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Research paper

Development of monoclonal antibodies for detection of Antisecretory Factor activity in human plasma ☆

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ABSTRACT

Antisecretory Factor (AF) is expressed in most tissues and can be demonstrated in plasma and other body fluids. Most of the AF in plasma is in an inactive form and activation of AF occurs after exposure to bacterial toxins or after intake of various dietary components. Patients with chronic diseases involving disturbances in inflammatory and secretory processes may benefit from an AF-inducing diet. The aim of the present study was to develop an *in vitro* assay for the analysis of AF-activity in human plasma. Monoclonal antibodies were raised against a native form of AF prepared from human placenta. Nine clones of the monoclonal antibodies recognizing AF and AF peptides were identified. With the aid of these antibodies, we developed a sensitive ELISA method for direct detection of AF-activity in human plasma. The AF activity in plasma from five healthy volunteers was low, 0.112±0.022 (absorbance at 405 nm), before intake of the AF-inducing diet with the SPC-Flakes®, and increased significantly ($p<0.05$) to 0.444±0.068 after ≥6 weeks on the diet. A comparison of the plasma-AF values, obtained by the bioassay and the immunogenic assay (indirect ELISA), shows that there is a significant correlation ($r=0.85$) between the values from the two methods. The results indicate that the ELISA measures AF-activity and has the potential to be an important tool for the analysis of AF-activity in further clinical studies on AF-therapy.

1. Introduction

Antisecretory Factor (AF) is a 41 kDa protein with potent antisecretory and anti-inflammatory activity expressed in most tissues (Johansson et al., 1995; Lange et al., 1999; Tateishi et al., 1999; Lange and Lönnroth, 2001; Davidson and Hickey, 2004). AF, also named S5a, is a component of the proteasome complex, but is also present in the free form (Ferrell et al., 1996). AF is secreted to and present in plasma and other tissue fluids, probably in a biologically inactive state in healthy individuals. Exposure to bacterial toxins induces activation of plasma AF, probably reflecting a natural defense mechanism to agents causing diarrhea, thereby contributing to a favorable clinical outcome and disease termination (Lönnroth et al., 2003).

Dietary intake of specially processed cereals (SPC-flakes®) can also increase AF-activity (Björk et al., 2000). Clinical studies have shown that SPC®-induced AF-activity is beneficial in the treatment of diseases in which inflammation and/or secretory imbalance are important parts of the pathophysiology, such as inflammatory bowel disease, enterotoxic diarrhea and Meniere's disease (Björk et al., 2000; Finkel et al., 2004; Zaman et al., 2007; Hanner et al., 2004).

In our previous experimental and clinical studies, we estimated AF activity by an *in vivo* assay, based on graded inhibition of cholera toxin induced hyper secretion in ligated loops of the rat small intestine (Lange, 1982). This method is reliable and

Abbreviations: AF, Antisecretory Factor; rAF, recombinant expressed AF; SPC, specially processed cereals; PBS, phosphate buffered saline; SDS-PAGE, sodium dodecyl sulfate-poly acryl amide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; AP, alkaline phosphatase; i.p., intraperitoneally; CNS, central nervous system; ENS, enteric nervous system; mAb, monoclonal antibody; BSA, bovine serum albumin; HRP, horseradish peroxidase; DAB, 3,3′-diaminobenzidine.

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reproducible, but is labor intensive, technically difficult, and furthermore, it consumes a large number of experimental animals. In order to evaluate the efficiency of AF-inducing treatment, it is necessary to perform extensive clinical studies with frequent sampling and measurement of AF activity. Consequently, it is of high priority to develop a reliable *in vitro*-assay for activated AF.

We have raised a number of polyclonal antibodies against AF and AF-peptides (Jennische et al., 2006) directed against linear epitopes of the AF protein. These antibodies serve as useful tools for the analysis of total AF when using immunoblotting and immunohistochemistry techniques. They do not however discriminate between the biologically inactive and activated forms of AF.

In the present study, we describe the preparation and characterization of monoclonal antibodies (mAbs) which we raised against a native and active form of AF prepared from human placenta. We used these antibodies in an enzyme-linked immunosorbent assay (ELISA) which selectively detects the biologically active form of AF.

2. Materials and methods

2.1. Animals

The rats for the activity test and the mice for immunization and production of mAbs were kept under approved standard conditions for experimental animals. All operative procedures were performed under isofluran anesthesia. The Animal Ethical Committee at the University of Gothenburg approved the experimental procedures described below.

2.2. Northern dot-blot analysis

To define tissues with high AF expression before antigen preparation, a human mRNA master dot-blot array (Clontech), containing human poly(A)$^+$ RNA from various adult and fetal human tissues, was pre-hybridized and hybridized in ExpressHyb™ Hybridization solution (Clontech) at 65 °C for the recommended time schedule. The filter was probed with an amplified 315-bp cDNA fragment from the 5′ end of the coding sequence of AF which was labeled with [α-^{32}P]dCTP, using the ready-prime DNA labeling system (GE Healthcare Bio-sciences AB) according to the manufacturer's protocol. After washing as recommended by the manufacturer, we exposed the blot to X-ray film at −70 °C for the appropriate time-period. The blot was scanned using a densitometer Phosphorimager (Molecular Dynamics), and the signals were quantified using an ImageQuant software (Molecular Dynamics). Values were given relative to the highest expressing tissue, arbitrarily set to 100. According to the results obtained, human placenta was chosen as the most suitable tissue for the preparation of immunogen and the production of monoclonal antibodies to AF.

2.3. Preparation of AF-immunogen using immuno-affinity chromatography

The rabbit polyclonal antiserum aM2, directed to the residue 326–335 at the C-terminal part of the AF protein (Jennische et al., 2006), was precipitated with caprylic acid and ammonium sulfate. Thereafter followed dialysis in 0.1 M $NaHCO_3$, before the coupling to CNBr-activated Sepharose 4B (8 mg of antibodies/ml of gel), as recommended by the manufacturer (GE Health care). To block the unbound activated sites, 0.1 M Tris–HCl (pH 8.0) was added to the mixture. Unbound proteins were removed by washing the gel with cycles of alternating pH, using 0.1 M acetate buffer (pH 4.0) followed by 0.1 M Tris–HCl (pH 8.0), both containing 0.5 M NaCl.

Human placenta was collected *post partum* at Sahlgrenska University hospital, Gothenburg. Phosphate buffered saline (PBS) was added to the tissue at a 1:8 w:v ratio, supplemented with a protease inhibitor cocktail (Roche Diagnostics), and the mixture was immediately frozen in liquid nitrogen before storing at −80 °C until use. After thawing, we homogenized the placenta sample in a glass homogenizer at 0 °C, then centrifuged it at 14,000 ×*g* for 5 min and the supernatant was collected. The placenta supernatant was then loaded onto the aM2-coupled Sepharose column. After washing in PBS, the bound antigens were eluted with 3 M NaSCN and dialyzed against PBS. The protein content was determined by measuring optical density at 280 nm, before storing at −20 °C in aliquots to be used as immunogen.

2.4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot

We assayed the content of AF in the placenta supernatant and eluted sample by SDS-PAGE followed by immunoblotting to a nitrocellulose membrane, as described previously (Johansson et al., 1995). The membrane was blocked with 1% bovine serum albumin in PBS at 4°C for 16 h. The primary polyclonal AF-antibody, aH3, directed against the residues 203–205 + 219–226 of the AF molecule (Lönnroth et al., 2003; Jennische et al., 2006), or pre-immune-serum, was then added to the membrane and incubated for 1.5 h. The blot was developed with alkaline phosphatase (AP)-conjugated goat anti-rabbit immunoglobulin (Jackson ImmunoResearch Europe Ltd.), followed by 5-bromo-4-chloro-3-indolylphosphate and 4-nitro blue tetrazolium (Roche Diagnostics).

2.5. Biological activity of AF prepared from placenta

We measured the antisecretory activity of AF, purified from the placenta preparation, in the previously described rat small intestinal ligated loop model, using cholera toxin as a secretagogue (Lange, 1982). Thus, a preparation inducing a 50% inhibition of cholera toxin-induced fluid secretion in the ligated loop was assigned an AF value of 1.0 unit.

2.6. Immunization schedule and hybridoma procedure

Six female BALB/c mice were immunized intraperitoneally (i.p.) with 25 μg of the human placental AF preparation, purified by affinity chromatography and emulsified in Freund's complete adjuvant. A booster i.p. injection of 12.5 μg in incomplete adjuvant was given on Day 21, followed by four booster-injections with the same amount of the antigen in PBS at 2-week intervals. Blood samples were collected from the tail vein of anaesthetized mice on Day 7 after the third booster dose and screened by ELISA against the purified human placental eluate, recombinant expressed human AF (rAF) (Johansson et al., 1995) and synthetically produced AF, residue 1–105, (Johansson et al., 2008) as