Effects of complexation with *in vivo* enhancing monoclonal antibodies on activity of growth hormone in two responsive cell culture systems

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ABSTRACT

We describe the properties of three monoclonal antibodies (MAbs) to ovine GH, two of which have previously been shown to enhance, *in vivo*, the biological activity of bovine and ovine growth hormone. We have examined the effects of these MAbs on GH activity in two appropriate GH-responsive cell culture systems, investigating both acute signalling effects (Janus-activated kinase (Jak)-2 tyrosine phosphorylation – 5 min) and longer-term (MTT-formazan production – 24 h) effects of hormone–antibody complexes. In the 3T3-F442A pre-adipocyte cell line (which has been demonstrated to be GH responsive), we show that complexation of recombinant bovine (rb) GH with either of the two enhancing anti-ovine GH MAbs (OA11 and OA15) and the non-enhancing MAb, OA14, attenuates the ability of GH to stimulate tyrosine phosphorylation of Jak-2 at a 5-min time point. Using the mouse myeloid cell line, FDC-P1,

stably transfected with the full-length ovine GH receptor (oGHR), we demonstrate that rbGH causes a dose-dependent increase in MTT-formazan production by these cells. Further, we demonstrate that OA11 and OA14, but not OA15, cause a decrease in this stimulatory activity of rbGH over a hormone concentration range of 5–50 ng/ml at both 24 and 48 h.

We conclude that the different *in vitro* activities of the two *in vivo* enhancing MAbs are most probably related to the time-courses over which these two assays are performed, and also to the relative affinities between antibody, hormone and receptor. In addition, the *in vitro* inhibitory activity of the enhancing MAb OA11 in both short- and long-term bioassay lends further support to an exclusively *in vivo* model for MAb-mediated enhancement of GH action.

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INTRODUCTION

The phenomenon of antibody-mediated enhancement of hormone activity has been extensively reported (Wallis *et al.* 1987, Holder *et al.* 1988, Holder 1992). Such observations have been made principally with the pituitary growth hormone (GH) and have almost exclusively examined activity *in vivo* in the GH-deficient Snell dwarf mouse. Although several theories have been advanced to explain this enhancing effect *in vivo* (Massart *et al.* 1993, van der Hel *et al.* 1994, Tans *et al.* 1994),

there are few data available that describe the activity of these antibodies in suitable GH-responsive cell culture models. Using the GH-responsive mouse pre-adipocyte cell line 3T3-F442A, we have recently demonstrated that the *in vivo* enhancing monoclonal antibody (MAb), OA15, allows for the interaction between GH and receptor (such that the tripartite complex MAb–hormone–receptor is formed), but appears to attenuate Janus-activated kinase (Jak)-2 tyrosine phosphorylation in response to recombinant bovine (rb) GH in these cells (Beattie *et al.* 1999). We rationalised such receptor

and biological activity on the previously described epitope defined by this enhancing antibody, which was removed from both sites 1 and 2 on the GH molecule, which are important for receptor interaction. We have now extended these studies with another GH-enhancing MAb (OA11) and have also examined the effects on rbGH activity of MAb OA14, which does not enhance *in vivo* the activity of ovine (o) GH. In addition to the 3T3-F442A pre-adipocyte cell culture system, we have examined the effect on longer-term (24–48 h) GH activity using the mouse promyeloid cell line, FDC-P1, transfected with the full-length oGH receptor (oGHR) and MTT-formazan dye production as an endpoint. We present results of experiments that report the effect of complexation of rbGH with each of these three MAbs on subsequent hormone activity in these two experimental models.

MATERIALS AND METHODS

Materials

Tissue culture plastic was from CoStar (Dursley, Glos, UK). Culture media (including phenol-red-free RPMI 1640), newborn and foetal calf serum and antibiotics were from Gibco (Inchinnan, Scotland). Mini-gel and blotting apparatus was from Bio-Rad (Herts, UK). rbGH (rbGH-Met¹...Leu¹²⁷, lot No. M-118-08308) was a gift from Dr R Collier (Monsanto Corp, St Louis, MO, USA). oGH (oGH-I-4), rat GH (rGH-I-6), rat prolactin (PRL; ratPRL-I-5) and oPRL (oPRL-I-3) were from NIDDK (Bethesda, MD, USA). Porcine (p) GH (pGH-B-1) and pPRL (pPRL-B-1) were from the United States Department of Agriculture. Polyclonal rabbit anti-mouse Jak-2 and the monoclonal anti-phosphotyrosine antibody 4G10 were both from Upstate Biotechnology (New York, NY, USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were from Sigma (Poole, Dorset, UK). Pansorbin (killed suspension of *Staphylococcus aureus*) was from Calbiochem (product No. 507861). Enhanced chemiluminescent (ECL) reagent and ECL Hyperfilm were from Amersham (Amersham, Bucks, England). Monoclonal antibodies OA11, OA14 and OA15 were raised to oGH and were used as dialysed 18% (NH₄)₂SO₄ fractions of hybridoma supernatants. Protein concentrations were approximately 0.6 mg/ml. These antibodies have been partially characterised previously (Aston *et al.* 1987). The mouse pre-adipocyte cell line, 3T3-F442A (Green & Kehinde 1976) was supplied by Dr Howard Green (Harvard Medical School, Boston, MA, USA). The mouse myeloid cell line, FDC-P1 (Hapel *et al.* 1984), stably

transfected with the full-length oGHR, oGHR-FDC-P1, was propagated as described in the Methods section. MTT reagent (3-(4,5-dimethyl thiazol-2yl)-2,5-diphenyl tetrazolium bromide), ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) and mouse interleukin-3 (mIL-3) were from Sigma.

Methods

Cell culture

3T3-F442A cells were propagated as described previously (Beattie *et al.* 1999). FDC-P1 cells stably expressing full-length oGHR grow in suspension and were maintained in RPMI 1640 containing 50 U/ml penicillin-streptomycin, 10% fetal calf serum (FCS), 2 mM glutamine and 50 U/ml recombinant mIL-3 (Sigma). Cells were grown in 225 cm² flasks and were split (1:10) when they reached a density of $1-2 \times 10^6$ per ml.

Stimulation of oGHR-transfected FDC-P1 cells

Stimulation of oGHR-FDCP1 cells was assessed using the MTT-formazan dye assay (Mosmann 1983). Cells were grown in complete medium as described above, washed by centrifugation at $500 \times g$ for 5 min and resuspended in the RPMI 1640. Washed cells were resuspended at a density of 8×10^5 cells per ml in RPMI 1640–0.5% FCS. Fifty microlitres of this cell suspension were dispensed into a 96-well microtitre plate (40 000 cells per well), followed by 100 µl hormone solution appropriately diluted in RPMI 1640–0.5% FCS. After incubation (3–72 h), 20 µl MTT at a concentration of 0.5 mg/ml in RPMI 1640 was added to each well, and after a further 3 h of incubation, cells were lysed with 150 µl 0.12 M HCl in isopropyl alcohol. The contents of microtitre wells were resuspended thoroughly by trituration and absorbance in each well was determined at 540 nm. In experiments that examined the effect of antibody complexation on the subsequent response to rbGH, hormone was incubated at concentrations of 1–50 ng/ml with MAbs at a dilution of 1:100. MAb and hormone were incubated at room temperature for 1 h before addition to cells. All hormone and hormone/antibody treatments were applied in triplicate and results are presented as means $\pm$ s.e.

ELISA

High-binding Immulon-2 microtitre plates were coated with 0.5 µg/well rbGH in 50 mM NaHCO₃ (pH 9.6). One hundred microlitres serial MAb dilutions in 50 mM Tris, 150 mM NaCl, 0.2% Tween 20, pH 8.0 (TBS-T) were incubated with microtitre plates for 2 h at 22 °C. Plates were then

washed in TBS-T and incubated for 2 h with anti-mouse-HRP conjugate (diluted 1:1000 in TBS-T). After the plates were further washed in TBS-T, colour was developed by the addition of 100 µl/well 0.5 mg/ml ABTS in 0.1 M phosphate-citrate buffer (pH 4), 0.01% (v:v) H₂O₂. After 30 min further incubation of the plates, absorbance was determined at 405 nm.

GH stimulation of Jak-2 tyrosine phosphorylation

GH stimulation of tyrosine phosphorylation of Jak-2 in monolayer cultures of 3T3-F442A cells was performed as described by Argetsinger *et al.* (1993). Effects of MAb complexation of GH on subsequent Jak-2 tyrosine phosphorylation was performed exactly as described previously (Beattie *et al.* 1999).

Receptor binding studies in transfected FDC-P1 cells

oGHR-transfected FDC-P1 cells (10⁷ per tube) were incubated overnight at 4 °C with 10⁶ c.p.m. ¹²⁵I-rbGH in a total volume of 300 µl binding buffer (10 mM Tris.Cl (pH 7.4), 10 mM CaCl₂, 0.1% BSA). After centrifugation (1800 g for 30 min), supernatants were decanted and bound radioactivity in the pellet was determined. Non-specific binding was assessed in the presence of 1 µg unlabelled rbGH and typically accounted for 3–4% of added c.p.m. Scatchard analysis was achieved by quantitation of ¹²⁵I-rbGH bound to cells in the presence of increasing concentrations of unlabelled rbGH.

RESULTS

In Fig. 1, we present the results of experiments that characterise in detail the response of the oGHR-FDC-P1-transfected cells to added hormones. We monitored the production of MTT-formazan as a result of GH treatment and it is important to note that this assay monitors the stimulation of both metabolic and mitogenic activity in these cells. In Fig. 1*a*, cells are shown to respond dose dependently to rbGH with an EC₅₀ of approximately 2.5 ng/ml. When we examined the specificity of the response of oGHR-FDCP1 cells to various species of GH, we observed the reactivity profile shown in Fig. 1*b*. In our hands, rbGH demonstrated a greater activity than the pituitary-derived oGH preparation that was available in our laboratory and, indeed, earlier studies have reported that bovine GH is active *in vivo* in sheep (Bass *et al.* 1991, Sauerwein *et al.* 1991). Porcine and rat GH displayed equivalent reactivities that were lower than that seen for the ruminant GH (Fig. 1*b*). As expected, none of the PRLs tested (o, r, p, or m) demonstrated activity in these cells (Fig. 1*c*). The time-course of

hormone stimulation with 10, 100 and 1000 ng/ml rbGH showed an effect of hormone 6 h after its addition, with the stimulatory effect of GH continuing to increase up to 72 h after the addition of rbGH (Fig. 1*d*). Scatchard analysis of rbGH binding to oGHR-FDCP1 cells indicated a K_d of 1×10^{-9} M for hormone-receptor binding, similar to previously reported values for the GH-GHR interaction. Estimation of total binding sites revealed 2200 per cell (Fig. 2).

Having clearly characterised the specificity of response of this stably transfected cell line to rbGH, we were able to undertake experiments that investigated the effects of three MAbs to oGH (OA11, OA14 and OA15) on the *in vitro* biological activity of rbGH. Two of these antibodies, OA11 and OA15, have been shown to enhance the activity of oGH when they are precomplexed with hormone and administered to hypopituitary Snell dwarf mice. Precomplexation of oGH with MAb OA14 did not enhance the activity of hormone (Aston *et al.* 1987). Initial characterisation of MAbs by ELISA indicated that they exhibited similar titres against rbGH (data not shown) and we therefore examined their activity at the same dilution over a range of rbGH concentrations. When used at final dilutions of 1:100, OA11 and OA14, but not OA15, inhibited the activity of rbGH in this bioassay (Fig. 3). For MAb OA11, inhibition of rbGH activity was evident over the entire range of hormone concentrations at a 24 h time point. For OA14, inhibition was overcome at the highest concentration of rbGH examined (50 ng/ml). Similar results were obtained at 48 h (data not shown).

In a separate *in vitro* bioassay system using the GH-responsive pre-adipocyte cell line, 3T3-F442A, OA11 and OA14—again at a final dilution of 1:100—appeared to attenuate the level of tyrosine phosphorylation of the tyrosine kinase Jak-2 caused by treatment of cells with 50 ng/ml rbGH for 5 min (Fig. 4). We have demonstrated previously that OA15 at the same dilution also attenuates GH-stimulated Jak-2 tyrosine phosphorylation (Beattie *et al.* 1999).

DISCUSSION

In the current study, we have clearly shown that the FDC-P1 cell line transfected with full-length oGHR responds specifically to ruminant GHs. Although there are no other published data on FDC-P1 cells transfected with ruminant GHRs, Fiddes *et al.* (1992) reported the functional expression of full-length oGHR in transfected Chinese hamster ovary (CHO) cells. The reported

FIGURE 1. (a) The response of oGHR-FDC-P1-transfected cells to increasing concentrations of rbGH. Cells were treated for 24 h with rbGH as described in Methods section. MTT assay revealed a dose-dependent effect of rbGH with an EC_{50} of approximately 2.5 ng/ml. Mean $\pm$ s.e. ($n=3$) for each hormone concentration. (b) Specificity of response of oGHR-transfected FDC-P1 cells to various species of GH. Cells were treated for 24 h with increasing concentrations (0.1–10 000 ng/ml) of rb (●), ovine (■), porcine (▲), or rat (▼) GH. Mean $\pm$ s.e. ($n=3$) for each hormone and concentration. (c) oGHR-transfected cells do not respond to PRL. Cells were treated for 24 h with rbGH (●; 0.1–1000 ng/ml) or with ovine (■), rat (▲), mouse (○) or porcine (▼) PRL. Mean $\pm$ s.e. ($n=3$) for each hormone and treatment. (d) Time-course of stimulation of oGHR-transfected FDC-P1 cells with rbGH. Cells were treated with 0 (○), 0.1 (□), 1 (△), 10 (●), 100 (■) or 1000 (▲) ng/ml rbGH and over the period 3–72 h. ABS, absorbance at 405 nm.

affinity of oGHR in their study, at 0.3 nM, was similar to that found in the current investigation. However, at 220 000 receptors per cell, the level of

expression was of the order of 100-fold greater than that in our experiments with FDC-P1 cells. Using increases in cellular protein synthesis as a measure

FIGURE 2. Scatchard analysis of rbGH binding to oGHR-FDC-P1 cells. Linear regression analysis gave a regression line represented by the equation $y = -0.0023x + 0.0844$ ($r^2 = 0.82$) estimating K_d at 1.4 nM and approximately 2200 binding sites per cell. For further details see Methods section. B, bound; F, free.

of GH activity, these authors reported a 27% increase in this parameter after GH treatment. They also reported that the GH-related hormone, ovine placental lactogen, was active in the oGHR-transfected CHO cells. We did not test the activity of this hormone in our transfected FDC-P1 cell line; however, there is preliminary evidence that, although not active on its own, it inhibits the activity of simultaneously added oGH (data not shown).

No studies have been reported on FDC-P1 cells transfected with the oGHR, but it is important to note that other species of GHR have been transfected into this cell line, either as full-length GHR proteins or as chimeric molecules containing the extracellular domain of the GHR linked to the cytosolic portion of the granulocyte-colony stimulating factor receptor (Fuh *et al.* 1992, Hackett *et al.* 1995, 1997, Rowlinson *et al.* 1995, 1998, Wang *et al.* 1995). Rowlinson *et al.* (1995), for example, described FDC-P1 cells stably expressing full-length human rabbit or rat GHR. When these cells were maintained in IL-3 containing (GH free) medium, as for the current study, they expressed a very similar number of receptors per cell (approximately 2100 and 2400, for the human GHR and rabbit GHR, respectively). When they examined

FIGURE 3. Effect of pre-incubation with MAbs on activity of rbGH. Varying doses of rbGH (0–50 ng/ml) were incubated for 1 h with OA11 (■), OA14 (▲) or OA15 (□) at a final dilution of 1 : 100 before addition to cells. Activities are compared with that seen for free rbGH (●) over the same concentration range. Incubations were for 24 h. Mean $\pm$ s.e. ($n=3$). ABS, absorbance at 405 nm.

FIGURE 4. Effect of precomplexation of rbGH with MAbs OA11 and OA14 on GH-stimulated Jak-2 tyrosine phosphorylation in 3T3-F442A pre-adipocytes. Cells were treated with 0, 50 or 500 ng/ml rbGH or with 50 ng/ml rbGH precomplexed with OA11 or OA14 as indicated in the figure. The location of tyrosine-phosphorylated Jak-2 is indicated by the arrow. This experiment was repeated three times with similar results in each instance. See Methods section for further details.

the activity of porcine, bovine and human GH on cells expressing full length rabbit GHR, using either thymidine incorporation or MTT assay, they reported that bovine and human GH had a threefold greater biopotency than the porcine molecule. As expected in our study, with the oGHR transfected cells we found rbGH to be more active than pGH at the ruminant receptor (Fig. 1B). In the study reported by Rowlinson *et al.* (1995) and in other studies (Fuh *et al.* 1992) using a similar *in vitro* bioassay system, a characteristic bell-shaped dose-response curve was observed, with a decrease in biological activity of hormone apparent at greater concentrations. This has, typically, been interpreted in the context of a productive 1 : 2 stoichiometry between GH and GHR, with high concentrations of GH driving the formation of non-biologically productive 1 : 1 ligand-receptor complexes. In our experiments, we observed a plateau of activity for rbGH and oGH at around 100 ng/ml of hormone and evidence for slight decreases in activity as the concentrations were increased to 10 000 ng/ml. The less active pGH and rGH showed little evidence for this bell-shaped activity – perhaps indicative of a failure to meet maximum potential biological activity by these hormones.

It is interesting to compare the results obtained after complexation of rbGH with the two *in vivo* enhancing monoclonal antibodies in both cell culture systems. OA11 inhibits hormone activity both in oGHR-FDC-P1 cells and in the

3T3-F442A pre-adipocytes. Our experiments in 3T3-F442A cells were carried out with a fixed concentration of rbGH – 50 ng/ml. At this concentration of hormone, the inhibitory effects of OA11 are partially overcome in the oGHR-FDC-P1 cells in the MTT assay. It is important to note, however, that this assay takes place over a 24-h period, and we believe that dissociation of hormone from MAb under these tissue-culture conditions may allow activity at the GHR. Indeed, such an equilibrium of hormone between receptor and MAb is favoured by reported affinity constants for MAb-hormone interaction of the order of 10^{-8} M (Aston *et al.* 1987), placing them at least one order of magnitude below the affinity of the GH-GHR interaction. GH stimulation of Jak-2 tyrosine phosphorylation in 3T3-F442A cells, however, is an acute event, and our incubations operate over a 5–10 min timescale. Kinetically, therefore, we believe that, under these conditions, the majority of hormone remains bound to MAb and results in attenuation of GH activity. In addition, affinity of rbGH for the mGHR, as presented by the 3T3-F442A cells, may be less than rbGH affinity for the largely homologous ruminant oGHR in the transfected FDC-P1 cells, again favouring inhibition of GH activity in the pre-adipocyte cell line.

For the other *in vivo* enhancing MAb, OA15, contradictory results seem to be apparent. This MAb does not appear to inhibit rbGH activity in the oGHR-FDC-P1 cell line, in which the activity profile of OA15-rbGH complexes was almost superimposable on that seen with rbGH alone. This can be rationalised with our previous demonstration that the epitope on rbGH defined by this MAb is removed from both the sites on GH responsible for GHR interaction (Beattie & Holder 1994) and, indeed, cell-based receptor assays and biosensor studies have confirmed that GH complexed with OA15 is still able to bind the extracellular domain of the ruminant GH receptor (Beattie *et al.* 1999). The results of the experiment described in Fig. 3 suggests that OA15-rbGH complexes are fully active. However, in our previous experiments with this antibody in 3T3-F442A cells, we have shown that (similar to OA11 and OA14 complexation) precomplexation of rbGH with OA15 attenuated the ability of the hormone to activate Jak-2. Although difficult to rationalise at present, it is clear that OA15 complexation with rbGH somehow affects the ability of hormone to activate GHR in the pre-adipocyte cell line, but not the oGHR-FDC-P1 cells. This may be related to receptor numbers per cell, or to some other differences in the spatial relationship between MAb-GH-GHR complexes in the two assay systems. Experiments in oGHR-FDC-P1 cells are currently

in progress to correlate the effect of MAb complexation on GH activity, as determined by 24-h MTT assay, with effects on short-term GH-stimulated signalling events.

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