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Modulation of the Effectiveness of Growth Hormone with a Monoclonal Antibody

Abstract

A monoclonal antibody (mAb), designated PS-11.2, was generated by immunizing mice with recombinant porcine growth hormone (pGH). This antibody recognized GHs of porcine, bovine (bGH) and chicken (cGH) origins, but not human GH, ovine prolactin, somatostatin S-14, and GH-releasing factor (1-29-NH₂). Western analysis indicated that PS-11.2 predominantly identified not only the 22.5-kD protein but also its 45-kD dimer. It also recognized the 4.5- and 10-kD fragments of pGH resulting from trypsin digestion. The binding kinetics of PS-11.2 to pGH was determined by the biospecific interaction analysis in a real-time mode. The association and dissociation rate constants were estimated as $1.4 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and $2.2 \times 10^4 \text{ s}^{-1}$, respectively, thus producing an overall affinity of $K_d = 1.6 \times 10^9 \text{ M}$. It partially inhibited the interaction of pGH and GH-binding protein in a competitive radioimmunoassay, suggesting that the pGH epitope recognized by PS-11.2 was closely related to the region responsible for engaging with GH receptors. Growth-deficient hypophysectomized rats were used for functional evaluation and shown to grow in response to the treatment of pGH. This effect was further augmented when pGH was administered together with PS-11.2, although antibody itself did not promote the growth of these animals. The antibody-mediated effect continued beyond the 5-day treatment period, indicating its long-lasting effect. Similar enhancement by PS-11.2 was also observed with bGH and cGH in this rat model. Therefore, the present findings clearly suggest that the somatogenesis of GH can be potentiated by PS-11.2 and that this mAb may serve as a useful tool for improving the growth performance of livestock.

Introduction

Growth hormone (GH) is a major pituitary factor consisting of a single polypeptide chain of 191 amino acids with two intrachain disulfide bonds. It has been well documented to increase milk yield in dairy cows and to improve muscle/fat ratio in cattle, sheep and pigs [1]. Although the rapid advances in biotechnology have resulted in several breakthroughs in the agricultural industry, novel strategies have been continuously explored for further improvement in the performance of farm animals.

Received: July 29, 1994

Accepted: October 10, 1994

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A number of laboratories including ours have investigated the possibilities to manipulate the endocrine system of livestock with immunological approaches. For instance, Aston et al. [2] reported that a monoclonal antibody (mAb) to ovine GH increased bone metabolism as evidenced by an accelerated incorporation of radioactive sulfate tracer into cartilage of treated animals. Holder et al. [3] demonstrated that an mAb raised against human GH augmented the GH effects not only on the growth and body composition of Snell dwarf mice but also on food conversion efficiency. We also obtained evidence to suggest that the somatogenesis of porcine GH (pGH) in GH-deficient hypophysectomized rats was enhanced by an anti-pGH mAb [4]. This type of antibodies appears useful for a better understanding in modulating GH effectiveness. Therefore, a search for additional

growth-promoting antibodies continues in our laboratory and we are describing such a new antibody in this report.

Materials and Methods

Reagents

Recombinant pGH, bovine GH (bGH), and chicken GH (cGH) were obtained from American Cyanamid Co., Princeton, N.J., USA. Pituitary human GH (hGH) and ovine prolactin (PRL) were purchased from Sigma Chemical Co., St. Louis, Mo., USA. Somatostatin S-14 (SRIF) was obtained from Peninsula Laboratories, Belmont, Calif., and GH-releasing factor (1-29NH_2) (GRF) was from Dr. D.H. Coy, Tulane University, New Orleans, La., USA. In some experiments, pGH was treated with immobilized trypsin (Boehringer-Mannheim, Germany) at a ratio of 10:1 for 18 h at room temperature in 25 mM NaHCO₃ (pH 9.4) and 50 mM phosphate buffer (pH 7.8). Trypsin was removed by centrifugation and the pGH digests in solution were stored at -20 °C prior to use. The native pGH was prepared from porcine anterior pituitary (Pel-Freez, Rogers, Ariz., USA). The tissue was homogenized at 4 °C in a dounce homogenizer in 1 mM sodium bicarbonate (pH 7.5), 1 mM EDTA, and 1 mM EGTA. A protease inhibitor cocktail consisting of 145 µg/ml phenylmethyl sulfonyl-fluoride, 76 µg/ml leupeptin, 30 µg/ml chymostatin, and 10 µg/ml pepstatin A was also added. Homogenate was centrifuged at 2,000 $\times g$ to remove debris, and the supernatant was frozen in aliquots at -80 °C.

Production of mAb

Balb/c mice (Charles River Breeding Laboratories, Wilmington, Mass., USA) were immunized with pGH emulsified in Freund's adjuvant followed by several boostings with the same antigen at a 4-week intervals. The sera of these animals were collected 3 days after the last boost for the determination of antibody titers in an enzyme-linked immunosorbent assay. The lymphocytes were harvested from mice demonstrating the highest antibody titers and fused with SP2/0 myeloma cells. These cells were cultured in wells of microtiter culture plates and the successfully fused hybridomas capable of releasing anti-pGH antibody were identified and immediately subcloned by a limiting dilution procedure. One such hybridoma, designated PS-11.2, was established and injected into the peritoneal cavity of Balb/c mice for ascites production. Antibody was purified from the ascitic fluid by ammonium sulfate precipitation and stored at -80 °C prior to use.

Competitive Radioimmunoassay

Microliter wells of RIA plates were coated with 1 µg/well of rabbit anti-mouse IgG Fc antibody (RAMFc; Pierce, Rockford, Ill., USA) and the remaining binding sites were blocked with 2% bovine serum albumin (BSA). PS-11.2 was subsequently added to these wells and its Fc was captured by the immobilized RAMFc antibody while its Fab was capable of binding ¹²⁵I-pGH (specific activity = 180—250 µCi/ng$\times$ New England Nuclear/DuPont Co., Boston, Mass., USA) in solution. When competitive RIA was performed, ²⁵I-pGH (30,000 cpm) was added to each well together with various competitors at a series of dilutions in phosphate-buffered saline (PBS) containing 2% BSA. The RIA plates were incubated at 37 °C for 2 h and the unbound ¹²⁵I-pGH was removed by washing. The radioactivity of the residual ²⁵I-pGH associated with PS-11.2 in each well was measured in a gamma counter.

GH Binding Protein (GHBP) RIA

An RIA was previously established to measure the interaction of pGH and recombinant rat GHBP [5]. Briefly, GHBP-4.3 mAb, which was raised against a 17-amino acid sequence corresponding to the C-terminus tail region of GHBP, was used to coat an RIA plate at 1 µg/well for capturing GHBP which in turn bound ¹²⁵I-pGH. PS-11.2 was added to this system to test its effect on GH-GHBP interaction. After a 60-min incubation, the unbound ¹²⁵I-pGH was removed by washing and the

remaining 125 I-pGH was measured in a gamma counter. The results were presented as the percent maximum binding under which no competitor was present.

Western Analysis

Homogenate of swine pituitary glands, recombinant pGH and the trypsin digests of recombinant pGH were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS/PAGE) under reducing conditions. The separated protein components were electroblotted onto a polyvinylidene difluoride (PVDF) microporous membrane (Millipore Corp., Bedford, Mass., USA) in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), pH 10.5. The membrane was blocked with 5% dry milk in 50 mM NaCl, 10 mM Tris (pH 7.5) and incubated with PS-11.2 at 10 μ g/ml for 1 h. After being washed three times with PBS, the PVDF membrane was exposed to peroxidase-linked goat anti-mouse Ig and subsequently to a Western blot chemiluminescence kit (New England SBHear/Du Pont Co.). Autoradiography was finally developed on an X-ray film.

Biospecific Interaction Analysis

Immobilization of pGH to a CM5 sensor chip of the BIAcore instrument was carried out with a procedure recommended by the manufacturer (Pharmacia Biosensor, Piscataway, N.J., USA). Thirty microliters of antibody was injected into the BIAcore at a flow rate of 5 μ l/min and antigen-antibody interaction was monitored by the changes of the refractive index which was registered in the sensor-gram in a real-time fashion. The surface of the sensor chip was regenerated with 10 mM HO between individual runs. Binding kinetics of antibody was determined by a method previously reported by Karlsson et al. [6] with software supplied by the manufacturer.

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1.000
800
600
400

• pGH
● bGH A cGH
■ 1 0.01

0.1 Competitors (nM)

Fig. 1. Antigenic specificity of mAb. Microtiter wells of RIA plates were coated with RAMFc antibody to capture PS-11.2. 125 I-pGH (30,000 cpm) was subsequently added to these wells together with various competitors at a series of dilutions. After a 2-hour incubation, these plates were washed and the radioactivity reflecting the amount of 125 I-pGH remaining associated with PS-11.2 in each well was measured in a gamma counter.

Fig. 2. Western analysis. Extracts from porcine pituitary glands (lane 1), recombinant pGH (lane 2), and trypsin-digested recombinant pGH (lane 3) were subjected to SDS-PAGE. After transfer to a PVDF membrane, the separated protein bands were sequentially exposed to PS-11.2, peroxidase-linked rabbit anti-mouse Ig, and chemiluminescence reagents for autoradiography.

Biological Assay for Animal Growth

Eight Sprague-Dawley female rats (Taconic Farm, Germantown, N.Y., USA), hypophysectomized at 21 days of age, were randomly allocated to each treatment group. These animals were injected intramuscularly with various GH (5 μ g/day) with or without antibody for 5 consecutive days. The growth of these animals was individually monitored and recorded as net weight gain. The significance of the differences between individual treatment groups was determined by the least-squares analysis of variance for randomized design using the General Linear Models procedure of the Statistical Analysis System [7].

Results

Antigenic Specificity of PS-11.2

The immunospecificity of PS-11.2 was examined with a competitive RIA. As demonstrated in figure 1, PS-11.2 was able to bind 125 I-pGH and this effect was, as expected, abolished in a dose-dependent manner by the presence of cold pGH. Similar competitions were observed with bGH and cGH, suggesting that PS-11.2 recognized an epitope shared by these three GHs. On the other hand, PS-11.2 was not specific to hGH, PRL, SRIF, or GRF, because these agents failed to compete with 125 I-pGH for the binding to PS-11.2.

Recognition of pGH by PS-11.2 was also examined by Western analysis. As illustrated in figure 2, PS-11.2 detected several forms of native pGH in swine pituitary extracts, including 90-, 68-, 45- and 22.5-kD proteins (lane 1). It also identified two forms of recombinant pGH with m.w. of 45 and 22.5 kD (lane 2). When recombinant pGH was digested with trypsin, two smaller fragments with m.w. of 10 and 4.5 kD were visualized by PS-11.2 (lane 3).

Binding Kinetics of PS-11.2 to pGH

A sensor chip of the BIAcore instrument was coated with pGH and PS-11.2 was injected to the chip at various concentrations. The interaction of PS-11.2 and pGH was continuously monitored and recorded as the increased resonance units (RU). The binding rate was plotted as a function of the amount of antigen-antibody complex formed at a given time. A series of binding plots with different slopes were obtained by injecting PS-11.2 at different concentrations (106-852 nM) (fig. 3). These slopes were used to calculate the association rate constant (k_a) as $1.4 \times 10^5 M^{-1} s^{-1}$. Dissociation of the complex was also observed by the decrease in RU signals when the injection of antibody was replaced by the eluting buffer flow. By

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Fig. Binding affinity of PS-11.2 to pGH. GH was immobilized on a sensor chip of the BIAcore instrument and PS-11.2 was injected to the instrument at various concentrations (106-852 nM). The binding of antibody to pGH was continuously monitored and registered in a sensorgram as the increased RU signals.

pGH/PS-11.2 together
 & pGH/PS-11.2 separate pGH

Q No treatment

400

6.25

Days

200 100 50 25 12.5
 Concentrations (ng/ml)

Fig. 4. Competition RIA. Being immobilized to wells of an RIA plate by anchoring to GHBP-4.3 mAb, GHBP was able to bind ^{125}I -pGH. PS-11.2 and control mouse IgG at a series of concentrations were added as competitors. Unbound ^{125}I -pGH was removed after incubation and the remaining ^{125}I -pGH bound to GHBP was measured in a gamma counter. Results are presented as percent maximum binding with respect to the controls lacking competitor.

fhfe* 5. Enhancement of pGH activity with PS-11.2. Hypophysectomized rats were injected with 5 μg pGH for 5 consecutive days and the weight gain of these animals was monitored daily. Some animals were treated with pGH which was either premixed with or separate from PS-11.2 (500 μg) prior to injections. Untreated rats were also included as the negative controls. Each treatment group contained 8 rats. * $p < 0.05$; ** $p < 0.01$ when compared to those receiving pGH alone treatment.

integrating these values with time, the dissociation rate constant (K_d) was determined as $2.2 \times 10^{-4} \text{ s}^{-1}$. Finally, the overall binding affinity of PS-11.2 to pGH represented by K_{ij} was calculated by the formula of k_j/k_a as $1.6 \times 10^{-9} \text{ M}$. The effect of PS-11.2 on the interaction of pGH and GHBP was examined in a GHBP RIA. The wells of an RIA plate were coated with GHBP-4.3 mAb to capture GHBP which subsequently bound ^{125}I -pGH. When PS-11.2 was added in this system, it partially competed with

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Fig. 6. Enhancement of hormonal effects with PS-11.2. Hypophysectomised rats were injected with 5 μg pGH (a), bGH or cGH (b) with or without 1 mg PS-11.2 for 5 consecutive days. The net weight gains were scored at day 9. Injection with PS-11.2 alone was also included (a). * p

< 0.05; ** p < 0.01; ***p < 0.001 when compared to those receiving GH alone treatment unless otherwise indicated.

GHBP for ¹²⁵I-pGH in a dose-dependent fashion, whereas normal IgG did not (Fig. 4). A 60% competition was demonstrated by PS-11.2 at a concentration of as much as 400 ug/ml.

Enhancement of pGH Activity with PS-11.2 The effects of GH and antibody on growth were measured in a hypophysectomized rat model. These animals were injected with 5 ug pGH for 5 consecutive days and shown to gain significantly more body weight than did untreated controls (fig. 5). This effect was further significantly enhanced by injecting these animals with a combination of pGH and PS-11.2 (500 fig) both of which were incubated together at room temperature for an hour prior to injection. However, animals showed a slight, but not significant, weight gain compared to those treated with pGH alone when PS-11.2 and pGH were administered separately (p = 0.101 at day 10). The difference in the weight gains of rats treated with pGH and PS-11.2 either together or separately was significant at day 10 (p = 0.026). In a separate experiment, PS-11.2 was again significantly increased the biological activity of pGH, although the antibody itself was not active (fig. 6a). Both bGH and cGH were also tested and found to promote the growth of rats (fig. 6b). PS-11.2 further elevated the somatogenic effects of these two GHs.

Discussion

PS-11.2 was raised by immunizing mice with pGH and shown to enhance the biological activity of pGH in promoting the growth of hypophysectomized rats. The antigenicity of PS-11.2 seemed rather specific to pGH, bGH and cGH, but not hGH. It also failed to recognize PRL, SRIF and GRF. The antigenic discrimination might be simply due to the differences in these molecular compositions. In fact, pGH was reported to differ from hGH by about 35% of their amino acid residues [8]. PRL, SRIF and GRF have no or very little similarity to pGH in amino acid sequences. Western analysis further indicated that PS-11.2 recognized two forms of recombinant pGH with m.w. of 45 and 22.5 kD. The 45-kD material might be a product of the dimerization of the 22.5-kD component. Additional proteins with larger m.w. were also seen in pituitary extracts, suggesting an incomplete extraction of tissues or aggregation of several minor components. PS-11.2 also recognized two smaller 10- and 4.5-kD fragments of pGH following trypsin digestion, indicating that the epitope for PS-11.2 might be present in these fractions. GH appeared to possess multiple enhancing epitopes as evidenced by several polyclonal antibodies specifically raised against different GH peptides [9-12]. Although the determination of the precise epitope recognizable by PS-11.2 is not fully ascertained yet, it might reside

within one of such reported sequences. Cunningham et al. [13] reported that each hGH molecule possesses distinct binding sites for two human GHBP molecules. The epitope of pGH recognized by PS-11.2 might reside within or be adjacent to one of these two binding sites because it partially inhibited ¹²⁵I-pGH from interacting with GHBP in the competitive RIA and failed to achieve complete competition even at an antibody concentration of as much as 400 µg/ml.

The binding affinities of PS-11.2 to bGH and cGH were not directly measured in this study, but similar competition curves presented in figure 1 suggested that the K_d's of the antibody to these two hormones were very close to the K_d of the antibody to pGH. Based upon the fact that PS-11.2 cross-reacted with bGH and cGH with similar binding affinities, it was not too surprising to demonstrate that the growth-promoting effects by these two GHs were significantly augmented by PS-11.2 in the hypophysectomized rat model. However, antibody itself was totally inactive.

Although the mechanism of action of PS-11.2 in growth enhancement is unknown, its inhibitory effect on GHBP-GH interaction might provide an explanation. Without a complete understanding of the physiological significance of GHBP, it was speculated that GHBP might have a negative modulatory role by competing with GH receptors for GH. PS-11.2 was shown in this study to prevent GH from engaging with GHBP by forming an antibody-pGH complex. Once the complexes reached the target tissues, a lower binding affinity between pGH and PS-11.2 (K_d=1.6 × 10⁻⁹ M) would preferentially deliver pGH to GH receptors which usually possessed a higher binding affinity. In fact, the K_d of hGH and its receptors was reported to be 3 × 10⁻¹⁰ M [14]. Our speculation was apparently explainable by the failure of PS-11.2 to augment pGH activity when both were administered separately.

Because under such a circumstance, pGH might be bound to circulating GHBP immediately upon injection thus becoming inaccessible for interaction with PS-11.2. Alternatively, the configuration of pGH might be altered following binding to PS-11.2 and such a conformational modification might make pGH interact favorably with tissue receptors rather than circulating GHBP. In fact, Mazza and Retegui [15] generated several mAb and reported that the interaction of these antibodies with hGH resulted in an allosteric alteration of hGH. Additional examples with influenza virus [16] and p-galactosidase [17] provided further support to the concept of antibody-mediated modulation of antigen molecules.

How the antibody enhances the hormonal effectiveness is not totally clear, but several possibilities have been documented. The antibody was shown to alter the pharmacokinetics of GH in circulation [4], protect GH from enzymatic degradation [18], accumulate GH deposits in tissues [4], restrict GH to engage with certain subtypes of receptors relevant to somatogenesis [19], target GH to the somatogenic receptors in hepatocytes [20], and increase the synthesis and release of somatomedin C/IGF-I by hepatic cells [20, 21]. In addition to the enhancement of GH activity, other immunological options have also been proposed to be feasible. For instance, antibodies were generated against SRIF to increase the synthesis of GH in pituitary glands [22,23], against adipose cells to decrease the body fat contents [24], against insulin receptor to induce insulin-like activities [25], against GH antibodies to elicit anti-idiotypes mimicking hormonal effects [26, 27], and against progesterone to prevent conception [28]. The concept of improving animal performance with immunological approaches may prove to be a novel and useful strategy to modify the current practice in managing the livestock industry.

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