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Growth hormone-albumin conjugates

Reduced renal toxicity and altered plasma clearance

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The effective therapeutic use of many small peptides such as growth hormone has been limited by their small molecular masses and rapid clearance by the kidneys. Moreover, various degrees of nephrotoxicity have been reported for small proteins which are readily filtered at the level of the glomerulus. We have attempted to circumvent this drawback by conjugating growth hormone (somatotropin) to serum albumin in an effort to alter the peptide's pharmacokinetics while retaining its biological activity.

Growth hormone; Albumin; Plasma clearance; Protein conjugate; Renal toxicity; Drug delivery

1. INTRODUCTION

Small polypeptides with molecular masses in the order of 20000 Da have produced significant interest in recent years in terms of their therapeutic potential. Cytokines have been examined as anti-tumor agents due to their ability to stimulate the immune system, interferons are being examined as anti-viral agents, small enzymes such as tissue plasminogen activator (TPA) are being examined for their lytic activity and a wide variety of hormones are being tested for their therapeutic efficacy in a range of clinical situations. This paper deals with growth hormone (GH), some of its clinical limitations and the possibility of circumventing some of these by chemically cross-linking the hormone to homologous albumin.

Human growth hormone (both native and genetically engineered) has been used for a number of years clinically for the stimulation of growth in children with growth hormone deficiencies [1]. Results have been encouraging, but given the rapid clearance of the hormone and the fairly high potential for immune reactivity, such hormone therapy is still considered a fairly experimental treatment. In the agriculture arena the possibility of administering growth hormone to cattle and swine has been widely considered [2]. This would lend itself not only to more rapid growth and weight gain but also to the production of a leaner animal as the growth hormone stimulates lipolysis. Several factors mitigate against the economic use of growth hormones in such instances. The rapid renal clearance of the peptide resulting in accumulation in the kidney will have to be resolved before growth hormone and other similar peptides are used effectively.

Our laboratory has for a number of years been involved in the use of albumin as a means of making enzymes more amendable for use in medicine. Our approach has been to conjugate enzymes to homologous albumin in an effort to: (i) stabilize the enzyme, (ii) alter the immunogenic nature of the enzyme, usually a foreign protein, and (iii) target the enzyme to specific tissue sites by the use of appropriately conjugated ligands [3,4]. In respect to the targeting issue, we have been successful in cross-linking insulin to either enzymes or to enzyme-albumin conjugates while retaining the insulin's biological activity and its ability to bind

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to and be internalized by its cell surface receptor [5]. We rationalize that if insulin can be conjugated to albumin while retaining its biological activity then the same should be true of a number of other small peptide hormones. Conjugation might therefore be used to dramatically alter the pharmacokinetics and potential usefulness of administered hormones such as growth hormone.

2. MATERIALS AND METHODS

2.1. *Preparation of growth hormone-albumin conjugates*

Porcine growth hormone was conjugated to either human or porcine serum albumin using glutaraldehyde as the cross-linking agent and using procedures [4] described for the conjugation of insulin to either enzymes or enzyme-albumin polymers. 1 mg of porcine growth hormone was dissolved in 1 ml of 50 mM PBS at pH 7.8. 1 mg of albumin in 3 ml of buffer was added with stirring at 4°C. 25 μl of 25% glutaraldehyde was added with stirring and the reaction allowed to proceed for 3 h at which time the cross-linking was quenched by the addition of 1 mg of glycine. The resultant mixture was dialyzed overnight against PBS (with 1% glycine) using dialysis tubing with a 35 kDa cut-off. The conjugate was purified by molecular sieve chromatography using Sephadex G-150.

2.2. *Lipolysis assay*

Lipolysis was measured according to described techniques [6] by determining glycerol production of tissue fragments of epididymal fat pads harvested from male Sprague Dawley rats. The tissue was incubated for 4 h in the presence or absence of varying concentrations of growth hormone or growth hormone-albumin conjugates.

2.3. *Insulin-like growth hormone activity*

Standard techniques [6] were used to determine the production of $^{14}CO_2$ from [^{14}C]glucose in the same adipose tissue preparation used for the lipolysis assay. The $^{14}CO_2$ was trapped using special tissue wells and 1 M hyamine hydroxide.

2.4. *In vivo experiments and blood urea nitrogen*

300–350 g male Sprague Dawley rats were anaesthetized using i.p. 'Somnotol' or 'Inactin'. Both femoral veins were cannulated one for the injection of GH and the second for blood sampling. Blood samples were drawn at regular intervals. At various times the animals were killed and tissue samples assayed for ^{125}I-albumin or ^{125}I-GH to determine tissue deposition of the GH or GH-albumin conjugates.

Blood urea nitrogen assays were performed on 250 μl blood samples using a standard 'Sigma' BUN assay kit (no.535).

3. RESULTS

The data presented below is for porcine growth hormone (GH) in its free form or for a GH-albumin conjugate with a molecular mass of 180 kDa representing a complex of, on average two molecules of albumin and six molecules of GH chemically cross-linked. Radiolabelled conjugates were prepared starting with ^{125}I-labelled GH and all conjugates were separated on Sephadex G150 to make sure that the preparation was free of non-conjugated GH. Fig.1 shows the elution profiles of free GH and purified GH-albumin conjugates.

Fig.2 and table 1 examine the plasma clearance and tissue distribution of labelled GH and GH-albumin. The clearance rates for the free GH ($t_{1/2}$ = 5 min) is similar to that previously reported [7] and fairly typical for small polypeptides that one would expect to be readily filtered by the glomerulus. The 180 kDa GH-albumin conjugate has an extended half life in the circulation of 2–3 h. This appears to be primarily a function of size since 120 kDa conjugates of GH alone (similarly cross-linked) also have protracted circulation times (fig.2). The tissue deposition of the free GH indicates a rapid renal clearance and the appearance of a large fraction of the labelled GH in the urine. The GH-albumin clearly bypasses the kidneys and is cleared from the circulation largely by the liver.

Tables 2 and 3 and fig.3 indicate that the conjugated GH retains much of the activity of the native hormone and may show increased potency

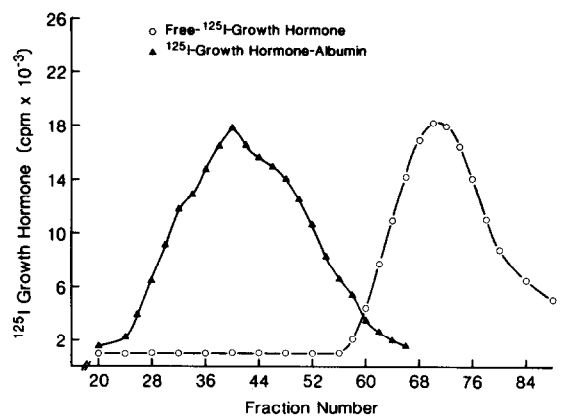

Fig.1. Molecular sieve chromatography of ^{125}I-growth hormone (o—o) and ^{125}I-growth hormone-albumin conjugates (▲—▲) on Sephadex G-150. The conjugate was prepared as described in the text, purified by column chromatography as described, stored for 72 h at 4°C and then rechromatographed. There is no evidence for any free growth hormone associated with the conjugate. Similarly in absorption data which is not shown, there is no evidence for any free albumin associated with the conjugate.

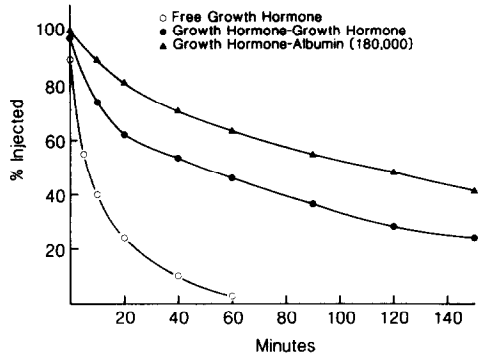

Fig.2. Plasma clearance of ^{125}I-labelled free GH (○—○), GH-albumin conjugates (▽—▽) and GH-GH conjugates (▲—▲). All animals received the equivalent amounts of growth hormone in the free or conjugated form. In each case the ^{125}I label is on the growth hormone but for the GH-albumin conjugate, identical results were obtained if the label was on the albumin or if the entire conjugate was labelled following the cross-linking procedure.

Table 2

Lipolytic activity of growth hormone and growth hormone-albumin conjugates

Preparation (ng/ml)	Glycerol (μmol/g per 60 min)
0	7.81
2.0 GH	8.98
3.0 GH	9.14
4.0 GH	10.97
8.0 GH	15.11
0.5 GH-albumin	9.55
1.0 GH-albumin	13.80
2.0 GH-albumin	14.98
4.0 GH-albumin	16.08
2.0 Albumin	8.01

All values refer to ng/ml of growth hormone whether it is in the free or the conjugated form. Each value represents the mean from 3 determinations of lipolytic activity. Standard deviations were less than 5% for each set of determinations

in the conjugated form. The conjugated form of GH is 2–10 times more active than the equivalent amount of free GH in terms of lipolysis (table 2) and glycerol release and in terms of insulin-like growth factor activity (table 3) and glucose utilization and CO_2 release.

Fig.3 examines the in vivo effects of GH on nitrogen metabolism and blood urea nitrogen (BUN) levels in anaesthetized rats receiving various doses of GH in either free (fig.3a) or conjugated form (fig.3b). Surprisingly we found that the higher concentrations of free GH resulted in very

Table 1

% tissue distribution of ^{125}I-labelled growth hormone and growth hormone-albumin conjugates

	GH (5 min)	GH (30 min)	GH-Alb. (30 min)	GH-Alb. (120 min)
Blood (total)	30	5	72	45
Heart	3	2	1	1
Lungs	3	5	1	2
Spleen	1	1	3	5
Liver	4	2	8	22
Kidneys	25	20	3	6
Bladder (urine)	10	28	2	4
Thyroid	1	1.5	0.5	1.5

The values represent the means from 3 separated experiments calculated as a percentage of the total amount of radiolabel injected. All of the values have been corrected for the amount of label still present in the blood contaminating the particular organ. In each of the organs, including the blood, better than 90% of the ^{125}I was found to be trichloroacetic acid precipitable. Values in brackets refer to the time (in min) when the animals were killed following the GH or GH-albumin administration

Table 3

Insulin-like growth hormone activity of free growth hormone and growth hormone-albumin conjugates

Preparation (ng/ml)	$^{14}CO_2$ (dpm/g per 60 min)
0 GH	5707 ± 250
100 GH	6150 ± 140
200 GH	7180 ± 200
400 GH	8560 ± 285
10 GH-albumin	6085 ± 320
50 GH-albumin	6850 ± 180
100 GH-albumin	8110 ± 220
200 GH-albumin	8430 ± 250
400 GH-albumin	8610 ± 250
100 albumin	5810 ± 180

All values refer to ng GH whether it is in the free or the conjugated form. Each value represents the mean and standard deviation from 3 determinations. The GH-albumin conjugate used in this experiment represents a different batch from that used in table 2 although each batch gave essentially similar data for both assays

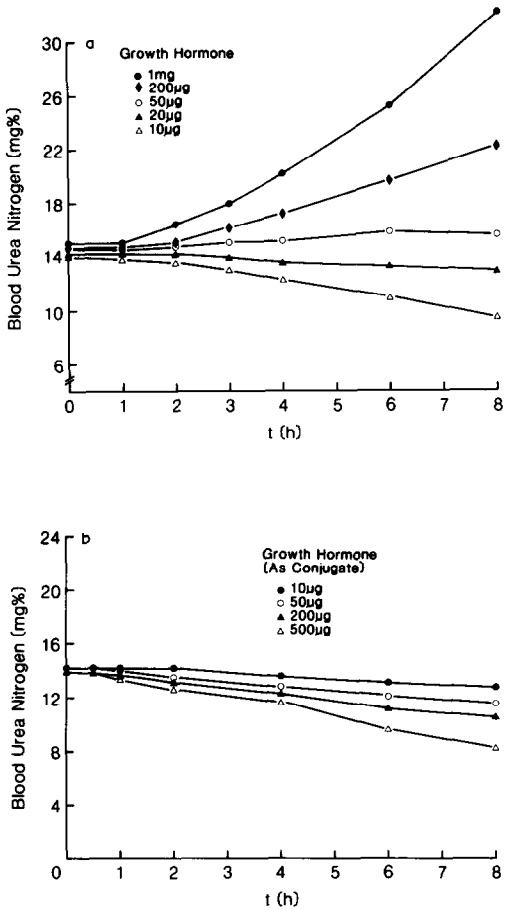

Fig.3. Blood urea nitrogen (BUN) levels in anaesthetized rats receiving various quantities of growth hormone in the free form (a) or equivalent amounts in the conjugated form (b). Values listed for amounts of growth hormone in b refer to the actual quantity of hormone and not the conjugate weight which is approximately double. Each point is the mean from 4 different rats. The standard deviations are in all cases 5% or less of the mean values.

significant increases in BUN levels whereas only small amounts (10 and 20 μg) of GH resulted in the expected decrease in BUN levels indicative of growth stimulation and a more effective energy utilization. We suggest that the increases in BUN levels seen for the higher concentrations of GH may be indicative of renal toxicity as large amounts of the free GH are filtered into the renal tubule [8,9]. On the other hand no such increases in BUN levels are observed when the rats were given similar amounts of GH conjugated with albumin (fig.3) where the decrease in BUN levels increased with the amount of GH-albumin administered. Evidence that nephrotoxicity was the explanation for the differences in BUN levels with free GH also comes from measuring serum creatinine levels (not shown) where levels rose only with higher concentrations of free GH and did not change for the smaller concentrations of GH or any of the concentrations of GH-albumin. (N.B. While lipolysis and insulin-like activities are demonstrated in vitro and nitrogen metabolism appears altered in vivo, unpublished data from another laboratory suggests that the GH-albumin conjugate is not active in growth promotion in hypophysectomized rats.)

4. DISCUSSION

While an increased understanding of bioactive peptides and developments in protein and genetic engineering are making these compounds therapeutically attractive, there are serious limitations to their use in medicine as well as agriculture. We and others have noted the rapid clearance from the circulation of proteins with molecular masses of 20 kDa as well as the potential for renal toxicity when large amounts of protein are cleared to the renal tubule. The incidence of serious renal toxicity for other small peptides such as the cytokines has also been reported.

This paper has demonstrated the possibility of altering small peptides by simple conjugation methods to larger molecules (such as homologous albumin) in the first instance to merely alter the pharmacokinetics by simply avoiding the kidneys. This has profound effects in terms of altered clearance, altered tissue deposition and reduced nephrotoxicity for GH and other bioactive peptides such as cytokines [10]. The fact that so many of the activities of growth hormone are maintained is both fortuitous and of great interest; although the trial and error nature of the cross-linking procedure required to find conditions to retain hormone activity should be emphasized. The fact that the GH activity appears higher in the conjugated form may reflect an allosteric synergy reported for insulin in the conjugated form by our laboratory [5] and by others [11]. The fact that reports suggest that our compound does not stimulate growth in hypophysectomized rats is disappointing but does not detract from the observation that small active

peptides may be engineered in such a way as to enhance their suitability as therapeutic agents. It may in fact reflect the multiple effects of GH [12] and the heterogeneity of GH receptors [13].

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