Development of Novel Recombinant Agonists and Antagonists of Glycoprotein Hormones Using Site-Directed Mutagenesis and Gene Transfer

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Abstract - One major issue regarding the clinical use of many peptides is their short half-life due to the rapid clearance from the circulation. To overcome this problem, we succeeded to ligate the signal sequence of *O*-linked oligosaccharides to the coding sequence of the hormones. The cassette gene that has been used contains the sequence of the carboxyl-terminal peptide (CTP) of human chorionic gonadotropin β (hCGβ) subunit. The CTP contains 28 amino acids with four *O*-linked oligosaccharide recognition sites. It was postulated that *O*-linked oligosaccharides add flexibility, hydrophilicity and stability to the protein. On the other hand it was suggested that the four *O*-linked oligosaccharides play an important role in preventing plasma clearance and thus increasing the half-life of the protein in circulation. Using this strategy we succeeded to ligate the CTP to the coding sequence of follitropin (FSH), thyrotropin (TSH), erythropoietin (EPO) growth hormone (GH) and thus to increase the longevity and bioactivity of these proteins *in-vivo*. Interestingly, the new analogs of FSH and GH were found not immunogenic in human and it is already passed successfully clinical trials phase III and phase II respectively. Moreover, FSH long acting was approved by the European Commission (EC) for treatment of fertility. In addition, our results indicated that long acting GH is not toxic in monkeys and the results from clinical trials phase I and phase II seem to be promising. Designing long acting peptides will diminish the cost of these drugs and perhaps reduce the number of injections in the clinical protocols.

Key wards- recombinant proteins, half-life, CTP, EPO, FSH, GH, TSH

1. Introduction

The family of glycoprotein hormones consist of Thyrotropin (TSH), Lutropin (LH), Follitropin (FSH) and Chorionic Gonadotropin (CG). These hormones are heterodimers, consisting of the noncovalent association of a common α subunit with a unique β subunit that confers biological specificity to the hormone [1,2]. The individual subunits have no known biological activity. Thus, formation of heterodimer is essential for activity [1-3]. The α subunit within a species has an identical amino acid sequence in all four members of this hormone family. The β subunit share considerable amino acid

homology with one another, indicating that most likely they evolved from a common precursor [4-6]. The subunits achieve their tertiary structures by the formation of internal disulfide bonds; 5 in the α subunit and 2-6 in the β subunits. The location of cystein residues, which determines the 3-dimensional structure of the subunits by predicating their folding, in the β subunits is highly conserved among the various hormones as well as different species [4].

The glycoprotein hormones activate the target cells via adenylate cyclase-linked receptors. Through binding to the membrane receptors, FSH stimulates follicular development in the ovary and gametogenesis in the testes. LH, acts primarily in promoting lueinization of the ovary and in stimulating Leydig cell function of the testes. CG maintains the corpus luteum in the ovary during pregnancy. TSH is a major regulator of thyroid hormone synthesis and secretion from the thyroid gland. The thyroid hormones triiodothyronine (T_3) and thyroxine (T_4) regulate the synthesis and secretion of TSH from the pituitary.

The subunits contain one (TSHβ and LHβ) or two (α, FSHβ and hCGβ) asparagine N-linked oligosaccharide chains [1,2]. One unique structural difference among the subunits is the sequence of the carboxy terminus. TSH and LH subunits contain short hydrophobic stretches at their termini, deduced from gene sequencing [7]. However, the mature subunit does not contain the sequence due to a final deletion process. hCGβ subunit is distinguish from the other human β subunits in that it contains a unique 29 amino acid carboxyl-terminal peptide bearing four O-linked oligosaccharide chains (Fig.1). It has been suggested that the O-linked oligosaccharide chains play an important role in the secretion of intact hCG from the cell, enhaced bioactivity and prolonged its circulating half-life [8]. Deletion of the O-linked oligosaccharide chains from hCG, didn't affect assemly of the subunits or secretion of the dimer from the cell. On the other hand, it was shown that truncated hCG without the CTP is 3 times less potent than intact hCG in vivo [9]. On the other hand, the O-linked oligosaccharide chains play a minor role in receptor binding and signal transduction. These findings indicate that the CTP of hCGβ and the associated O-linked oligosaccharides are not important for receptor binding or in

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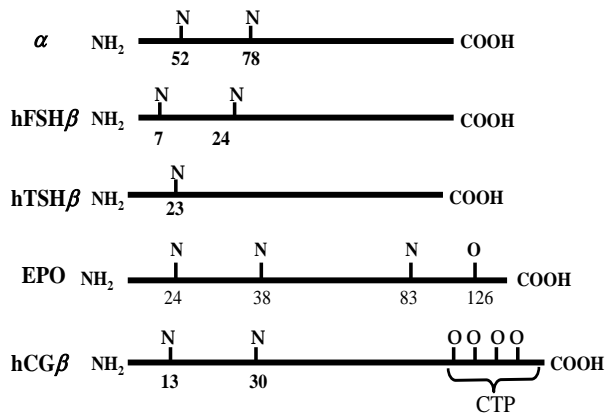University of Haifa, Israel

Fig.1. Human Glycopritein subunits. Location of asparagine - linked carbohydrates(N) in the hormone subunits is indicated. The hCGb subunit contain four sites of O-linked oligosaccharid (O) in the carboxyl-terminal peptide (CTP) region.

vitro signal transduction, but are critical for *in vivo* bioactivity and half-life [8] . It was reported that the kidney is the main site of clearance for glycoprotein hormones [10]. On the other hand much less hCG, which contains the CTP associatioted with the four O-linked oligosaccharide chains, is cleared by the kidney [11]. Other studies indicated that sialic acid plays an important role in the survival of glycoproteins in the circulation [1,2]. It has been suggested that more negatively charged forms of glycoprotein hormones have longer half-lives, which may be related to a decrease glomerular filtration [12]. Thus, the presence of the CTP with its sialylated O-linked oligosaccharides may prolonged the circulating half-life of the hormone secondary to a decrease in renal clearance.

Erythropoietin, (EPO) is a glycoprotein hormone produced primarily by cells of the peritubular capillary endothelium of the kidney [13,14]. EPO is a member of an extensive cyto-kine family which also includes growth hormone, prolactin, interleukins 2 through 7, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), oncostatin-M, leukemia inhibitory factor and cili-aryneurotrophic factor. EPO production is stimulated by reduced oxygen content in the renal arterial circulation. Circulating EPO binds to EPO receptors on the surface of erythroid progenitors resulting in replication and maturation to functional erythrocytes by an incompletely understood mechanism.

The gene encoding human erythropoietin was cloned in 1985 leading to the production of recombinant human EPO (rhuEPO) [15]. The rhuEPO has been used successfully in a variety of clinical situations to increase production of red blood cells. Currently, this agent is licensed for use in the treatment of the anemia of renal failure, the anemia associated with HIV infection in zidovudine (AZT) treated patients, and anemia associated with cancer chemotherapy. Administration of rhuEPO has become routine in the treatment of anemia secondary to renal insufficiency where doses of 50-75 U/Kg given three times per week are used to gradually restore heamatocrit and eliminate transfusion dependency.

2. Research topics

One major issue regarding the clinical use of glycoprotein hormones is their relatively short half-life *in vivo* due to their rapid clearance from the circulation when it's injected intra-venously. Thus, the therapeutic protocol used in the treatment of glycoproteins, required frequent injections. The recommended therapy with rhuEPO is 2-3 times per week by subcutaneous or intravenous injections. Similarly, FSH is injected daily. Therefore, we anticipated that ligation the CTP to the coding sequence of glycoprotein hormones will increase the half-life and bioactivity *in vivo*. This may reduce the number of injections per week. On the other hand, deletion of the N-linked oligosaccharides does not affect receptor binding; however, it reduced the bioactivity of the hormone. Therefore, designing deglycosylated hormones by site-directed mutagenesis of the N-linked oligosaccharide sites, will lead to the development of new antagonists.

3. Designing glycoprotein analogs

To address the issue of the relatively short half-life of FSH and EPO, the CTP of hCGβ was fused to the carboxyl-terminal of hFSHβ [16, 17] and hEPO [18] coding sequence using site-directed mutagenesis (Fig.2). It was hypothesized that FSH dimmer or EPO containing the CTP would have a prolonged half-life and higher bioactivity *in vivo*. The chimeric gene, EPO-CTP was transfected into CHO
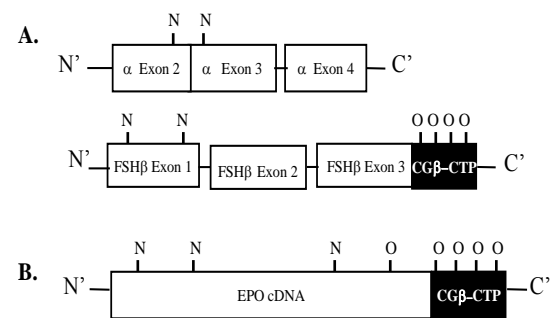

Fig.2. Construction of FSH-CTP (A) and EPO-CTP (B) chimeric genes. The coding sequence of CTP was ligated to the 3rd end of hFSHβ subunit using overlapping PCR mutagenesis. Similarly, the CTP coding sequence was ligated to the 3rd end of human EPO cDNA. The position of N-linked (N) and O-linked oligosaccharides (O) is indicated.

cells and hFSHβ-CTP was transfected into CHO cells that expressed α wild-type subunit. Stable clones expressing either EPO-CTP or hFSH-CTP dimer were selected.The results indicated that fusing the CTP of hCGβ to human FSH or to human EPO did not affect secretion, receptor binding affinity or *in vitro* bioactivity. However, the *in vivo* potency of the proteins containing CTP and their circulatory half-lives were substantially increased [16-18]. Ligation of the CTP to the coding sequence of the proteins increased the half-life in vivo by 2-3 folds. In addition, it was shown that the use of FSH–CTP in human is safe and does not lead to detectable formation of antibodies [19, 20]. Moreover, pregnancy and live birth was achieved after ovarian stimulation using FSH-CTP for *in vitro* fertilization [21]. An interesting observation from the present study was the ability of a single injection once a week of EPO-CTP (15 μg/kg) to increase

the levels of haematocrit, whereas the same effect was achieved by administration of the same total dose of rhuEPO administered three times a week as 5 μg/kg per injection. These results indicated the importance of sustained blood levels, rather than total dose of EPO. These findings are consistent with the hypothesis that the ability of a single injection of EPO-CTP to increase haematocrit, results from its increased stability in the circulation.

The increased biopotency of the chimera may reflect a change in their metabolic clearance *in vivo*. Therefore, the circulatory half-lives of the hormones were determined. EPO-WT or EPO-CTP chimera were injected IV into immature male mice and RIA monitored the plasma half-lives. The results indicated that a higher level of the chimera is still detectable in serum after 24 h. The half-life of EPO CTP is increased 2-3 fold comparing to EPO-WT. These data suggest that the mechanism of EPO metabolic clearance is affected by the presence of CTP. Previously, a long acting EPO was developed by increasing the carbohydrate content using site – directed mutagenesis [22]. These studies indicated that addition of two more N-linked glycosylation sites to EPO backbone increased significantly the in vivo half-life and bioactivity. However, this analog binds to human EPO receptor with a lower affinity comparing to the binding of EPO wild – type (EPO-WT). However, it seems that ligation of CTP to the coding sequence of EPO is more promising although; clinical studies are needed in order to examine the immunogenicity of the chimeric gene.

It was reported that the *N*-linked oligosaccharide chains are critical for bioactivity of the glycoprotein hormones. Deletion of the oligosaccharide chain at Asn 52 of the α-subunit resulted in a significant reduction in bioactivity of hCG or hFSH [23, 24]. Interestingly, deletion of the *N*-linked oligosaccharide units from glycoprotein hormones increases receptor binding affinity [25]. Assembly of the glycoprotein hormone subunits is the rate-limiting step in the production of functional heterodimers. Mutations in the glycoprotein hormone subunits significantly affected assembly of heterodimer subunits [26]. To bypass the problem of dimerization of deglycosylated subunits, the subunits β and α were genetically fused in a single chain hormone with or without the CTP as a linker between the subunits. Single chains of hTSH [27, 28] retained a biologically active conformation similar to that of the wild-type heterodimer.

The hTSHβ-CTP-α single chain was used to investigate the role of *N*-linked oligosaccharides of hTSH in the secretion and function of hTSH. Two variants were prepared; one lacking the *N*-linked oligosaccharide chains of α subunit (hTSHβ-CTP- α_{1+2}), and the other lacking the oligosaccharide chains of both subunits, β and α (hTSHβ-CTP-α(deg) (Fig. 3). Both mutants expressed and secreted from CHO cells. Pulse-chase analysis and immunoprecipitation studies indicated that wild-type hTSHβ- CTP-α and hTSHβ-CTP-α_{1+2} were secreted efficiently with a similar $t_{1/2}$ of 2h. However, the secretion rate of hTSHβ-CTP-α (deg) was significantly slower with $t_{1/2}$ of 17h. Human dimeric hTSH as

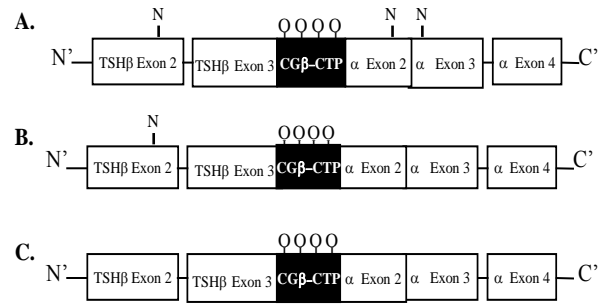Fig.3. Mutant forms of hTSH single peptide chain, hTSHβ CTPα wild-type contains the *N*-linked glycosylation sites at Asn23 of the hTSHβ subunit and at Asn52 and Asn78 of the α subunit. hTSHβ CTP$\alpha1+2$ contains only the *N*-linked glycosylation site on Asn23 of the β subunit. hTSHβ CTPα(deg) lacks the N-linked oligosaccharide chains from β and α subunits.

well as *N*-linked free carbohydrate-free single chain variants bind to hTSH receptor with high affinity.

However, deletion of the *N*-linked oligosaccharide chains of hTSHα subunit or also of β subunit resulted in a significant reduction in *in vitro* bioactivity. These findings led to the concept that *N*-linked carbohydrate-free variants may serve as hTSH antagonists. In competition experiments, cells grown in the presence of hTSH or human thyroid-stimulating immunoglobulins (hTSI) and different concentrations of deglycosylated hTSH variants, significantly blocked the activity of hTSH and hTSI. Moreover, the hTSH variants significantly inhibited TSH activity with respect to thyroid hormone secretion *in vivo*. The results thus indicated that deglycosylated hTSH variants behave as hTSH antagonists. Moreover it was shown that the effect of these variants is on the receptor binding site and not on the post receptor level [28]. The deglycosylated variants of hTSH do not affect the bioactivity of forskolin in the *in vitro* systems. These results suggest that the inhibition of TSH and TSI activity by deglycosylated variants, was not due to non-specific effects but is due to the receptor binding site.

The receptors for the glycoprotein hormones have been cloned and sequence analysis indicated that it belongs to the superfamily of G – proteins coupled receptor. The hormone binds the extracellular domain of the receptor and activates G proteins. Possible, amino acid motifs in the extracellular domain of the receptor recognize *N*-linked oligosaccharides and mediate G protein coupling and induce glycosylated TSH function. Deglycosylated TSH variants still bind to the extracellular domain with high affinity. However, absence of the *N*-linked oligosaccharides keeps the *N*-linked extracellular site free and do not mediate G protein coupling.

4. Conclusions

Ligation of the CTP bearing four sites for O-linked oligosaccharide chains to different proteins indicated that the *O*-linked glycosylation recognition sites of the CTP are preserved. Moreover, this ligation is not involved in secretion, receptor binding and *in vitro* bioactivity. However, both the *in vivo* bioactivity and half-life in circulation of proteins bearing the CTP were significantly enhanced. Proteins containing the CTP could serve as long acting agonists for clinical use. This strategy may have wide applications for en-

hancing the *in vivo* bioactivity and half-life of diverse proteins.

The *N*-linked oligosaccharides have a minor role in receptor binding but they are critical for bioactivity. Mutant glycoprotein hormones lacking the *N*-linked oligosaccharides bind with high affinity to the receptor have low biological activity and behave as antagonists. In the case of hTSH, deglycosylated variants blocked the activity of hTSH as well as of hTSI which responsible for the most common cause of hyperthyroidisim, Graves' disease. Thus hTSH deglycosylated variants behave as potential antagonists, offering a novel therapeutic strategy in the treatment of hyperthyroidism caused by Graves' disease and TSH secreting pituitary adenomas.

5. Acknowledgments

6. References

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