AACC Annual Meeting, July 29, 2008, Washington, DC, Poster B-122

Development of sandwich immunoassays for measuring total and high-molecular weight human adiponectin

Vladimir L. Filatov (1), Alexander E. Kogan (2), Olga V. Kolosova (3), Tatiana I. Solovyova (1), Ivan A. Katrukha (2), Andrey N. Kara (2), Alexey G. Katrukha (1).

1- HyTest LTD, Turku, Finland; 2 – Department of Biochemistry, Moscow State University, Moscow, Russia; 3 – Moscow Institute of Medical Ecology, Moscow, Russia.

Introduction

Adiponectin is a protein hormone that belongs to a family of so-called 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 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 occurs 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. Most of the biologic action of adiponectin is mediated by high-molecular weight form. It has been established recently, that concentration of HMW form of adiponectin or ratio HMW/total adiponectin(sum of three types of oligomers) in serum correlates better then total adiponectin with measures of type 2 diabetes (1). We aimed at raising anti-adiponectin monoclonal antibodies (MAbs) suitable for measuring total and high-molecular weight form of adiponectin in human serum and at designing prototype immunoassay suitable for measuring adiponectin concentration in human serum.

Materials and methods

Native adiponectin was obtained from HyTest Ltd.

Sandwich immunofluorescent assay (IFA) *(Fig.1)*
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 (3+) chelate in assay buffer were added. After 30 min incubation the plates were washed, then enhancement solution was added, and fluorescence was measured.

MAbs raising
Native adiponectin isolated from normal human plasma was used as an immunogen to stimulate immune response in Balb/c mice. B-lymphocytes from immune mice were fused with sp 2/0 myeloma cells. Antibody-producing hybridoma cells were subjected to positive selection on purified adiponectin and negative selection on complement component C1q, adiponectin closest structural homolog. MAbs selected were labeled with stable Eu^{3+} chelate and pairs combinations were tested in sandwich immunofluorescent assay

Size-exclusion chromatography
1.2 ml of normal human serum was 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 on the elution. Immunoreactivity in the fractions was measured using sandwich immunofluorescent assay with coating and detection MAbs as specified in the legends to figures.

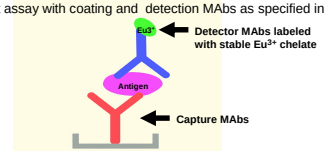

Fig.1. Schematic depiction of sandwich immunofluorescent assay

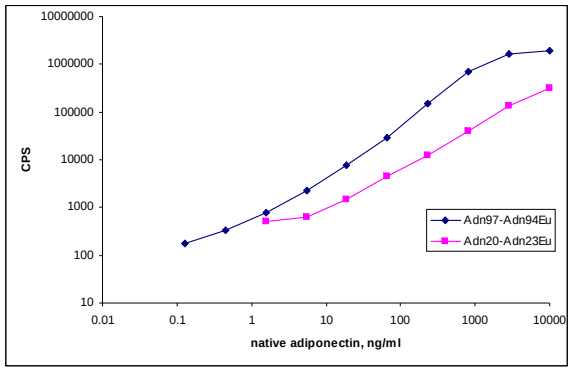

Fig. 2. Calibration curve for measuring adiponectin concentration.
Adn97 or Adn20 MAbs were used as a coating, Adn94 or Adn23 were labeled with stable Eu3+ chelate and were used as a detector.

Results and discussion

Using standard hybridoma technology and native human adiponectin as an immunogen we'd raised a panel of MAbs specific to human adiponectin. Those MAbs showed no cross-reactivity to C1q, closest structural homolog of adiponectin (results not shown). To test those MAbs in sandwich immunofluorescent assay, we labeled them the with stable Eu3+ chelate. Several of MAb pairs showed good linearity when probed with purified human adiponectin (Fig. 2). The same MAb pairs demonstrated excellent linear titration curve when normal human serum was taken as an antigen (Fig. 3). The latter observation could be interpreted as ability of given MAb pair to interact with adiponectin in the highly complex protein mixtures. This observation led us to conclusion that MAb pairs Adn20-Adn23Eu and Adn97-Adn94Eu are suitable for adiponectin measuring in human serum.

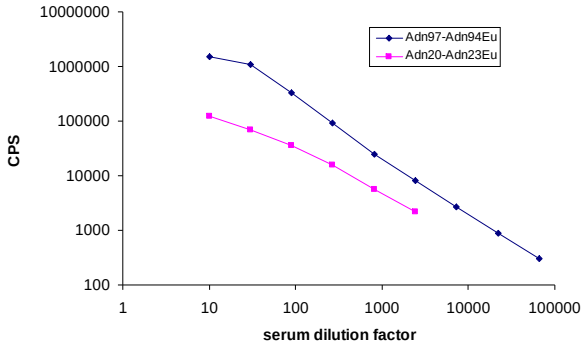

Fig. 3 Normal human serum titration in sandwich immunofluorescent assay. Adn97 or Adn20 MAbs were taken as a coating antibodies, Adn94Eu and Adn23Eu, respectively, were taken as a detector. Normal human serum, serially diluted with phosphate-buffered saline (10 mM K-phosphate, pH 7.4, 150 mM NaCl, 0.1% Tween-20), was used as an antigen.

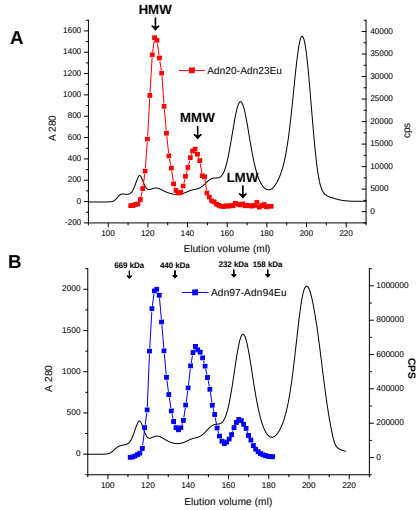

Fig.4 Immunoreactivity in protein fractions after size-exclusion chromatography, measured with MAb pair Adn20-Adn23Eu (A) and with MAb pair Adn97-Adn94Eu (B) Positions of oligomeric forms of adiponectin and molecular weight markers are depicted on the picture. Black line is optical density at 280 nm.

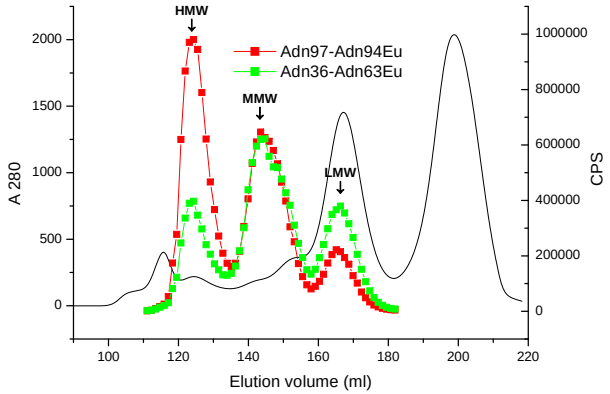

Fig. 5 Immunoreactivity in the proteins fraction after serum separation by size-exclusion chromatography measured with Adn97-Adn94Eu MAb pair (red line) and Adn36-Adn63Eu MAb pair (green line). Black line is optical density at 280 nm.

To further examine specificity of our MAbs, we'd separated adiponectin oligomers in normal human serum by means of size-exclusion chromatography and tested fractions from chromatography column for immunoreactivity in certain MAbs pairs. It turned out that pair Adn97-Adn94Eu demonstrated three peaks of immunoreactivity (Fig. 4) whereas pair Adn20-Adn23Eu was capable of detecting only two peaks of immunoreactivity in human serum fraction after size-exclusion chromatography. We assumed that first peak of immunoreactivity with highest MW represents HMW form of adiponectin, second peak of immunoreactivity – MMW form of protein (hexamers) and third peak – LMW form of adiponectin (trimers). Our data about 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) are in good accordance with recently published data of Nakano et al (2). Therefore, we've selected MAbs pair Adn20-Adn23Eu which predominantly recognizes HMW and, to a lesser extent, MMW forms of adiponectin. Another selected MAb pair Adn97-Adn94Eu, interacts with all three oligomeric forms of adiponectin and therefore capable of measuring total adiponectin in human serum.

Intriguingly, testing the same protein fractions after size-exclusion chromatography with pair Adn97-Adn94Eu as well as another pair Adn36-And63Eu showed that both of those pairs recognize all three oligomeric forms of adiponectin, but relative intensity of immunoreactivity is different (Fig. 5). MAb pair Adn97-Adn94Eu interacts with HMW form of adiponectin more readily then with other two forms while MAb pair Adn26-Adn63Eu interacts most strongly with MMW form of adiponectin. It is evident that ratio of peaks intensity doesn't necessarily reflect mass ratio of oligomeric forms. This phenomenon might mean that results of adiponectin forms measurement in human serum by means of immunoassay should be interpreted with caution. Issue of different forms of adiponectin ratio in human serum definitely needs further research

Conclusions

1. **We've raised a panel of monoclonal antibodies specific to human adiponectin. Several of MAbs when combined in immunofluorescent assay were capable of detecting human adiponectin in serum with good sensitivity.**
2. **We've selected MAb pair Adn20-Adn23Eu capable of detecting primarily HMW oligomeric form of adiponectin. Another MAb pair selected, Adn97-Adn94Eu was shown to interact with all three oligomeric forms of adiponectin – total adiponectin.**
3. **Anti-total adiponectin MAb pairs recognize different oligomeric forms of adiponectin with varying specificity**

References
1. Fisher et al., Diabetologia, 2005, 48, 1084-1087
2. Nakano et al., J. Lipid Research, 2006, 47, 1572-1582

For further information

Phone: +358-2-512-09-00
Fax: +358-2-512-09-09

hytest@hytest.fi
www.hytest.fi

HyTest