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Sandwich immunoassay prototypes for human LMW adiponectin and for complex of adiponectin with serum albumin

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Introduction

Adiponectin is a protein hormone that belongs to a family of adipokines. Adiponectin is produced by adipocytes and is important regulator of lipid and glucose metabolism. Decreased serum adiponectin might serve as a predictor of future type 2 diabetes mellitus (T2DM) and cardiovascular disorders. Human monomeric adiponectin consists of 244 amino acid residues and has distinct domain structure: it contains both collagen-like and globular C1q-like domains. Native adiponectin exists in the blood stream as a mixture of oligomers: trimers (low-molecular weight form, LMW), hexamers (medium molecular weight form, MMW) and higher order multimers (high molecular weight form, HMW). It is believed that those oligomeric forms exist in the bloodstream as a separate moieties and do not convert into each other. Information about a ratio of oligomeric forms in human blood, about their involvement in Adn hormonal activity and about their possible utilization as markers is contradictive and incomplete. We have developed of a panel of adiponectin-specific monoclonal antibodies (MAbs) and by using such Mabs have studied different Adn forms as well as their possible utilization as diagnostic markers of T2DM.

Materials and methods

Native adiponectin and anti-HSA MAb HSA20 were obtained from HyTest Ltd.

Monoclonal antibodies

Native adiponectin, containing all three adiponectin forms, was used as an antigen for Balb/c mice immunization. Splenocytes of immune animals were fused with sp 2/0 myeloma cells. Antibody-producing hybridoma cells were subjected to positive selection using purified adiponectin and negative selection using complement component C1q, adiponectin closest structural homolog. Selected MAbs were labeled with stable Eu^{3+} chelate and used as detection antibodies in two-site combinations with unlabeled MAbs, to find best combinations for adiponectin sandwich immunofluorescent assay.

Sandwich immunofluorescent assay (IFA)

Capture antibodies, 1 µg per well in 100 µL of PBS, were incubated in immunoassay plates for 30 min at room temperature. After washing, 50 µL of tested sample or calibrator and 50 µL of detection antibodies labeled with stable europium (Eu^{3+}) chelate in assay buffer were added. After 30 min incubation the plates were washed, then enhancement solution was added, and fluorescence was measured.

Size-exclusion chromatography

1.2 ml of normal human serum were applied onto Superdex 200 26/60 column and proteins were eluted with phosphate-buffered saline (10 mM K-phosphate, pH 7.4, 150 mM NaCl). 1.2 ml fraction were collected. Immunoreactivity in the fractions was measured using sandwich immunofluorescent assay with coating and detection MAbs as specified in the legend to figures.

Serum samples

Venous blood from healthy volunteers (n=37) and T2DM patients (n=69) was collected into sterile Vacuette tubes and incubated at RT for 1 hour. Samples were further centrifuged at 3000g for 15 minutes at 4ºC. Serum samples were stored -20 until use. T2DM was diagnosed as fasting glucose level greater than 7 mmol/L.

Results and discussion

Using standard hybridoma technology and native human adiponectin as an immunogen we'd raised 11 MAbs specific to human adiponectin. Those MAbs showed no cross-reactivity to C1q, closest structural homolog of adiponectin (results not shown). All MAbs were tested in sandwich IFA as capture and detection (labeled with stable Eu^{3+} chelate) antibodies. Several of MAb combinations showed good linearity when probed with purified human adiponectin (Fig. 1). MAb pairs Adn94-Adn63Eu and Adn214-Adn27Eu demonstrated excellent linear titration curve when normal human serum was used as a source of antigen (data not shown).

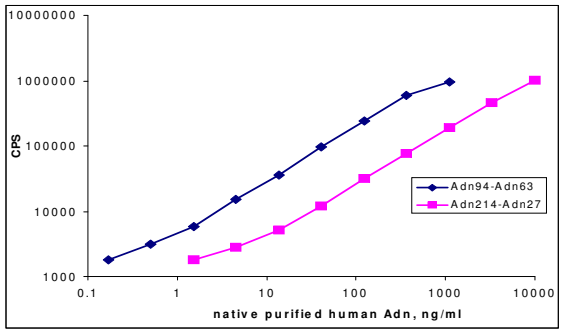

Fig. 1 Calibration curve for sandwich IFAs.
MAbs Adn94 or Adn214 MAbs were used as a capture (1 µg/well), whereas MAbs Adn63 or Adn27 were labeled with stable Eu^{3+} chelate and were used as a detection antibodies (0.2 µg/well). Native Adn isolated from normal human serum was used as a calibrator.

The latter observation could be interpreted as ability of given MAb pairs to interact with adiponectin in the highly complex protein mixtures. Assays utilizing MAb pairs Adn94-Adn63Eu and Adn214-Adn27Eu could be used for adiponectin measurements in human serum

To further examine specificity of obtained MAbs, adiponectin oligomers in normal human serum were separated by means of size-exclusion chromatography and fractions were tested for adiponectin immunoreactivity by two-site MAbs combinations utilizing all obtained Mabs as capture or detection antibodies. It was found that assay Adn94-Adn63Eu was able to detect in fractions three peaks of immunoreactivity (Fig. 2) - first peak of immunoreactivity with highest MW representing HMW form of adiponectin, second peak of immunoreactivity – MMW form of protein (hexamers) and third peak – LMW form of adiponectin (trimers). Apparent molecular masses of different oligomeric forms of adiponectin (510 kDa for HMW form of adiponectin, 310 kDa for MMW form of adiponectin and 178 kDa for LMW form of adiponectin) were in good agreement with published data of Nakano et al (1).

It was shown that assays Adn214-Adn27Eu recognizes predominantly LMW, as well as interacts with proteins eluting slightly earlier than LMW Adn (Fig.2, blue line). Since it was demonstrated earlier (2) by Hada et. al., that part of the LMW form of Adn may form complexes with human serum albumin (HSA) during Adn isolation from serum, we hypothesized that smaller-size immunoreactivity peak detected with assay Adn214-Adn27Eu represents a complex of LMW Adn with HSA. To check this hypothesis, immunoreactivity in the same fractions was tested with assay Adn214-HSA20Eu in which MAb HSA20 is a HAS-specific antibody, and such assay detects only LMW Adn-HSA complex (Fig 2, green line). As it could be seen from Fig 2, peak of immunoreactivity, detected by Adn214-Adn27Eu assay coincided with the smaller peak detected by assay Adn214-Adn27Eu. It was concluded that LMW adiponectin – BSA complex exists in human blood and it can be detected by the assay Adn214-HSA20Eu.

To check applicability of LMW Adn measurement for diagnosing of T2DM we'd measured LMW Adn as well as total Adn and LMW-Adn-HSA complex levels in serum samples of healthy donors (n=37) and T2DM patients (n=69). It was shown that LMW Adn and LMW Adn-HSA complex levels were decreased in the blood of T2DM patients (2.9±0.8 µg/ml vs 3.3±0.7 µg/ml for LMW Adn and 6.9±1.0 artificial units, a.u. vs. 9.1±2.6 a.u. for LMW Adn-HSA complex; T2DM patients vs. healthy donors, respectively). Total Adn levels measured with assay Adn94-Adn63, were also lower in T2DM patients than in healthy donors (9.3±8.5 µg/ml vs 13.0±8.6 µg/ml, respectively).

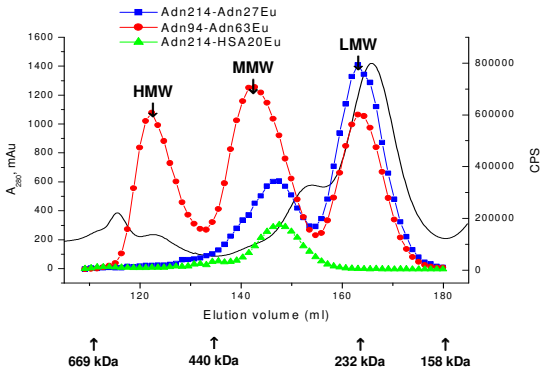

Fig. 2 Adiponectin immunoreactivity in the proteins fraction after serum separation by size-exclusion chromatography.

Immunoreactivity was measured with: Adn94-Adn63Eu MAb assay (red line), Adn214-HSA20Eu MAb assay (green line), Adn214-Adn27Eu MAb assay (blue line). Black line indicates absorbance at 280 nm. Molecular weight markers positions are indicated.

Since assay Adn214-Adn27 was shown to detect both LMW Adn and LMW Adn-HSA complex and it is unclear at the moment which part of LMW Adn is engaged in the complex formation, we cannot conclude that it is only LMW form of Adn level that is changed in T2DM patients comparing to healthy controls. Most likely, both LMW Adn and LMW Adn-HSA complex levels are reduced in serum of T2DM patients.

Conclusions

1. **Panel of monoclonal antibodies specific to different forms of human adiponectin was obtained. Some of these antibodies could be used for the immunodetection of different adiponectin forms in human blood.**
2. **We've demonstrated that LMW Adn-HSA complex exists in human serum.**
3. **Small-scale clinical studies have shown reduction of LMW Adn and/or LMW Adn-HSA levels in serum of type 2 diabetes patients in comparison with healthy donors.**

References

1. *Nakano et al., J. Lipid Research, 2006, 47, 1572-1582*
2. *Hada et. al., Biochem. Biophys.Res.Commun., 2007, 356(2), 487-493.*

For further information

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