Cross-linked growth hormone dimers have enhanced biological activity

James W Mockridge, Roger Aston¹, David J Morrell² and Andrew T Holder

The Babraham Institute, Babraham, Cambridge CB2 4AT, UK, ¹Peptec (UK Ltd), 60 Dyer Street, Cirencester GL7 2PF, UK and ²Reproductive Medicine Laboratory, University of Edinburgh, Centre for Reproductive Biology, 37 Chalmers Street, Edinburgh EH9 3EW, UK

(Correspondence should be addressed to J W Mockridge, Department of Cardiology, The Rayne Institute, St Thomas's Hospital, Lambeth Palace Road, London SE1 7EH, UK)

Abstract

In this study we have investigated the effect on the bioactivity of pituitary-derived human growth hormone (hGH) and recombinant bovine (b) GH after the addition of various concentrations of the water soluble cross-linking agent 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC; 6.25–100 mg/ml). The biological activity of resulting cross-linked reactions were determined by its ability to promote incorporation of $^{35}\text{SO}_4^{2-}$ into costal cartilage of hypopituitary Snell dwarf mice *in vivo*. Administration of EDC-treated hGH solutions resulted in a significant enhancement of hormone activity *in vivo* compared with non-cross-linked samples. A similar significant enhancement of bGH activity *in vivo* was also observed when solutions containing recombinant bGH were cross-linked using EDC. For both hGH and bGH the degree of enhancement appears to be dose-dependent for the concentration of EDC (6.25–100 mg/ml for hGH; 6.25–50 mg/ml for bGH) present in the cross-linking reactions. SDS-PAGE analysis of EDC cross-linked solutions containing hGH and bGH spiked with ^{125}I -hGH and ^{125}I -bGH respectively revealed that dimeric GH was the primary cross-linked component. Increasing the concentration of EDC in cross-linking reactions resulted in increased formation of dimeric hGH and bGH. There was a significant correlation between the amount of GH dimer present and the increase in biological activity, suggesting that GH dimers were responsible for the enhanced biological activity. This was confirmed by the enhanced biological activity of a purified preparation of EDC cross-linked dimeric hGH.

In conclusion, covalently cross-linked GH dimers reported here have enhanced bioactivity *in vivo*. However, since naturally occurring GH dimers are known to have reduced biological activity, this work suggests that the structure of EDC cross-linked GH dimers differs fundamentally from that of native dimeric hGH.

European Journal of Endocrinology 138 449–459

Introduction

Pituitary growth hormone (GH) is a multifunctional polypeptide hormone that exhibits somatogenic, lactogenic and diabetogenic activity (1). The structures of both porcine GH (pGH) and human GH (hGH) have been determined by high resolution X-ray crystallographic studies; they are 4 α -helix bundle proteins with connections between the helices formed by varying lengths of loop/coil structure (2, 3). This structural arrangement is thought to be shared by the GH-related proteins prolactin and placental lactogen and by other members of the larger family of haematopoietins (4–6). GH receptors are also structurally related to receptors for other haematopoietins; this structural homology is most apparent in the extracellular domain of these single transmembrane proteins (7). Other emerging features of this receptor family are ligand-induced receptor dimerisation which has been observed for

both GH (3) and prolactin (8), and tyrosine phosphorylation and activation of the JAK family of receptor-associated tyrosines (8–11) and members of the STAT family of transcription factors (12–14).

Growth hormone is polymorphic comprising the predominant 22 kDa form, a 20 kDa variant, which lacks residues 32–46 (15), and 45 kDa dimeric GH (16–19); there are also a large number of less well characterised variants (20, 21). Dimeric GH is thought to represent about 10–20% of GH in pituitary extracts. About 70% of naturally occurring dimers are non-covalently linked; of the 30% which are covalently linked the majority are joined via inappropriate intermolecular disulphide bridges (18, 21). For example, Lewis *et al.* (18) reported a disulphide dimer where cys^{35} and cys^{182} in one molecule are linked to cys^{165} and cys^{189} respectively in a second GH molecule. The somatogenic activity of such dimers, whether covalently linked or otherwise, appears to be around 10% of that

observed for monomeric 22 kDa GH (18, 22). Little is known of the three-dimensional structure of dimeric GH and how it may differ from that proposed for monomeric GH.

In a previous study we attempted to examine the biological activity of bovine GH (bGH) when cross-linked to BSA. The cross-linking process, which utilised carbodiimide, resulted in a significant increase in the ability of bGH to promote cartilage metabolism in hypopituitary Snell dwarf mice *in vivo*. However, the preliminary data also suggested that it was cross-linked GH dimers rather than GH/BSA complexes which were responsible for the enhanced activity (23). This paper reports an examination of this phenomenon and demonstrates that chemically cross-linked GH dimers have enhanced biological activity.

Materials and methods

Materials

Outdated MRC clinical grade pituitary GH was rechromatographed on Sephadex G-100 followed by Sephadex G-50 (Pharmacia LKB Biotech., St Albans, Herts, UK) and the monomer peak was retained and freeze-dried. SDS-PAGE analysis of this peak revealed a single band at 22 kDa. Recombinant bGH was obtained from the Monsanto Corporation (St Louis, MO, USA). The cross-linking agent 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was obtained from Pierce Chemicals, Life Science Laboratories Ltd (Luton, Beds, UK). All chemicals and molecular weight (MW) markers used in polyacrylamide gel electrophoresis were obtained from Sigma (Poole, Dorset, UK); radiochemicals (^{125}I and $^{35}\text{SO}_4^{2-}$) were obtained from Amersham International plc (Amersham, Bucks, UK). All other reagents were purchased from BDH (Poole, Dorset, UK).

Iodination of hGH

Monomeric hGH, prepared as above, and recombinant bGH were iodinated to a specific activity of 50–60 $\mu\text{Ci}/\mu\text{g}$ using Iodogen (1,3,4,6-tetrachloro-3 α ,6 α -diphenyl glycoluril; Pierce Chemicals) by the method of Fraker and Speck (24). Iodinated hormone was separated from free iodine by column chromatography on a PD10 column (Pharmacia LKB Biotech).

Cross-linking reaction and purification of GH dimer

In all cross-linking reactions equal volumes of GH and EDC were mixed such that the final concentration of GH was 1 mg/ml. EDC, which was added last, was dissolved in PBS, diluted appropriately (final concentration 0–100 mg/ml) and added immediately. Non-cross-linked control solutions were treated identically except that buffer was substituted for EDC. Samples (100 μl) for PAGE analysis were taken from EDC reaction mixtures

immediately after the addition of EDC and added to tubes containing 5 μl ^{125}I -hGH (370 000 c.p.m.). All cross-linking reactions were incubated for 2 h at room temperature. Solutions used to treat mice were dialysed extensively against PBS and diluted to give the stated concentration of GH in an injection volume of 100 μl .

Where hGH was cross-linked in order to purify GH dimers, 200 mg EDC were dissolved in 1 ml PBS and added to 15 mg hGH in 3 ml PBS and incubated for 2 h at room temperature. Column chromatography on a 50 $\times$ 3.5 cm Sephadex G-50 column, equilibrated with PBS, was used to separate monomeric and dimeric hGH. An initial column run gave a partially pure preparation of dimer; this peak was re-run on Sephadex G-50 and the dimer peak gave a single band on PAGE (see Fig. 5). After each Sephadex G-50 run the dimer peak was concentrated using a Vivapore concentrator (Viva Science, Lincoln, Lincs, UK). Doses of purified dimer or monomer hGH used in the dwarf mouse assay were calculated from protein estimations using Coomassie Protein Assay Reagent (Pierce Chemicals) according to the manufacturer's instructions.

SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli (25) on a Bio-Rad vertical slab gel unit with 1.5 mm thick gels. Samples of the cross-linking reactions were mixed with an equal volume of buffer containing SDS (4% w/v), glycerol (20% v/v), bromophenol blue (0.5% w/v) and mercaptoethanol (4% v/v) and heated at 100 $^{\circ}\text{C}$ for 2 min prior to loading. Samples (20 μl) were applied to a 5–15% gradient gel with a 3% stacking gel and run at 30 mA/gel. Gels were stained with 0.1% Kenacid blue in 10% acetic acid (w/v) and 20% methanol (v/v) and destained using 7% acetic acid and 20% methanol. Bands in each lane were mapped by comparison with MW standards obtained from Sigma. Each lane was cut into 60 $\times$ 2 mm slices and the ^{125}I -hGH present in each section was determined by counting on a Packard Cobra gamma counter.

Animal experiments

The bioactivities of the various cross-linked preparations of GH and the equivalent non-cross-linked controls were assessed by their effects on cartilage metabolism in hypopituitary Snell dwarf mice. Small size in these animals (30% of normal) is a consequence of defective differentiation of the anterior pituitary gland (26) which secretes negligible amounts of GH, prolactin and thyrotrophin; these primary defects result in low circulating levels of the thyroid hormones and insulin-like growth factor-I (27–30). Dwarf mice (aged 9–11 weeks and weighing 8–11 g at the start of the experiment) were bred, maintained, marked and caged as described previously (31). No differences have been detected between the responses of male and female mice to GH

treatment and they were therefore pooled and used at random. Mice were injected (0.1 ml) subcutaneously in the back of the neck once per day for two days with GH either cross-linked or non-cross-linked ($n = 6$ /treatment group). They received $^{35}\text{SO}_4^{2-}$ on the third day ($0.5 \mu\text{Ci/g}$ body weight, i.p.) and were killed 20 h later. Rib cages were removed, costal cartilages were dissected out, pooled for each animal, dried, weighed, solubilised in $200 \mu\text{l}$ 98% formic acid, mixed with 3.5 ml scintillation fluid and counted on a Packard TriCarb 2500TR liquid scintillation counter as described previously (31). Results were expressed as d.p.m./mg cartilage. All procedures and care of animals were carried out in accordance with Home Office (UK) regulations.

Statistics

All values are expressed as means $\pm$ S.E.M. ($n = 6$). Differences between groups were assessed using two-tailed Student's *t*-test. Correlation coefficients, slopes and significances of slopes were calculated using standard statistical procedures. For investigation of dose dependency these statistical procedures were carried out after log transformation of EDC doses (expressed as d.p.m./log dose unit).

Results

In the initial investigation, groups of mice were treated with cross-linked complexes produced in reactions

containing pituitary-derived hGH plus increasing concentrations of EDC (6.25–200 mg/ml). The effects of the various cross-linked complexes on cartilage metabolism in hypopituitary Snell dwarf mice are illustrated in Fig. 1. Uptake of $^{35}\text{SO}_4^{2-}$ was significantly enhanced ($P < 0.01$) by hGH after the addition of EDC concentrations of 25 mg/ml and above with a maximum enhancement being obtained with hGH cross-linked with 100 mg/ml EDC. The response of hGH plus 6.25–100 mg/ml EDC appeared to be dose-dependent for the concentration of EDC (b (regression coefficient) = 1459 d.p.m./log dose unit; $P < 0.001$). The response of animals receiving complexes formed in reactions containing hGH plus 200 mg/ml EDC declined; however, in this case a precipitate was observed after the cross-linking reaction. Non-cross-linked hGH promoted a significant increase in cartilage metabolism compared with animals receiving PBS ($P < 0.001$).

SDS-PAGE was used to determine the molecular weights of the various complexes produced in cross-linking reactions (Fig. 2) the biological activities of which are illustrated in Fig. 1. The size distribution of ^{125}I -hGH in samples obtained from cross-linking reactions containing GH and 0, 6.25, 12.5 or 25 mg/ml EDC are illustrated in Fig. 2a and hGH and 0, 50 and 100 EDC in Fig. 2b. Molecular weight determinations were carried out on a 5–15% gradient gel by comparison with commercially available MW standards. It is evident from Fig. 2a and b that the vast majority of GH (over

Figure 1 The effect of cross-linked complexes produced in reactions containing hGH plus increasing concentrations of EDC (0–200 mg/ml) on $^{35}\text{SO}_4^{2-}$ uptake into costal cartilage of hypopituitary Snell dwarf mice *in vivo*. All cross-linked and non-cross-linked reaction solutions were diluted to give a final concentration of $40 \mu\text{g}$ hGH/0.1 ml (injection volume); control groups injected with PBS containing 50 mg/ml EDC and PBS alone were also included. Values are means $\pm$ S.E.M.; $n = 6$ per group. Mice received one injection/day for two days. ** $P < 0.01$, *** $P < 0.001$ compared with the group receiving non-cross-linked hGH.

Figure 2 PAGE analysis of EDC cross-linked reactions containing hGH spiked with ^{125}I -hGH. Each lane was cut into 2-mm sections and counted on a gamma counter. The MWs of the 'peaks' of ^{125}I -hGH containing complexes were assessed by comparison with MW standards run on the same gel. (a) PAGE analysis of hGH non-cross-linked ($\blacklozenge$), hGH plus 6.25 mg/ml EDC ($\blacksquare$), hGH plus 12.5 mg/ml EDC ($\circ$) and hGH plus 25 mg/ml EDC ($\square$). (b) PAGE analysis of hGH alone non-cross-linked ($\blacklozenge$), hGH plus 50 mg/ml EDC ($\blacksquare$) and hGH plus 100 mg/ml EDC ($\circ$). Dimeric hGH was found in gel slices 38–43 and monomeric hGH in gel slices

90%) remains uncross-linked and has a relative mobility (R_f) appropriate to monomeric hGH. Also, non-cross-linked control contained a small but significant amount (3.4%) of GH dimer (44 kDa) which was not converted to monomer by the presence of mercaptoethanol. This dimer was presumably formed either during freeze drying or storage (at -30°C) of our 'monomeric' hGH preparation, or is a product of the iodination process. This dimer has been isolated and has reduced bioactivity compared with monomeric GH (results not shown). The presence of increasing concentrations of EDC in reaction mixtures containing hGH was associated with increasing amounts of ^{125}I -hGH appearing in the GH dimer region. For all samples the GH dimer peak was found in gel slices 38–43. Counts in these sections (minus background) were summed and expressed as a percentage of counts loaded onto the gel in order to calculate the percentage cross-linking of ^{125}I -hGH in each cross-linking reaction; these values are given in Table 1. Relatively little ^{125}I -hGH was found at MWs higher than 44 kDa. A highly significant correlation between increased biological activity and the percentage EDC cross-linked hGH dimer present in each daily injection was observed ($r = 0.95$; $P < 0.001$). Similar observations have been obtained from three separate experiments with similar patterns being observed in each.

In order to determine whether this enhancing phenomenon is peculiar to hGH or simply the preparation used, cross-linking reactions using recombinant bGH were investigated. For bGH, the cross-linking reactions were repeated with EDC concentrations between 0–100 mg/ml and ^{125}I -bGH as tracer instead of ^{125}I -hGH. The addition of EDC at concentrations of 12.5–50 mg/ml to the samples stimulated significant increases in $^{35}\text{SO}_4^{2-}$ uptake compared with bGH alone, with a maximum enhancement being produced by bGH plus 50 mg/ml EDC (Fig. 3). As for hGH, the response (bGH plus 6.25–50 mg/ml EDC) appeared to be dose-dependent for

the concentration of EDC ($b = 1406 \text{ d.p.m./log dose unit}$; $P < 0.01$). However, bGH cross-linked with 100 mg/ml EDC produced a decrease in $^{35}\text{SO}_4^{2-}$ uptake compared with bGH plus 50 mg/ml; in this case a precipitate was observed after the cross-linking reaction. Non-cross-linked bGH control groups promoted a significant increase in cartilage metabolism compared with animals receiving PBS ($P < 0.001$).

Examination of the size distribution of ^{125}I -bGH in samples obtained from the subsample cross-linking reactions showed they were similar to those described for hGH and are illustrated in Fig. 4a and b. For all the samples the bGH dimer peak was found in gel slices 28–34 with the monomeric peak found in gel slices 42–47. The majority of bGH remained in the non-cross-linked monomeric form. In this preparation of bGH a small proportion (approx. 1%) of 'naturally occurring' dimer was found in the non-cross-linked sample but this was considerably less than in the pituitary-derived hGH preparation (Table 2). As for hGH, addition of increasing concentrations of EDC in the reaction mixtures was associated with increasing amounts of ^{125}I -bGH appearing in the dimer region; a highly significant correlation was observed between the increased biological activity induced by cross-linking and the percentage EDC cross-linked bGH ($r = 0.97$; $P < 0.001$). Similar observations have been obtained from three separate experiments with similar patterns being observed in each. Treatment of Snell dwarf mice with EDC-treated solutions of ovine GH and pGH also resulted in a significant increase in $^{35}\text{SO}_4^{2-}$ incorporation compared with non-cross-linked samples (results not shown).

The enhanced biological activity of the 'unpurified' cross-linked complexes in all the species of GH studied appeared to be due to an increase in the amount of dimeric GH formed rather than to the source (pituitary or recombinant) of the material used. Therefore, in order to investigate whether covalently cross-linked GH dimers themselves have enhanced biological activity,

Table 1 The effect of various concentrations of the cross-linking agent EDC on the biological activity of hGH and the formation of cross-linked hGH dimers. (A) The uptake of $^{35}\text{SO}_4^{2-}$ into costal cartilage of Snell dwarf mice (d.p.m./mg cartilage) promoted by GH cross-linked by various concentrations of EDC (0–100 mg/ml). (B) The increase in $^{35}\text{SO}_4^{2-}$ attributable to the cross-linking process (values for non-cross-linked hGH alone were subtracted from the appropriate EDC cross-linked values). (C) Dimeric hGH present in each reaction as calculated from gel slices from Fig. 2a and b. (D) The percentage of EDC GH dimer attributable to cross-linking with EDC (percentage of hGH dimer in EDC cross-linked reactions minus that in non-cross-linked hGH alone reaction).

hGH (40 µg/day)	+	+	+	+	+	+	–	–
EDC (mg/ml) present in cross-linking reactions	–	6.25	12.5	25	50	100	50	–
(A) $^{35}\text{SO}_4^{2-}$ uptake (dpm) (plotted values Fig. 1)	1552	1987	1973	2505	2935	3670	510	491
(B) Increased $^{35}\text{SO}_4^{2-}$ uptake due to cross-linking	–	435	421	953	1383	2118	–	–
(C) % dimer (total)	3.40	6.81	7.36	8.07	8.10	8.19	–	–
(D) % EDC dimer	0	3.41	3.96	4.67	4.70	4.79	–	–

Figure 3 The effect of cross-linked complexes produced in reactions containing bGH plus increasing concentrations of EDC (0–100 mg/ml) on ³⁵SO₄²⁻ uptake into costal cartilage of hypopituitary Snell dwarf mice *in vivo*. All cross-linked and non-cross-linked reaction solutions were diluted to give a final concentration of 40 μg bGH/0.1 ml (injection volume); control groups injected with PBS containing 50 mg/ml EDC and PBS alone were also included. Values are means ± S.E.M.; *n* = 6 per group. Mice received one injection/day for two days. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared with the group receiving non-cross-linked bGH.

hGH was cross-linked to itself using EDC and the resulting covalently linked GH dimers purified and assayed for biological activity. Cross-linking reactions were carried out using 50 mg/ml EDC and the degree of cross-linking (about 10%) was established as described above. Purification of cross-linked dimeric GH was achieved by column chromatography on Sephadex G-50. SDS-PAGE analysis of the hGH monomer and hGH dimer peaks after the first Sephadex G-50 run showed that the monomer peak was contaminated by dimer and vice versa. In addition, the dimer peak also had a small band of what appeared to be GH trimer. The hGH dimer peak from the first Sephadex G-50 run was then rechromatographed on Sephadex G-50. SDS-PAGE analysis of the resulting monomer and dimer peaks showed complete separation of monomeric and dimeric hGH (Fig. 5); however, a small amount of what may be trimeric hGH was still present. It is interesting to note that a significant amount of the dimer present in our in-house preparation of naturally occurring dimer (also run on the gel) is not converted to monomeric hGH by SDS-PAGE under reducing conditions.

The biological activity of purified cross-linked hGH dimers was compared with that of purified EDC-exposed monomeric GH in the hypopituitary Snell dwarf mice model (Fig. 6). Monomeric hGH (5, 20 and 80 μg/day) promoted a significant dose-dependent increase in cartilage metabolism (*b* = 988 d.p.m./log dose unit; *P* < 0.001). The response to 5 μg dimeric GH did not differ significantly from that observed with the same dose of monomeric GH; this is probably due to the concentra-

tion being the lowest point on the dose-response curve. However, the response to 20 μg cross-linked dimer was significantly greater than that observed for the same dose of monomeric GH (*P* < 0.001). All groups which received GH (monomer or dimer) promoted a significantly greater increase in cartilage metabolism than the PBS-injected group (*P* < 0.001 in all cases). No difference in biological activity was observed when EDC-exposed monomer was compared with our monomer preparation of hGH (results not shown).

Discussion

From the data presented here it is clear that the cross-linking agent EDC can be used to enhance the biological activity of either hGH or bGH (Figs 1 and 3) irrespective of the origin (pituitary or recombinant) of the hormone. Furthermore, the degree of enhancement appears to be dose-dependent for the concentration of EDC present in the cross-linking reaction. PAGE analysis of cross-linking reactions which contained hGH (Fig. 2) and bGH (Fig. 4) revealed a significant correlation between the amount of EDC cross-linked dimeric GH and biological activity (Table 1 for hGH and Table 2 for bGH). This suggests that GH dimers are the active component. The enhanced biological activity of purified EDC cross-linked hGH dimers (Fig. 6) clearly supports this view.

In contrast to the dimers reported here native GH dimers have a reduced bioactivity compared with monomeric GH (18, 20, 21) and thus it appears that

Figure 4 PAGE analysis of EDC cross-linked reactions containing bGH spiked with ^{125}I -bGH. Each lane was cut into 2-mm sections and counted on a gamma counter. The MWs of the 'peaks' of ^{125}I -bGH containing complexes were assessed by comparison with MW standards run on the same gel. (a) PAGE analysis of bGH non-cross-linked ($\blacklozenge$), bGH plus 6.25 mg/ml EDC ($\blacksquare$), bGH plus 12.5 mg/ml EDC ($\circ$). (b) PAGE analysis of hGH alone non-cross-linked ($\blacklozenge$), hGH plus 25 mg/ml EDC ($\blacksquare$), hGH plus 50 mg/ml EDC ($\circ$). Dimeric bGH was found in gel slices 28–34 and monomeric bGH in gel slices 42–47.

Table 2 The effect of various concentrations of the cross-linking agent EDC on the biological activity of bGH and the formation of cross-linked bGH dimers. (A) The uptake of $^{35}\text{SO}_4^{2-}$ into costal cartilage of Snell dwarf mice (d.p.m./mg cartilage) promoted by bGH cross-linked by various concentrations of EDC (0–100 mg/ml). (B) The increase in $^{35}\text{SO}_4^{2-}$ attributable to the cross-linking process (values for non-cross-linked bGH alone were subtracted from the appropriate EDC cross-linked values). (C) Dimeric bGH present in each reaction as calculated from gel slices from Fig. 4a and b. (D) The percentage of EDC GH dimer attributable to cross-linking with EDC (percentage of bGH dimer in EDC cross-linked reactions minus that in non-cross-linked bGH alone reaction).

bGH (40 µg/day)	+	+	+	+	+	+	–	–
EDC (mg/ml) present in cross-linking reactions	–	6.25	12.5	25	50	100	50	–
(A) $^{35}\text{SO}_4^{2-}$ uptake (dpm) (plotted values Fig. 4)	2082	2523	2878	3611	3686	3027	1179	1059
(B) Increased $^{35}\text{SO}_4^{2-}$ uptake due to cross-linking	–	440	796	1528	1604	945	–	–
(C) % dimer (total)	1.470	1.826	2.079	2.378	2.639	nd	–	–
(D) % EDC dimer	0	0.356	0.609	0.908	1.170	nd	–	–

nd, not determined.

Figure 5 SDS-PAGE analysis of a purified preparation of EDC cross-linked hGH dimers. Lanes 1 and 6 are MW standards. Lane 2 represents the monomeric hGH peak after the second column chromatography run on Sephadex G-50. Our in-house standard preparations of naturally occurring dimeric and monomeric hGH were run in lanes 3 and 4 respectively. Lane 5 represents the purified preparation of EDC cross-linked dimeric hGH.

the nature of these dimers (naturally occurring and EDC cross-linked) are fundamentally different. In essence EDC is a heterobifunctional cross-linking reagent that reacts with available $-\text{COOH}$ groups (found on the terminal residues and aspartic acid and glutamic acid) to form an unstable active *O*-acylisourea intermediate; this intermediate subsequently reacts with free $-\text{NH}_2$ groups on amino acids (e.g. lysine) to form an amide bond with the release of a soluble urea derivative as a by-product (32–34). These by-products are unlikely to have a role in the enhancement as the reaction mixtures were thoroughly dialysed and thus removed prior to administration into dwarf mice.

As the cross-linking process is random the structure of the EDC cross-linked dimers and the precise location of the residues involved remains to be determined. Nevertheless, it is likely that they form between residues on the surface of the protein and are postulated not to cause significant disruption of hormone structure or block receptor recognition sites. As the location of covalent linkage is non-specific a number of different molecular species are formed in response to the cross-linking agent (35). These may include a number of different forms of GH dimer plus monomeric GH with intramolecular bonds and insoluble protein aggregates. However, given the similar biological response of purified EDC-exposed monomer and our monomer preparation it is unlikely that modification of this species contributes to the enhancement. In addition, the data from Fig. 6 demonstrate that purified cross-linked GH dimers have enhanced bioactivity compared with EDC-exposed monomeric hGH.

From Tables 1 and 2 the maximum cross-linking attributable to EDC has been calculated to be only 5% for hGH and 1% for bGH. This low level of cross-linking suggests that out of the monomer pool only a small proportion of GH is present that either has a propensity to dimerise or is in close enough proximity to be cross-linked by EDC even at a high protein concentration.

Figure 6 The effect of purified EDC cross-linked dimeric hGH and purified EDC-exposed monomeric hGH on $^{35}\text{SO}_4^{2-}$ uptake into costal cartilage in hypopituitary Snell dwarf mice *in vivo*. Control mice were injected with PBS (hormone diluent). Values are means $\pm$ S.E.M.; $n = 6$ per group. All mice received one injection/day for two days. Equivalent doses of monomeric and dimeric hGH were compared: *** $P < 0.001$.

Alternatively, naturally occurring dimers, as identified in the GH preparations in Fig. 2 and Fig. 4, may be replaced by EDC cross-linked dimers. Thus the cross-link would be proposed to stabilise the conformation found in native dimers fundamentally altering its structure and altering its bioactivity.

Enhancement of GH action has previously been reported when laboratory animals (dwarf mice and dwarf rats) are treated with the hormone complexed to anti-GH monoclonal or polyclonal antibodies (36–41), the GH binding protein (42) and when covalently linked to polyethylene glycol (43). A number of mechanisms have been suggested to account for this enhancement but as yet the precise mechanism(s) underlying the phenomenon remains unclear. One common mechanism proposed by a number of these workers is that of prolongation of the half-life of the hormone within the circulation (40, 42, 43). It is suggested that the increased size of the hormone complex (whether the hormone is attached to an antibody, binding protein or covalently linked to another molecule) prevents or

delays hormone clearance from the circulation via glomerular filtration and receptor-mediated internalisation and protects from enzymatic degradation. Thus the result would be a circulating pool of bioactive hormone with prolonged half-life and thus prolonged bioactivity. Although there is no direct evidence, it is possible that a similar mechanism may be operating to account for the enhancement of GH activity by EDC cross-linked hGH dimers. Indeed, this mechanism is supported by the finding in the rat that native hGH dimers have an extended serum half-life when compared with monomeric hGH (44). However, although this mechanism may account for the enhanced bioactivity of purified EDC cross-linked dimers it is unlikely that such a mechanism could fully explain the enhanced biological activity of the 'unpurified' EDC-induced dimeric GHs (containing both monomer and dimer).

An alternative mechanism suggests that the binding of GH, whether complexed to an antibody, binding protein or covalently linked to itself, may slow or disrupt the rate of receptor-mediated internalisation of hormone receptor

complexes (45, 46). This delayed receptor internalisation may explain why purified EDC cross-linked dimeric GH (Fig. 6) was less biologically active than expected. In Fig. 1, GH cross-linked to itself by EDC (50 mg/ml) gave rise to a 136% increase in biological activity yet only a small proportion was dimer. In Fig. 6, 20 μ g purified dimer only gave rise to a 48% increase in biological activity over that observed for 20 μ g monomeric GH. Thus it seems on first inspection that the presence of monomeric GH somehow amplifies the response to EDC cross-linked dimeric GH. A plausible explanation would be that delayed receptor internalisation occurs which may also mean delayed receptor recycling and delayed receptor synthesis (47–49); thus the presence of monomeric GH, which is internalised, may facilitate a healthy turnover of, and possibly synthesis of, GH receptors. Much further work on the mechanistic details is necessary.

In this paper we have clearly demonstrated that EDC cross-linked GH dimers have markedly enhanced biological activity *in vivo* compared with monomeric GH. As yet the mechanism underlying this phenomena is unknown but it appears to operate predominantly via prolongation of the half-life of the hormone and delayed receptor internalisation. With the classification of GH as a member of the haematopoietin superfamily (7) it is possible that these techniques could be used to enhance the biological activity of other structurally related 4 α -helix bundle proteins, including a number of cytokines.

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Received 13 August 1997

Accepted 17 November 1997