Receptor-binding and down-regulatory properties of 22000- M_r human growth hormone and its natural 20000- M_r variant on IM-9 human lymphocytes

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Our earlier binding studies of the 22000- and 20000- M_r variants of human growth hormone (somatotropin) to pregnant-rabbit liver and mammary receptors [Closset, Smal, Gomez & Hennen (1983) *Biochem. J.* **214**, 885–892] suggested that the 20000- M_r variant was a lower-affinity analogue of the 22000- M_r molecule. Since the receptor population in these tissues is not fully characterized, we have now investigated the binding of both variants to the well-characterized and highly specific human-growth-hormone receptor of the human lymphocyte IM-9 cell line. The maximum bindability of radioiodinated 22000- and 20000- M_r to IM-9 cells was 60 and 45% respectively. Both hormone variants have essentially the same binding characteristics: slow association (equilibrium reached in 8–10 h at 30°C), poor reversibility ('tight binding'), linear Scatchard plot, same specificity as shown by lack of competition by bovine, porcine or equine growth hormones or human growth hormone-(32–46)- (missing in the 20000- M_r variant), -(1–134)- and -(141–191)-peptides. Both unlabelled hormones inhibit binding of both tracers completely, with the 20000- M_r variant being only half as potent as the 22000- M_r one. The apparent affinity is $2.8 \times 10^9 \text{ M}^{-1}$ for the 22000- M_r variant and $1.6 \times 10^9 \text{ M}^{-1}$ for the 20000- M_r variant. This decreased affinity of the 20000- M_r variant appears to be due to a lower association rate constant. Concentrations (5 ng/ml) of the two variants that occupy about 15% of the total sites induce a marked down-regulation of the receptors after 18 h incubation, but the 20000- M_r variant (50% decrease) has a smaller effect than the 22000- M_r variant (75% decrease). Thus the only consequence of the residues-32–46 deletion in the 20000- M_r variant is a lower association rate and affinity for the IM-9 lymphocyte human-growth-hormone receptor. The close binding characteristics of the two forms suggest that the known differences in their insulin-like effects cannot be explained by differences in the nature of their interaction with the human-growth-hormone receptor.

The 20000- M_r variant of hGH was recently discovered and characterized by Lewis *et al.* (1978). In contrast with the other forms of hGH, this variant is not an enzymically degraded form or an aggregate, but consists of a molecule that has exactly the same sequence as hGH (22000 M_r), except that it lacks the sequence of amino acids 32–46 (Lewis *et al.*, 1980). Moreover, there is evidence for a processed mRNA coding for the 20000- M_r

variant (De Noto *et al.*, 1981). The biological activity of the 20000- M_r variant of hGH has been tested in various bioassays [weight-gain test (Lewis *et al.*, 1978), tibia test (Spencer *et al.*, 1981; Closset *et al.*, 1983), somatomedin-production test (Spencer *et al.*, 1981), pigeon-crop-sac assay (Lewis *et al.*, 1978)]. There was no statistically significant difference between the biological activities of the two variants. The only discrepancy between the activities of hGH (22000 M_r) and its 20000- M_r variant is that, in contrast with hGH, the 20000- M_r variant appears to lack the early insulin-like activity (Frigeri *et al.*, 1979).

Abbreviations used: hGH, bGH, pGH and eGH, human, bovine, porcine and equine growth hormones (somatotropins) respectively; h.p.l.c., high pressure liquid chromatography; SDS, sodium dodecyl sulphate.

To understand better the molecular basis of the differences in activity of the 20000- M_r and 22000- M_r variants, it is interesting to compare the interaction of both variants with the growth-hormone (GH) receptor, i.e. to see if hGH and its 20000- M_r variant exert their effects by binding to the same receptor and, if so, whether the binding activity is comparable.

Sigel *et al.* (1981) and Wohnlich & Moore (1982) have published preliminary data on the binding of hGH and its 20000- M_r variant to receptors of rat and rabbit liver, where the 20000- M_r variant appeared to be a poor inhibitor of ^{125}I -hGH (22000- M_r) binding to these GH receptors. We have previously presented quantitative data on the binding sites of pregnant-rabbit liver membranes (Closset *et al.*, 1983). Our results suggested that hGH and its 20000- M_r variant bind to the same receptor with a lower difference in affinity than that suggested by the above-mentioned authors. During the course of this work, Hughes *et al.* (1983) published data that confirm our hypothesis that the 20000- M_r variant of hGH might bind with high affinity to a GH receptor present on rat and rabbit liver membranes. However, the GH receptor of the rabbit liver appeared to be a complex model for GH binding in that the population of binding sites is seemingly heterogeneous, both lactogenic and somatogenic (Cadman & Wallis, 1981; Hughes *et al.*, 1983).

Therefore we have continued our investigation using as a source of receptors the human lymphoid cell line IM-9. Indeed, the hGH receptor of these cells, described in 1973 by Lesniak *et al.* and since then widely used as a model for hGH binding, is unique in that it possesses absolute specificity for hGH and that the interaction of hGH with this receptor has been extensively characterized (Lesniak *et al.*, 1974; De Meyts, 1976).

Materials and methods

Materials

hGH (22000 M_r) and its 20000- M_r variant were purified as previously described (Closset *et al.*, 1983), except that the final ion-exchange step was performed by h.p.l.c. on a Mono Q HR 5/5 column (Pharmacia) with a linear (conductivity) gradient of 1.1–20 mS- NH_4HCO_3 .

bGH, pGH and eGH were prepared as described by Closset *et al.* (1983) for hGH. The bovine and porcine preparations were compared with the standard preparations NIH-GH-B-18 and USDA-pGH-B-1 respectively, by using a radio-receptor assay with pregnant-rabbit liver membranes as a source of GH receptors and as tracer the relevant bovine or porcine preparation. Both bGH and pGH displayed displacement curves

parallel with their standards and exhibited high potency.

The peptides corresponding to residues 1–134 and 141–191 were obtained by digestion of a purified preparation of hGH by human plasmin (Sigma) (Li & Graf, 1974) in 0.05 M- Na_2HPO_4 , pH 8.0, with an enzyme/substrate ratio of 1:250 (w/w) at 37°C for 4 h, reduction of the disulphide bridges with a 5 M excess of dithiothreitol (Calbiochem) over disulphide content in 1.4 M-Tris/HCl, pH 8.1, for 2 h, and carbamidomethylation by addition of recrystallized iodoacetamide (Janssen Pharmaceutica, Beerse, Belgium) in a 6-fold excess over reducing agent in the dark for 30 min. Excess reagents were removed by gel filtration on Sephadex G-25 in 0.05 M- NH_4HCO_3 , pH 8.0, and the plasmin fragments separated and purified by gel filtration on a column (200 cm $\times$ 2.5 cm) of Sephadex G-75 in 10% (v/v) formic acid. The peptides obtained were characterized by SDS/polyacrylamide-gradient (5–25%, w/v)-gel electrophoresis (Laemmli, 1970) and N-terminal-residue analysis.

The peptide corresponding to hGH residues 32–46 was synthesized by UCB-Bioproducts, Brussels, Belgium.

Both the 22000 and the 20000- M_r proteins were labelled with Na^{125}I (IMS-30; Amersham International) by the Iodogen (1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril; Pierce Chemicals) method as previously described (Closset *et al.*, 1983) and purified on Sephadex G-100. The specific radioactivity ranged between 60 and 70 $\mu\text{Ci}/\mu\text{g}$ and 20 and 30 $\mu\text{Ci}/\mu\text{g}$ for ^{125}I -22000- M_r -GH and the variant ^{125}I -20000- M_r -GH respectively. About 60% of ^{125}I -22000- M_r -GH and 45% of ^{125}I -20000- M_r -GH were specifically bound to an excess of lymphocyte receptors in terms of (bound-non specific)/total.

Growth of cultured lymphocytes

Human lymphocytes (line IM-9) were maintained at 37°C in RPMI 1640 medium supplemented with 10% (v/v) foetal-calf serum, penicillin at 100 units/ml and streptomycin at 100 $\mu\text{g}/\text{ml}$, by feeding three times a week (De Meyts, 1976; Gavin *et al.*, 1974). The cells were used in the binding studies when they were in stationary phase of growth (Fahey *et al.*, 1971).

Lymphocyte binding assays

^{125}I -22000- M_r -GH and ^{125}I -20000- M_r -GH binding to the IM-9 lymphocytes was assayed essentially as previously described (De Meyts, 1976). Centrifuged cells were washed three times in assay buffer (100 mM-Tris/120 mM-NaCl/1.2 mM- MgSO_4 /1 mM-EDTA/10 mM-Tris/120 mM-sodium acetate/bovine serum albumin (10 mg/ml),

pH 7.0), then resuspended for cell counts and binding experiments. Cell viability was determined by the Trypan Blue-exclusion method (Rosselin *et al.*, 1966). For each comparative experiment, a single batch of cells was used for the binding of both ^{125}I -22000- M_r -GH and ^{125}I -20000- M_r -GH tracers. Steady-state binding of the tracers in the presence of increasing concentrations of hormones and peptides was determined after 10 h at 30°C. Binding of ^{125}I -22000- M_r -GH and ^{125}I -20000- M_r -GH in the presence of unlabelled 22000- M_r and 20000- M_r hormone at 10 $\mu\text{g/ml}$ was measured and subtracted as 'non-specific binding' (De Meyts, 1976) to yield specific binding data.

Scatchard analysis of binding data

Scatchard (1949) analysis was performed by using binding competition experiments in which a constant amount of receptor was incubated with increasing concentrations of unlabelled hormones.

The maximum bindability of the tracers (percentage of radioactively labelled hormone specifically bound to an excess of receptors) was used for correction. The analysis of binding data was performed by using the MLAB curve-fitting program described by Knott & Schragar (1972) run on a PDP-20 time-sharing digital computer.

Down-regulation

Starting from the same lymphocyte batch, three flasks each containing 15×10^7 cells in 150 ml of culture medium were preincubated with, respectively, 5 ng of 22000- M_r -GH/ml, 5 ng of 20000- M_r -GH/ml and no hormone for the control flask. The cells were then collected by centrifugation and washed four times in assay buffer. We determined the residual binding capacity of the preincubated cells and the control binding capacity as previously described (De Meyts, 1976).

Results

Association and dissociation kinetics

The association of both ^{125}I -22000- M_r -GH and ^{125}I -20000- M_r -GH to IM-9 cells (2×10^7 cells/ml) at 30°C was slow (Fig. 1a). Even though the initial rate of association was identical for the two forms, the overall rate of association was found to be slower for the 20000- M_r form (Fig. 1b). Specific binding reached a plateau after 7–8 h for the 22000- M_r and 10 h for the 20000- M_r variant. Therefore the time of incubation was prolonged to 10 h in all comparative binding experiments in order to ensure that a steady state was reached by both the 22000- and the 20000- M_r hormones.

The dissociation kinetics of the two tracers from

Fig. 1. Kinetics of association of 22000- M_r -GH and 20000- M_r -GH with IM-9 lymphocytes. ^{125}I -22000- M_r -GH (approx. 7500 c.p.m.) (○) and ^{125}I -20000- M_r -GH (approx. 7500 c.p.m.) (●) were incubated with IM-9 lymphocytes (2×10^7 cells/ml) at 30°C. At the times indicated, the specifically bound radioactivity (a) and the percentage of specific binding reached at the steady state (b) were determined for the two hormones. Inset: normalized initial rates of association of ^{125}I -22000- M_r -GH (○) and ^{125}I -20000- M_r -GH (●) (expanded scales).

Fig. 2. Kinetics of dissociation of 22000- M_r -GH and 20000- M_r -GH from IM-9 lymphocytes

In this case, ^{125}I -22000- M_r -GH (○) and ^{125}I -20000- M_r -GH (●) were incubated with IM-9 lymphocytes (8.5×10^7 cells/ml) for 3 h at 30°C. Dissociation was initiated by a 1:40 dilution of 50 μl samples in 2 ml of the test buffer, and for the time of dilution the residual specific binding was determined for the two hormones.

IM-9 lymphocytes at 30°C at 1:40 dilution were comparable. The binding of the two forms of the hormone was not first-order. It is in effect poorly reversible in both cases by dilution (Fig. 2) and by addition of unlabelled hormone ($2 \mu\text{g}$ of 22000- M_r -GH/tube).

Competition curves

Each of the two labelled forms of hGH was incubated to steady state at 30°C in the presence or absence of increasing concentrations of unlabelled 22000- M_r -GH, 20000- M_r -GH, bGH, pGH, eGH and hGH peptides representing residues 1-134, 141-191 and 32-46. Under our conditions of incubation, the initial specific binding of the tracers was, respectively, 16% for the hGH (22000 M_r) and 9% for the 20000- M_r variant. When tracer binding was normalized, the competition curves determined with both unlabelled hormones were the same with either tracer (Figs. 3a and 3b). The 22000- and 20000- M_r -GH completely displaced

Fig. 3. Competition curves of binding to IM-9 lymphocytes

Tracers are (a) ^{125}I -22000- M_r -GH (approx. 7500 c.p.m.) and (b) ^{125}I -20000- M_r -GH (approx. 7500 c.p.m.). IM-9 lymphocytes (2×10^7 cells/ml) were incubated for 10 h at 30°C with each of the tracers plus increasing concentrations (0.1–10000 ng/ml) of unlabelled 22000- M_r -GH (○), 20000- M_r -GH (●), pGH (▼), bGH (■), eGH (◆) and (1-134)-peptide (◇), (141-191)-peptide (□) and (32-46)-peptide (△), and B/B_0 (specific radioactivity bound)/(specific radioactivity bound in the absence of unlabelled hormone) values were determined.

Fig. 4. Scatchard analysis of 22000- M_r -GH (a) and 20000- M_r -GH (b) binding to IM-9 lymphocytes. Binding experiments similar to those of Figs. 3(a) and 3(b) were performed with cells of a single batch (2×10^7 cells/ml). ^{125}I -22000- M_r -GH and ^{125}I -20000- M_r -GH were equilibrated with increasing concentrations (as for Fig. 3) of their homologous unlabelled forms. The bound/free radioactivity is plotted as a function of bound hormone.

Fig. 5. Down-regulation of hGH receptors of IM-9 lymphocytes by 22000- and 20000- M_r -GH.

Cells were preincubated with 5 ng/ml of 22000- M_r -GH, 20000- M_r -GH or no hormone (control) for 18 h, washed, resuspended (2×10^7 cells/ml) and incubated for 2 h at 30°C with ^{125}I -22000- M_r -GH and increasing concentrations of unlabelled 22000- M_r -GH (as in Fig. 3). In comparison with the control curve ($\blacktriangle$) (no hormone preincubated), ^{125}I -22000- M_r -GH binding to cells preincubated with 20000- or 22000- M_r -GH was decreased by respectively 50% ($\bullet$) and 75% ($\circ$).

both tracers, the 22000- M_r -GH having twice the potency of the 20000 M_r -GH in both systems. By contrast, pGH, bGH and the (32–46)-peptide (the peptide sequence absent from the 20000- M_r variant) did not inhibit the binding of either tracer to the hGH IM-9 receptor at concentration of 10 $\mu\text{g}/\text{ml}$. eGH and the (1–134)- and (141–191)-peptides competed with the tracers with a very low potency relative to the 22000- and 20000- M_r -GH. The inhibition of binding by the peptides can be

accounted for by the minor contamination of these preparations with the native hormone [0.6% for the (1–134)-peptide and 0.1% for the (141–191)-peptide].

These results are in agreement with the high specificity of the IM-9 lymphocyte receptor for hGH (Lesniak *et al.*, 1974, 1977).

Scatchard plots

Competition curves with both tracers in homologous systems, with identical concentrations of lymphocytes (2×10^7 cells/ml) from the same pool of cells, were analysed as described by Scatchard (1949). Linear plots were obtained, revealing a single class of binding sites with a high affinity for the two forms of hGH (Figs. 4a and 4b). The affinity constant was $2.8 \times 10^9 \text{ M}^{-1}$ for the 22000- M_r and $1.6 \times 10^9 \text{ M}^{-1}$ for the 20000- M_r hormone. There is a ratio of 0.6 between the observed K_a for 20000- M_r -GH and that of the 22000- M_r -GH. The receptor concentration was the same for the two proteins, namely 2700 sites/cell.

Down-regulation

The abilities of the 22000- and 20000- M_r forms of hGH to down-regulate the receptors in IM-9 cells during 18 h preincubation at 37°C were compared. Concentrations (5 ng/ml) of the two variants occupying only 15% of the total sites induced a marked down-regulation of the receptors after preincubation. However, the 20000- M_r variant, which decreased the binding of ^{125}I -22000- M_r -GH to 50% of the control number of sites, had a smaller effect than the 22000- M_r -GH (75% decrease) (Fig. 5).

Discussion

Lesniak *et al.* (1973, 1974, 1977), and subsequently other groups, have studied the characteristics of the hGH receptor of the IM-9 cell line. Given the high specificity of this system, we have used the IM-9 cell line in order to compare the interaction of hGH (22000 M_r) and its 20000- M_r variant with a hGH receptor.

The results obtained in the present study show that hGH and its 20000- M_r variant interact with this receptor in a similar manner. Both 22000- M_r and 20000- M_r variants show slow association kinetics and poor reversibility of binding. The observed time course of association is in agreement with the data reported by Rosenfeld & Hintz (1980) (>8 h) for hGH binding to the same cell line, although Retegui *et al.* (1982) (5 h) and Lesniak *et al.* (1974) (90 min) have reported a faster time course of association.

The dissociation kinetics obtained are consistent with those from studies on the internalization of ^{125}I -hGH in cultured human lymphocytes (Rosenfeld & Hintz, 1980; Barazzone *et al.*, 1980). hGH binding to rat hepatocyte receptors has also been shown to be poorly reversible (Donner *et al.*, 1980). Each form is able to inhibit completely the binding of the other from the IM-9 cell receptor, with 22000- M_r -hGH exhibiting twice the potency of the 20000- M_r variant. Scatchard analysis of competitive binding curves gives apparent affinity constants of $2.8 \times 10^9 \text{ M}^{-1}$ for the 22000- M_r form and $1.6 \times 10^9 \text{ M}^{-1}$ for the 20000- M_r variant, with a capacity ($R_0 = 2700$ sites/cell) that is identical for the two forms. The affinity constants and receptor number are in the same range as the values previously published for hGH by Lesniak *et al.* (1974) ($K_a = 1.3 \times 10^9 \text{ M}^{-1}$; $R_0 = 4000$ sites/cell) and Retegui *et al.* (1982) ($K_b = 2.43 \times 10^9 \text{ M}^{-1}$; $3.45 \times 10^9 \text{ M}^{-1}$; $R_0 = 3200$ sites/cell; 2070 sites/cell). We are aware that the estimation by Scatchard analysis of the affinity constants of a hormone that exhibits poor reversibility of binding to its receptor must be treated with caution, since the simple equilibrium model on which it is based may not apply (Ketelslegers *et al.*, 1984). However, our aim was not to define accurately an affinity constant for hGH, but to compare the apparent affinity constants of the two growth-hormone variants, and also to compare the values obtained with those previously reported for hGH binding to the same receptor.

In our study, the labelled ^{125}I -20000- M_r variant showed reduced binding when compared with the ^{125}I -22000- M_r tracer. There is evidence that this reduced binding was not due to a greater iodination damage than that created by the process for the 22000- M_r form. Indeed, both labelled ^{125}I -20000- and ^{125}I -22000- M_r -hGH possessed the

same elution volume on gel filtration, and parallel curves were recorded with the unlabelled 20000- M_r variant and the labelled one in autodisplacement experiments (results not shown). Moreover, the unlabelled 20000- M_r hGH exhibited half the potency of that of the 22000- M_r form in competition studies. That correlates well with the reduced binding of its labelled form to the IM-9 lymphocyte receptor.

The hGH-receptor concentrations on the IM-9 cell line are known to be regulated by the ambient concentrations of GH (Lesniak & Roth, 1976). We found that both 22000- and 20000- M_r -hGH induce a decrease in ^{125}I -hGH binding. Since the binding of both forms of hGH to the IM-9 cells was poorly reversible, it is possible that some or all of the decrease in binding is due to residual occupancy of the site. This explanation, however, is not likely, since the doses used for preincubation were chosen to occupy not more than 15–20% of the sites at apparent equilibrium. Indeed the reduction in the binding (50% for the 20000- M_r -GH, 75% for the 22000- M_r -GH) was much greater than the occupied fraction, suggesting that a true down-regulation accounted for at least part of the observed decrease. This down-regulation may be explained by the internalization of hGH in IM-9 cells (Hizuka *et al.*, 1981). However, our data confirm that receptor binding is the first step in the down-regulation process, since 22000- M_r -GH was found to be 1.5 times more potent in this process than its 20000- M_r counterpart. This correlates well with the higher affinity constant of the 22000- M_r -GH, which can be calculated to occupy 1.7 times as many specific binding sites as its 20000- M_r variant at steady state.

Taken together, our results show that the only apparent consequence of the deletion of residues in 20000- M_r -hGH is that this variant is recognized by the receptor on the IM-9 cell line as an analogue of lower affinity. This difference in affinity is possibly due to slight conformational changes. These results confirm our previously published data on the binding of both 22000- and 20000- M_r molecules to pregnant-rabbit liver and mammary-gland receptors (Closset *et al.*, 1983). In that work, 20000- M_r -GH was found to have half the potency of 22000- M_r -GH in the inhibition of binding of the ^{125}I -22000- M_r form and the ^{125}I -20000- M_r variant to the rabbit liver receptor. However, the two variants did not recognize the same number of sites. This can be explained by the heterogeneity of the binding sites present in that tissue. Hughes *et al.* (1983) have proposed a model in which rabbit liver contains a low-capacity GH receptor, which binds hGH (22000- M_r) and the 20000- M_r variant with comparable affinities, and a second receptor, which binds hGH (22000 M_r)

with high affinity and the 20000- M_r variant with low affinity. In contrast, the IM-9 model for hGH binding presents a unique receptor for the 22000- and 20000- M_r -GH, which allows a better control in the comparison of the two forms of hGH in a homologous system.

The differences in the binding of 22000- and 20000- M_r -GH to the IM-9 cell receptor are not as marked as suggested by Sigel *et al.* (1981) and Wohnlich *et al.* (1982). Nevertheless, the affinity of 20000- M_r -GH is only 60% that of 22000- M_r -GH, which is in good agreement with the findings of Hughes *et al.* (1983), who used rat liver membrane as a source of receptors. This must be compared with the reported identical biological properties of both variants. The slight discrepancy may be due to the fact that these were bioassays 'in vivo', where factors other than receptor binding *per se* may contribute, such as half-life and degradation 'in vivo', which have not been measured.

On the other hand, Frigeri *et al.* (1979) have demonstrated that the 20000- M_r variant has lost the insulin-like activity normally associated with the 22000- M_r hormone. In fact, the 20000- M_r variant is not able to decrease either serum glucose or non-esterified fatty acids 1 h after injection into fasted hypophysectomized rats. Even so, the 20000- M_r variant does not accelerate glucose utilization in epididymal adipose tissue from hypophysectomized rats (Frigeri *et al.*, 1979). Since both variants do bind to the hGH receptor, a logical hypothesis would be that the differences between the insulin-like effects of both variants reside at the post-receptor level, that is, in the coupling between binding and biological activity.

However, we cannot exclude the alternative hypothesis that the receptors directly implicated in the production of the insulin-like effects by hGH are different from those studied so far, and do not recognize the 20000- M_r variant of the hormone. Hughes *et al.* (1983) reported recently that a subset of rabbit liver receptors recognize the 20000- M_r variant with only a slight affinity. Gavin *et al.* (1982) have also shown that the specificity of the GH receptor of the rat adipocyte is different from that of the hGH receptor on IM-9 human lymphocytes.

A more appropriate model with which to study this problem would be the adipocyte of the hypophysectomized rat, for which the correlation between binding and insulin-like effect *in vitro* could be determined (Grichting *et al.*, 1983).

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