT-IGFBP-4 cannot be detected by IGFBP-4 fragments immunoassays utilizing neo-epitope specific antibodies. The goal of our study was to investigate biochemical properties, the level of degradation and truncation of NT- and CT-IGFBP-4 in blood.

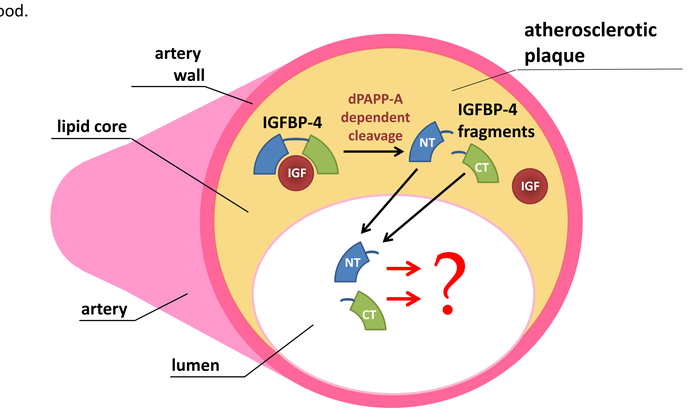

Figure 1. The scheme of dPAPP-A dependent cleavage of IGFBP-4 in the atherosclerotic plaque. The cleavage leads to two proteolytic fragments formation. The fragments are released from atherosclerotic plaque and can be identified in blood. dPAPP-A cleaves IGFBP4 in the high-specific site between M135 and K136. The consequent state of NT- and CT-IGFBP-4 in blood is still unrevealed.

Materials and methods

Affinity purification of endogenous NT- and CT-IGFBP-4 from human ACS plasma. Mouse monoclonal antibodies IBP180 and IBP185 (HyTest, Finland) were used for immunosorbents preparation. The extraction of endogenous NT- and CT-IGFBP-4 was realized utilizing pooled human plasma collected from 15 individual ACS patients.

Sandwich immunoassays. The concentrations of purified NT- and CT-IGFBP-4 were measured using sandwich assays specific to the epitopes located distantly from the ends of each protein, and using recombinant NT- and CT-IGFBP-4 expressed in human cell line (HyTest, Finland) as calibrators.

Immunochemical activities of endogenous and recombinant NT- and CT-IGFBP-4 were compared using fragment-specific sandwich immunoassays IBP3-IBP180^{Eu} and IBP182-IBP163^{Eu} (Fig. 2), respectively. MAbs IBP3 and IBP163 are specific to the neo-epitopes of NT- and CT-IGFBP-4, respectively, and do not cross-react with full-length IGFBP-4 as well as with truncated neo-epitopes [1]. The native state of the standard proteins' neo-epitopes was demonstrated using MALDI mass-spectrometry analysis (Fig. 3).

Western blotting. Extracted proteins were analyzed by WB using MAbs IBP3, IBP180 (NT-IGFBP-4), IBP163 and IBP185 (CT-IGFBP-4).

Size-exclusion chromatography on Superdex 75 column was utilized for characterization of IGFBP-4 fragments in ACS patients' plasma.

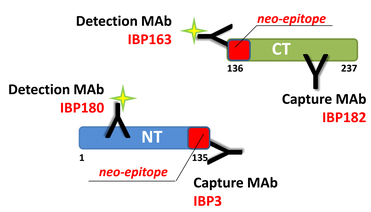

Figure 2. Schematic representation of the IGFBP-4 fragments specific immunoassays utilizing MAbs to the neo-epitopes of NT- and CT-IGFBP-4. These assays have no cross-reaction to full-length IGFBP-4 as well as to the fragments with truncated neo-epitopes [2].

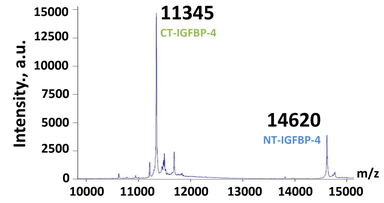

Figure 3. Mass-spectra of recombinant NT-IGFBP-4 and CT-IGFBP-4. The molecular masses of the proteins are identical to calculated masses of NT-IGFBP-4 (14628 Da) and CT-IGFBP-4 (11347 Da). These data suggest that the recombinant proteins contain non-truncated neo-epitopes and can be used as calibrators in the fragments specific sandwich immunoassays.

Results and discussion

In Western blotting studies (Fig. 5) NT- and CT-IGFBP-4 bands had the same apparent molecular masses as their recombinant analogues' bands: 18 and 14 kDa, respectively. Endogenous NT- and CT-IGFBP-4 were detected using core- (IBP180 and IBP185) and neo-epitope-specific MAbs (IBP3 and IBP163, respectively). Thus we can suggest that the extracted endogenous NT- and CT-IGFBP-4 fractions contain the products of the proteolytic activity of PAPP-A, and do not contain measurable amounts of degraded fragments.

Mass-spectrometry studies of endogenous NT- and CT-IGFBP-4 (Fig. 4) confirmed the data obtained by WB analysis revealing the only one peak for both proteins corresponding to the products of dPAPP-A dependent cleavage of IGFBP-4 (14615 and 11349 Da, respectively; accuracy of mass measurements <0.05%). Peaks corresponding to the truncated forms of NT- and CT-IGFBP-4 were not detected.

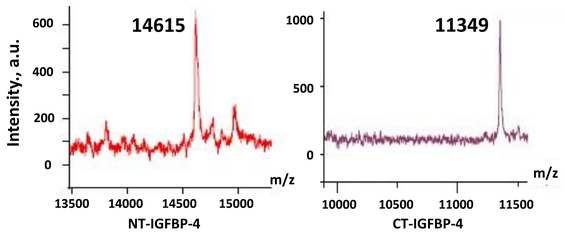

Figure 4 (↑). The mass-spectra of endogenous NT-IGFBP-4 and CT-IGFBP-4 purified from human plasma.

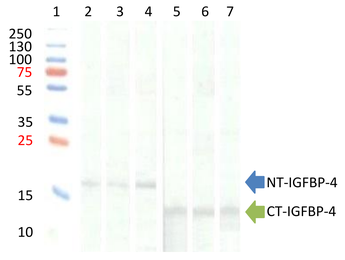

Figure 5 (↑). Western blotting of endogenous NT- and CT-IGFBP-4 purified from human plasma. Lanes: 1 – molecular mass standard, 2 – recombinant NT-IGFBP-4, IBP180 stained; 3 – endogenous NT-IGFBP-4, IBP180 stained; 4 – endogenous NT-IGFBP-4, IBP3 stained; 5 – recombinant CT-IGFBP-4, IBP185 stained; 6 – endogenous CT-IGFBP-4, IBP185 stained; 7 – endogenous CT-IGFBP-4, IBP163 stained.

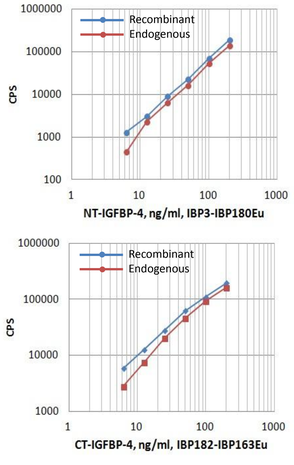

Figure 6 (→). Comparison of immunochemical activities of recombinant (blue) and endogenous (red) NT- and CT-IGFBP-4 using fragments specific sandwich immunofluorescent assays. The similar slopes of the curves reflect the similar immunochemical properties of recombinant and endogenous proteins.

Immunochemical activities of recombinant and endogenous NT- and CT-IGFBP-4 were similar (Fig. 6). These data demonstrate that endogenous NT- and CT-IGFBP-4 are presented by non-truncated proteolytic fragments.

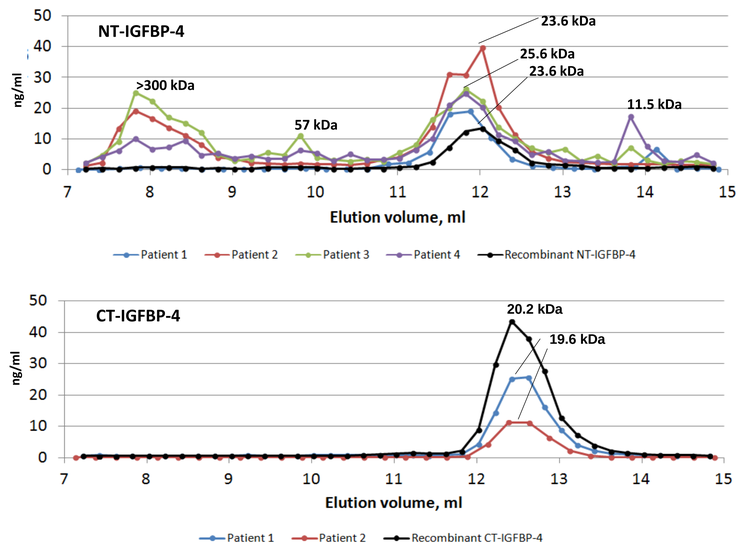

Figure 7. Size-exclusion chromatography of individual ACS plasma samples. Immunochemical activities of NT-IGFBP-4 and CT-IGFBP-4 were identified by IBP3-IBP180^{Eu} and IBP182-IBP163^{Eu} sandwich immunoassays, respectively. Apparent molecular weights of the peaks are indicated.

Size-exclusion chromatography analysis of individual plasma samples of ACS patients using fragment-specific immunoassay IBP3-IBP180^{Eu} revealed several peaks of NT-IGFBP-4 immunochemical activity (Fig. 7). The main peak (23.6 – 25.6 kDa, 70 – 80% of immunochemical activity) corresponded to the position of the peak of recombinant NT-IGFBP-4 (23.6 kDa). Several additional minor peaks (11, 57, and >300 kDa) of NT-IGFBP-4 immunochemical activity were also identified. Immunochemical activity in these minor peaks varied significantly (up to 30%) in individual samples. The presence of these NT-IGFBP-4 forms could reflect partial degradation from the N-terminal site (low molecular weight peaks) as well as formation of higher molecular weight complexes with other proteins in human plasma (high molecular weight peaks).

For CT-IGFBP-4 the single peak of immunochemical activity was identified using fragment-specific immunoassay IBP182-IBP163^{Eu} (Fig. 7). Its apparent molecular mass was very close to that of corresponding recombinant fragment.

Conclusions

Here we for the first time describe the endogenous NT- and CT-IGFBP-4 from ACS patients' blood. Both proteins display the biochemical and immunochemical features similar to the recombinant proteins expressed in mammalian cell lines.

References

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