Vol. 134, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 14, 1986 Pages 159-165

THE RECEPTOR BINDING PROPERTIES OF THE 20K VARIANT OF HUMAN GROWTH HORMONE EXPLAIN ITS DISCREPANT INSULIN-LIKE AND GROWTH PROMOTING ACTIVITIES

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Received November 21, 1985

Summary : The 20K variant of native (22K) hGH is a full agonist for the growth promoting and lactogenic properties of the hormone in vivo but has been reported to have weak or absent insulin-like properties. To explore if these differences may be explained at the receptor level, we compared the ability of 22K and 20K hGH to inhibit the binding of ^{125}I-22K hGH to receptors in isolated rat adipocytes, a target for the insulin-like effects of the hormone and in IM-9 cultured human lymphocytes, more specific for growth effects. Our data show that while 20K hGH is a potent agonist of native 22K hGH in the IM-9 lymphocyte assay, its potency in the rat adipocyte binding assay is only 3%, even when both cells are incubated together in identical conditions. Thus, the receptors for hGH appear to be different on various target cells, explaining why the 20K variant has different relative biological potencies at different sites of action.

A variant of human growth hormone (hGH) with a molecular weight of 20,000 daltons (20K) instead of 22,000 (22K), due to a 15 amino acid deletion (32 to 46) has been found in both the pituitary (1,2) and the circulation (3,4). The consequence of the deletion on the biological properties of the hormone depends on the effect considered. The growth promoting and lactogenic activities of the two forms are not significantly different in various bioassays (1,2,5,6), while there is controversy concerning their diabetogenic and insulin-like properties (7-9).

The precise biochemical mechanisms by which growth hormone exerts these various effects are still unclear. The first step in the action of polypeptide hormones is binding to a specific cell surface receptor. So far, few authors have attempted to compare the binding properties of 22K and 20K hGH in various systems in order to explain the discrepant properties of the GH variants. We showed recently (10) that the human IM9 lymphocyte receptor, probably the most specific (11) for human growth hormone recognizes the 20K variant with 50-60 % of the affinity for 22K, using competition studies as well as direct binding of ^{125}I-20K hGH. We had demonstrated previously (6) that 22K and 20K hGH had a similar affinity for the receptors of pregnant rabbit liver and mammary glands, probably related to both growth and lactogenic activities. The high affinity of 20K hGH for these various receptors was in agreement with the data of Hughes et al (12) in pregnant rat and

0006-291X/86 $1.50

adult female rabbit liver membranes. In contrast, much lower relative affinities of the 20K hGH had been reported earlier in rat and rabbit liver and mammary glands by Sigel et al (13) and Wohnlich et al (14).

In the present work, we compare for the first time the binding of 20K hGH and 22K hGH in a model more relevant to their insulin-like effects, the isolated rat adipocyte, previously used by several authors for studying 22K hGH binding (15-17). We found that in contrast to the IM-9 lymphocyte receptor, the rat adipocyte receptor recognizes poorly the 20K hGH variant, a finding consistent with its potent growth promoting but poor insulin-like agonist properties.

MATERIALS AND METHODS

Hormones and tracers The 20K and 22K hGHs were purified as described previously (10). The contamination of the 20K by 22K hGH was < 0.4 % as measured by a specific monoclonal radioimmunoassay (6). 22K and 20K hGHs were labelled as described in (10).

Chemicals and reagents Bacitracin was from Aldrich (Milwaukee, Wis, USA), Trasylol from Bayer (Leverkusen, W.Germany), collagenase (Clostridium histolyticum) from Boehringer (Mannheim, W.Germany), bovine serum albumin (BSA) fraction V from Sigma Chemical Company (St Louis, Mo, USA), Dinonylptalate from Merck (Darmstadt, W.Germany). Other chemicals were of reagent grade and obtained from Merck. Bovine serum albumin was freed of fatty acids by charcoal treatment (18).

Lymphocyte culture and binding assay IM-9 human lymphocytes were cultured and used in binding assays (10 hours, 30°C, 2 X 10^7 cells/ml) as described previously (10).

Animals Male Wistar rats (140-150 g) were maintened in light-dark cycles of 12 hrs and fed ad libitum.

Adipocytes preparation and growth hormone binding assays We adapted the procedure of Gliemann and Sonne (19). Briefly, dissected epididymal and retroperitoneal fat pads were digested under vigourous shaking at 37°C for 30-60 min with collagenase (0.5 mg/ml) in Krebs-Ringer-Hepes (KRH) buffer pH 7.4, 35 mg/ml delipidated BSA, 5.5 mM glucose and 0.5 mg/ml bacitracin. After filtration on cheesecloth and 4 washes in KRH with 10 mg/ml BSA, the adipocytes were suspended in the binding buffer (KRH with 50 mg/ml BSA, glucose 5.5 mM, bacitracin 0.5 mg/ml and Trasylol (10,000 units KI/liter). The concentration of adipocytes was adjusted to 25 % (V/V) by measuring the cell volume in a IEC-MB microhematocrit centrifuge.

The growth hormone binding assays were performed in 5 ml polypropylene tube (75 X 12 mm) in duplicate, by adding successively 50 ul ^{125}I-22K hGH (100,000 cpm corresponding to 40 pM final), 50 ul assay buffer containing unlabelled hormones (0 - 10 ug/ml final) and 400 ul of adipocyte suspension, in a total volume of 0.5 ml.

By counting the cells on a Burker plate, we determined that the final concentration of 20 % (V/V) = 1.6 X 10^6 cells/ml.

For steady state binding studies, the incubation time was 75 min at 37°C under vigourous shaking (120 cycles/min).

Incubations were interrupted by adding successively 3 ml of ice-cold 9 ‰ NaCl and 1 ml of dinonylphtalate to separate the cell layer from the aqueous medium. The tubes are immediately centrifuged at 4°C, 5000 rpm for 5 min. The floating cells are recovered with a 500 ul Eppendorf pipette fitted with a truncated tip. The tips are placed in 5 ml plastic tubes and centrifuged 5 min at 3000 rpm before counting. Cell-associated radioactivity in the presence of 10 ug/ml unlabelled hormone is

subtracted as non-specific binding. Binding data were treated by computer as described in (10).

Simultaneous binding of hGH to lymphocytes and adipocytes To study the binding of hGH to IM-9 lymphocytes and adipocytes under strictly comparable conditions, we took advantage of the different densities of the two cell types to incubate them simultaneously in the same medium and to separate them by centrifugation. Most hormone remains free in our assay conditions so that the presence of one binding component is not expected to affect the other significantly. Binding was conducted in the adipocyte assay conditions described above, except that the final volume was 700 ul, due to the addition of 600 ul of a mixture of adipocytes and lymphocytes. The final concentration of adipocytes was 1.6 X 10^6 cells/ml and lymphocytes 2 X 10^7 cells/ml. At the end of the incubation (75 min at 37°C under vigourous shaking), 200 ul aliquots were removed from each tube and layered upon microfuge tubes containing 200 ul of ice-cold assay buffer. The microfuge tubes were centrifuged immediately for one minute in a Beckman microfuge. The supernatants were discarded by aspiration and the tips containing the lymphocyte pellets cut and counted.
The remaining 500 ul of incubation were treated according to the adipocyte binding assay protocol.

RESULTS

Kinetics of association of ^{125}I-22K and 20K hGHs to rat adipocytes

^{125}I-22K hGH (40 pM) bound rapidly, with an apparent steady state at 75 min at 37°C (Fig. 1). Specific binding was usually 5 - 8% of tracer with only 0.4 -0.9 % non specifically bound. Comparatively, specific binding of ^{125}I-20 K hGH (data not shown) was hardly detectable (200-300 cpm bound from a total of 100,000 cpm), suggesting that in the adipocyte the affinity of ^{125}-I-20K hGH was at least 25 times lower than ^{125}I-22K hGH. Thus, the properties of the 20K hGH could only be examined in competition studies using 22K hGH as a tracer. To ensure that the competition curve with 20K would also be at steady-state, we studied the

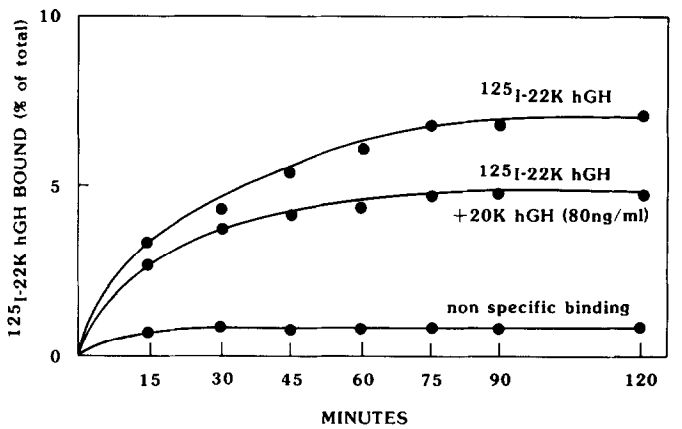

Fig. 1. Kinetics of association of ^{125}I-22K hGH to rat adipocytes.
^{125}I-22K hGH (40 pM) was incubated as described in "Materials and methods" with rat adipocytes (1.6 X 10^6/ml) in the absence of competing hGH (upper curve), in the presence of 80 ng/ml of 20K hGH (middle curve), or in the presence of 10 ug/ml of 22K hGH (lower curve = non-specific binding). The cell associated radioactivity was measured at various times. The fraction (in %) of tracer bound is expressed as a function of incubation time.

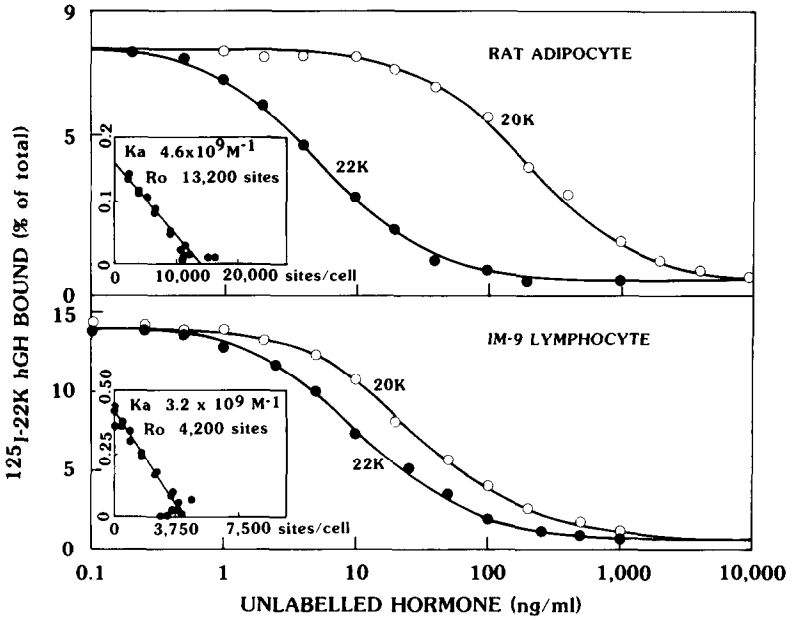

Fig. 2. Inhibition of ^{125}I-22K hGH binding to rat adipocytes (upper panel) and IM-9 lymphocytes (lower panel) by increasing concentrations of unlabelled 22K and 20K hGH.
The ^{125}I-22K hGH (40 pM) was incubated separately in the respective assay buffers for 75 min at 37°C with 1.6 X 10^6 adipocytes/ml or 10 hr at 30°C with 20 X 10^6 lymphocytes/ml, in the presence of 0-10 ug/ml of unlabelled 22K or 20K hGH.
The fraction of tracer bound is plotted as a function of the concentration of competing hGH. Insets : Scatchard plots of the 22K competition data. The solid line is drawn according to the computer-fitted parameters (apparent affinity constant (Ka) and binding capacity (Ro)).

association kinetics of ^{125}I-22K hGH in the presence of 80 ng/ml 20K hGH. An apparent steady state was obtained at 75 min. After that time, the inhibition of tracer binding by 20K hGH was constant amounting to about 30 %. The kinetics of association of ^{125}I-hGH (22K and 20K) to IM-9 lymphocytes had been shown previously (10) to require much longer times of association (6 to 10hrs) to reach an apparent steady state.

Competition curves and Scatchard analysis of rat adipocyte binding

The specific binding of ^{125}I-22K hGH to rat adipocytes was completely inhibited by both 22K and 20K hGH (Fig. 2 top). A concentration of 200 ng/ml of 20K hGH was required for 50 % inhibition versus only 6 ng/ml of 22K hGH. The 20K hGH thus has a relative potency of 3 %. The Scatchard plot of the 22K hGH competition data is linear, suggesting a single class of sites with an apparent affinity constant of 4.6 X 10^9 M^{-1} and a binding capacity of 13,200 sites/adipocyte[1].

[1]The capacity value is valid, but since hGH binding is not a simple reversible process (22), the slope derived from Scatchard analysis is given here as an apparent Ka value only for comparison purposes. A more appropriate quantitative computer analysis of hGH binding based on a more complex model is in progress (Ilondo and De Meyts, in preparation).

Competition curves of IM-9 lymphocyte binding

Control competition binding experiments in IM-9 cells (Fig. 2, bottom) are presented with the same hormone preparation batches as used in the adipocyte system. 20K hGH was found 40% as potent as 22K hGH in competing for ^{125}I-22K hGH binding, close to our previously reported 50 % (10). Scatchard analysis of the 22K competition curve in IM-9 cells shows a single class of sites with an apparent affinity constant of 3.2 X 10^9 M^{-1}. The concentration of 22K hGH needed to inhibit tracer binding by 50% was 11 ng/ml in the case of the lymphocyte versus 6 ng/ml for the adipocyte, suggesting a higher affinity for the adipocyte receptor.

Simultaneous incubation of rat adipocytes and IM-9 lymphocytes

In order to ascertain that the observed differences in binding of 22K and 20K hGH to adipocytes and lymphocytes were not due to the differences in the incubation conditions, the binding assays were done with IM-9 lymphocytes and rat adipocytes incubated simultaneously in the same tube under adipocyte assay conditions as described in "Materials and methods". The competition curves of 20K and 22K hGH were superimposable to those observed when each cell type had been studied separately under different conditions, with the exception of a slight shift to the right of the curve for 20K hGH with IM-9 lymphocytes (fig. 3). This is likely due to the short incubation time and slower association kinetics for 20K hGH in IM-9 cells (10), given that the tracer used here is ^{125}I-22K hGH.

The discrepancy between the relative potencies of 22K and 20K hGH in adipocytes versus lymphocytes remains striking in the simultaneous incubation conditions, 20K

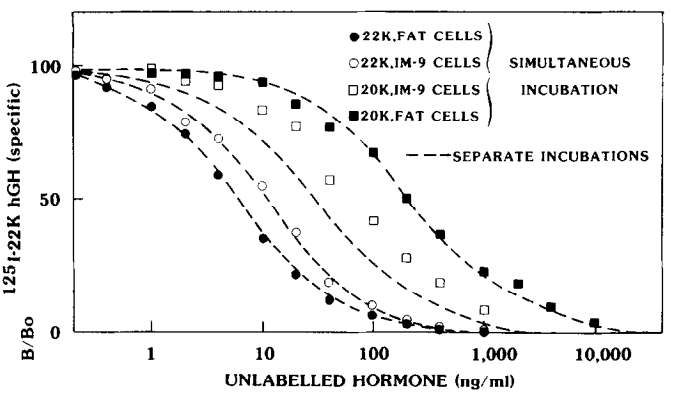

Fig. 3. Inhibition of ^{125}I-hGH binding to rat adipocytes and IM-9 lymphocytes incubated together in the same tube, by unlabelled 22K and 20K hGH.
^{125}I-22K hGH was incubated with a mixture of rat adipocytes (1.6 X 10^6/ml) and IM-9 lymphocytes (20 X 10^6/ml) in adipocyte assay buffer for 75 min at 37°C, in the presence of 0 - 10 ug/ml of unlabelled 22K or 20K hGH.
At the end of incubation, lymphocytes were isolated from an aliquot by centrifugation, and adipocytes separated from the remaining volume by flotation on dinonylphtalate. The fraction of tracer specifically bound (B/Bo in %) to each cell type is plotted as a function of the concentration of competing ligand. Binding in the presence of 10 ug/ml of unlabelled hGH has been subtracted as non-specific.

hGH being only 3% as potent as 22K hGH in adipocytes versus 25% in the IM-9 cells. The higher affinity of the 22K hGH for the adipocyte than for the IM-9 lymphocyte receptor is also apparent in the simultaneous binding experiments.

DISCUSSION

While 22K and 20K hGH have similar growth promoting and lactogenic properties, they differ markedly in their effects on glucose metabolism, although there is some controversy concerning the latter aspect. Thus, Lewis et al (7) reported that 20K hGH lacked the diabetogenic activities (induction of hyperglycemia and glucose intolerance in dogs), while more recently, Kostyo et al (8) showed that a 20K hGH prepared by recombinant DNA methods has essentially the same diabetogenic activity as 22K hGH when given chronically to ob/ob mice. As for the acute insulin-like actions of hGH, Frigeri et al (9) reported that 20K hGH lacked this property, while Kostyo et al (8) reported that recombinant 20K hGH has a weak (20 %) in vitro insulin-like activity (stimulation of glucose oxidation in epididymal adipose tissue of hypophysectomized rats).

Our new data help explaining the discrepancy between the strong growth promoting effects and weak insulin-like effects of 20K hGH.

Indeed, our data demonstrate several differences between the adipocyte and the IM-9 receptor, suggesting that they may be of a different nature.

First, the potency in competition and apparent affinity of binding of the 22K hGH is higher in the adipocyte than in the IM-9 cell. This is true both in binding conditions optimal for each cell type and in simultaneous incubation of both cells.

Second, the association kinetics of ^{125}I-22K hGH is much faster with the adipocytes, although this was determined at 37°C for adipocytes versus 30°C for IM-9 cells.

Third, most importantly, the adipocyte receptor recognizes significantly less the 20K variant than the IM-9 lymphocyte receptor. The potency of 20K hGH relative to 22K hGH is only 3% in adipocytes whether the cells are studied in separate or in simultaneous conditions. This low potency is intrinsic to the 20K hGH since contamination with 22K hGH was $< 0.4\%$. We have obtained quantitatively similar data with adipocytes from hypophysectomized rats (data not shown).

The lower relative affinity of the 20K hGH for rat adipocyte versus human lymphocyte receptors could be due to the species specificity rather than the tissue difference. However, Hughes et al (12) have reported that the affinity of the 20K hGH for rat liver receptors is only slightly lower than that of 22K hGH. Moreover, 20K hGH has a growth promoting activity similar to that of 22K hGH in the rat tibia test (1,6).

The relative affinity of 20K hGH for the human IM-9 lymphocyte receptor is consistent with its potent growth promoting activity, especially since the lower

metabolic clearance rate in vivo of 20K hGH (21) would compensate for the slight decrease in receptor binding.

Conversely, the very low relative binding affinity of 20K hGH for the rat adipocyte receptor is consistent with its reported low insulin-like activity.

Thus, the receptors for hGH appear to be different on various target cells, explaining why the 20K variant has different relative biological potencies at different sites of action.

ACKNOWLEDGEMENTS

We are grateful to Mr. J. Freson for expert technical assistance in preparing the hormone and Mrs. A. Dukers for excellent secretarial assistance. P. De Meyts is a qualified investigator of the Belgian National Fund for Scientific Research and recipient of FRSM grant No 3.4537.84.

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