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The activation and differential signalling of the growth hormone receptor induced by pGH or anti-idiotypic monoclonal antibodies in primary rat hepatocytes

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ABSTRACT

In this report, we have developed a panel of monoclonal anti-idiotypic antibodies to pGH by immunising BALB/c mice with a purified monoclonal anti-pGH antibody (1A3), among which one mAb, termed CG-8F, was selected for further characterisation. We found that CG-8F behaved as a typical Ab2β, not only conformationally competing with pGH for 1A3 but also exhibiting recognition for GHR in a rat hepatocyte model. We next examined the resulting signal transduction pathways triggered by this antibody in rat hepatocytes and found that both pGH and CG-8F could trigger the JAK2-STAT1/3/5-mediated signal transduction pathway. Furthermore, the phosphorylation kinetics of pSTAT1/3/5 induced by either pGH or CG-8F were remarkably similar in the dose–response and time course rat hepatocyte experiments. In contrast, only pGH, but not CG-8F, was capable of inducing ERK phosphorylation. Further experimental studies indicated that the two functional binding sites on CG-8F are required for GHR activation. This study partially reveals the mechanism of action of GH anti-idiotypic antibodies and also indicates that monoclonal anti-idiotypic antibodies represent an effective way to produce GH mimics, suggesting that it is possible to produce signal-specific cytokine agonists using an anti-idiotypic antibody approach.

1. Introduction

Growth hormone (GH) is a protein hormone composed of 191 amino acids that is predominantly synthesised and secreted by the anterior pituitary (Abdel-Meguid et al., 1987). GH has long been recognised as a regulator of body growth and metabolism (Waxman and O'Connor, 2006), and it acts by binding to its cognate receptor that is expressed on the surface of target cells. Binding of GH to these receptors triggers a cascade of signalling events that include the phosphorylation of the GH receptor and the activation of the JAK–STAT and MAPK–ERK pathways (Zhu et al., 2001). These signalling pathways act in concert to contribute to the overall actions of GH; many of the actions and the relative importance associated with the various individual GH-induced signalling pathways are dependent on the target tissues involved. The liver is a major target tissue for GH, and GH has been demonstrated to predominantly activate the JAK–STAT and MAPK–ERK pathways in rat hepatocytes (Beauloye et al., 2002; Murray et al., 2004; Ram et al., 1996).

Over the past few decades, the use of antibodies as GH mimics has been extensively studied. One approach has been to generate anti-idiotypic antibodies to GH; this approach is based on immune network theory (Jerne, 1974). Several studies have produced GH anti-idiotypic antibodies to mimic the biological functions of GH as previously described for pGH (Wang et al., 1994), rGH (Gardner et al., 1990), bGH (Roberge et al., 1999) and hGH (Elbashir et al., 1990). Another approach has been to generate anti-GH receptor antibodies; this approach is mainly based on X-ray crystallographical studies of hGH-GHR complexes (De Vos et al., 1992), which have revealed that GHR appears to be activated by GH-induced receptor dimerisation through two asymmetric binding sites on GH. Many researchers have therefore chosen to develop antibodies raised against GHR as GH mimics because antibodies (IgG) are bivalent, contain two antigen binding sites, and might simultaneously bind

Abbreviations: pGH, porcine growth hormone; mAb, monoclonal antibody; PRL, prolactin; GHR, growth hormone receptor; CLSM, confocal laser scanning microscope; FITC, fluorescein isothiocyanate; Ab2, anti-idiotypic antibody; PBS, phosphate-buffered saline; TMB, tetramethylbenzidine; JAK, Janus kinase; STAT, signal transducers and activators of transcription; MAPK, mitogen-activated protein kinase; TPO, thrombopoietin.

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to two antigen molecules (GHR). Based on these characteristics, they should therefore be able to induce receptor dimerisation and trigger receptor activation. There have been numerous reports that antibodies raised against GHR elicit agonist-like properties (Carlsson et al., 1994; Fuh et al., 1992; Mellado et al., 1997; Rowlinson et al., 1998; Wang et al., 1996; Wu et al., 2010). However, more recent studies suggest that the GHR is dimerised even in the absence of the ligand (Frank, 2002; Yang et al., 2008), and GHR conformational changes induced by ligand are required for GHR activation (Brown et al., 2005). Mechanisms of GHR activation have been extensively studied but are still incompletely understood.

In a previous study, we produced polyclonal anti-idiotypic antibodies against pGH by immunising goats with purified monoclonal anti-pGH antibody (clone 1A3). The polyclonal anti-idiotypic antibodies specifically bound to the cell surface GHRs expressed by rat hepatocytes (in vitro) and also increased weight gain of hypophysectomised rats (in vivo) (Zheng et al., 2006). Several similar studies have been previously conducted (Elbashir et al., 1990; Gardner et al., 1990; Roberge et al., 1999; Wang et al., 1994). Although these anti-idiotypic antibodies exert GH-like biological effects in experimental animal models, the mechanisms underlying their actions remain unclear until now.

In this study, we developed a panel of monoclonal anti-idiotypic antibodies against pGH and examined their ability to induce downstream signal transduction pathways in a rat hepatocyte model. Among these antibodies, one mAb, termed CG-8F, specifically binds to GHR and triggers differential signalling in primary rat hepatocytes. This study provides an explanation for understanding the mechanism of action of anti-idiotypic antibodies against GH and also substantiates the possibility of creating pathway-specific agonists for GHR-mediated signalling by using an anti-idiotypic antibody approach.

2. Materials and methods

2.1. Antigen and antibodies

Clone 1A3 was raised against pGH; its properties have been described in detail previously (Zheng et al., 2006). Our previous study demonstrated that 1A3 is a neutralising monoclonal anti-pGH antibody that can inhibit the binding of pGH to its receptor and that polyclonal antibodies produced by the immunisation of goats with purified 1A3 could specifically bind to cell surface receptors expressed by rat hepatocytes. Therefore, we used clone 1A3 as the antigen in our subsequent experiments. The $F(ab')_2$ or Fab fragments of 1A3 were prepared using the Pierce ImmunoPure Preparation kit according to the manufacturer's protocols, and coupled to keyhole limpet haemocyanin (KLH).

Phospho-JAK2(Tyr 1007/1008) and total JAK2, phospho-STAT5(-Tyr 694) and total STAT5, phospho-STAT3(Tyr 705) and total STAT3, phospho-STAT1(Tyr 701) and total STAT1, and phospho-ERK1/2(Thr 202/Tyr 204) and total ERK1/2 antibodies were obtained from Cell Signalling Technology (USA). MAb263 was obtained from Santa Cruz (USA). HRP-conjugated goat anti-rabbit and anti-mouse antibodies, pGH and PRL were obtained from Sigma (USA). The ImmunoPure Fab Preparation kit, Cell Lysis Buffer, IP kit, Enhanced chemiluminescence (ECL) and BCA kit were obtained from Pierce (USA). Williams E (WE) medium and fetal bovine serum (FBS) were obtained from Gibco (USA). Unless otherwise stated, other reagents were from Sigma (USA).

2.2. Preparation of monoclonal anti-idiotypic antibodies

Female BALB/c mice (6-to-8-week-old, Changchun Institute of Biological Products) were immunised intraperitoneally with 0.2 mg of purified 1A3-$F(ab')_2$–KLH emulsified in Freund's complete adjuvant, at 14-day intervals. The mice were administered similar booster injections of 1A3-$F(ab')_2$–KLH in incomplete Freund's adjuvant until suitable antibody titres were obtained. Three days after the final injections, splenocytes from immunised mice were fused with sp2/0 myeloma cells using 50% PEG1500. The resulting fused cells were seeded into 96-well cell culture plates and grown in 1640/HAT/20%FCS selection medium. The culture supernatants were tested by ELISA to determine the secretion of antibodies against the 1A3-$F(ab')_2$ fragments, and the cells producing monoclonal anti-idiotypic antibody were isolated by single-cell cloning at least twice by limiting dilution.

2.3. Indirect ELISA

The presence of anti-idiotypic antibodies in hybridoma culture medium was assayed using ELISA. Microtitre plates (96 wells) were coated with 100 μl of 1 μg/ml 1A3-$F(ab')_2$ and incubated overnight at 4 °C. Next, the wells were washed twice with PBST, blocked with 2% BSA at 37 °C for 60 min, and then washed twice again with PBST. The culture medium from the hybridomas was added, incubated for 120 min, and the wells were washed 3 times again with PBST. Next, goat anti-mouse IgG-FC HRP secondary antibody (1:2000 dilution) was added and incubated for another 60 min. The wells were washed again, and the TMB substrate was used to develop the colorimetric reaction for 15 min at 37 °C. Reaction was stopped by the addition of 3 M H_2SO_4 into each well (50 μl/well) and was read at OD 450 nm on an automatic ELISA plate reader (Multiskan FC, Thermo Fisher Scientific Inc.).

2.4. Fluorescent labelling

pGH or PRL was conjugated to FITC as previously described (Badolato et al., 1994; Rapaport et al., 1995). Briefly, pGH or PRL that had been previously dialysed overnight against a 0.1 mol/L sodium bicarbonate buffer (pH 9.5) was incubated with FITC (in dimethylsulfoxide, 4 mg/ml) for 2 h at 20 °C with continuous magnetic stirring in the dark. Free unconjugated FITC was removed by gel filtration using Sephadex G-25 (Pharmacia), followed by dialysis against PBS (pH 7.2) for 20 h. The FITC/protein ratio was calculated from the absorbance at 280 nm and 495 nm using the following formula: molar ratio = $2.87 \times A495/A280 - (0.35 \times A495)$. Conjugates with F/P ratios of 1.5 exhibited the greatest sensitivity and reproducibility and were chosen for further use in this study.

2.5. Primary rat hepatocyte culture

Rat hepatocytes were isolated using the two-step collagenase perfusion technique by Seglen (1976) with slight modifications as previously described (Ribaux et al., 2002). Briefly, the rats (male Wistar; 200–220 g body weight; provided by the Experimental Animal Center, Jilin University) were anesthetised using sodium pentobarbital (100 mg/kg), and the portal vein and inferior vena cava were exposed and cannulated. The rat livers were initially perfused with calcium- and magnesium-free Hank's buffer (pH 7.4) at 37 °C for 10 min, followed by perfusion with collagenase solution (1 mg/ml) at 37 °C for 20 min. The livers were excised, minced and passed through a series of nylon mesh filters. The resulting cell suspensions were centrifuged twice at 50 g. The viability of the isolated hepatocytes was shown to be approximately 90–95% by trypan blue dye exclusion assay. The cells were resuspended with WE medium supplemented with glutamine, penicillin plus streptomycin, and 10% FBS. Unless otherwise indicated, the freshly isolated hepatocytes were plated onto collagen-coated plastic culture dishes at a density of 2×10^6 and allowed to attach