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Review

Involvement of equine chorionic gonadotropin (eCG) carbohydrate side chains in its bioactivity; lessons from recombinant hormone expressed in insect cells

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Abstract – Natural eCG consists of as much as 45% carbohydrate side chains. The present paper deals with the analysis of the roles of the N- and O-linked saccharides of this hormone in the different steps of its activity and its possible repacement by recombinant eCG expressed in baculovirus – insect cell systems.

half-life / gonadotropin / carbohydrate / recombinant hormone / baculovirus

1. INTRODUCTION

Equine chorionic gonadotropin (eCG) formerly named pregnant mare serum gonadotropin (PMSG) belongs to the family of glycoprotein hormones. It is produced by trophoblast cells of endometrial cups in pregnant mares and plays a major role in the maintenance of early gestation [1]. As the other members of the family including luteinizing hormone (LH), follicle-stimulating hormone (FSH) and thyroid stimulating hormone (TSH), eCG is composed of two dissimilar and noncovalently associated α-and β-subunits. Within the same species, the α-subunit is encoded by a single gene and is common to all glycoprotein hormones whereas different genes encode β-subunits, which confer specificity to the glycoprotein hormone heterodimers [2, 3]. In horses, in contrast to primates, both LH and CG β-subunits are encoded by the same gene [4] and consequently the recombinant hormone is called eLH/CG. Both placental eCG and pituitary eLH exhibit dual LH and FSH in non equine species with identical FSH/LH activity ratios [5]. Although eCG and eLH exhibit identical α and β polypeptide chains, their carbohydrate contents are different. Indeed, eCG is the most heavily glycosylated of all glycoprotein hormones with 45% carbohydrate by weight versus 30% for eLH. The α-subunit, composed of 96 amino acids, bears two complex-type N-linked oligosaccharide chains located at asparagines (Asn) 56 and 82 whereas the β-subunit composed of 149 amino acids has only one at Asn 13. In addition to N-glycans, both eLH and eCG β-subunits possess a carboxy-terminal peptide (CTP) of 29 amino acids (β121–149), which is O-glycosylated at the same twelve serine or threonine residues [6]. Placental and pituitary hormones also strongly differ by their N-glycan termination with sialic acids (Siaα2, 3Gal) on eCG and sulfated N-acetylgalactosamines (SO_4-4-GalNac) in eLH [7, 8].

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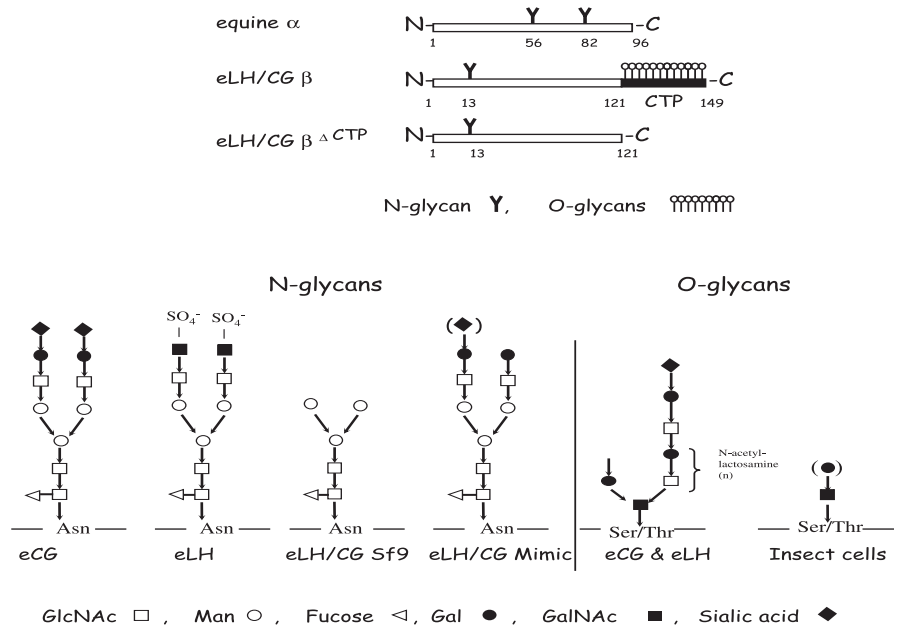

Figure 1. Positions and structures of carbohydrate chains of equine gonadotropins. Upper panel: Positions of N- and O-glycans in the α- and β-subunit of eLH and eCG, and in the recombinant β-subunit lacking its CTP. Lower panel: Typical structures of N-carbohydrate side chains (left) and O-carbohydrate side chains (right) of natural eCG and eLH and of recombinant eLH/CG expressed in Sf9 and Mimik cell lines respectively. In O-glycans, the number of N-acetyl-lactosaminyl motifs (n) is much larger in eCG than in eLH.

The remarkable difference in their molecular weight is essentially due to the presence of longer disialylated poly-N-acetyllactosaminyl O-glycans on eCG with a greater percentage of O-glycans attached at serine and threonine sites [6, 9] (Fig. 1). These structural differences explain why eCG has such an exceptional half-life compared to eLH. Owing to its biological properties, eCG has been used for a long time in fertilization programs. Nevertheless, commercial preparations of partially purified eCG from pregnant mare sera (PMSG) could contain contaminants with potential sanitary risks.

It is of great interest to produce in large quantities a bioactive substitute for eCG and other gonadotropins used as therapeutic agents. Only recombinant gonadotropins produced in mammalian cells have been shown so far to exhibit in vivo biological activity. Such hormones like hFSH expressed in CHO cells [10] have even been put on the market (Gonal-F® Serono, Geneva, Switzerland; Puregon® NV Organon, Oss, The Netherlands). However, the great advantage of non-mammalian systems like yeasts, insect cell lines or plants would be to produce recombinant proteins in large amounts, at a lower cost, and in the absence of fetal calf serum. Some gonadotropins of zootechnical interest have already been expressed in baculovirus systems [11–14], but also in the methylotropic yeast *Pichia pastoris* [15–17], and in plants [18]. However, only in vitro biological activities have been reported in these studies with no information concerning in vivo potencies.

Recently, single-chain eLH/CG [19] and bFSH [20] have been produced in milk of transgenic rabbits. In vivo biological activity was determined only for eLH/CG, and it was found to be inactive because of its

too-short half-life, likely because the carbohydrate moieties of recombinant glycoproteins in milk [21] are unusual compared to those found in serum glycoproteins.

In three recent papers we have described the long half-life of natural eCG in rats [22], the production of recombinant eLH/CG by Sf9 cells and Mimic cells and compared the biological [23] and structural [24] properties of these hormones with those of natural eLH and eCG.

2. TOMOGRAPHIC STUDY OF eCG HALF-LIFE

In contrast to ^{123}I-pLH that is trapped by kidneys in less than 5min time, ^{123}I-eCG remains in the circulation over hours in the rat [22]. This huge difference is due to the higher molecular weight and negative charge of eCG that impedes its glomerular filtration. The acidic nature of eCG is due in large part to its high sialic acid content and therefore, this point must be taken into account in the production of recombinant eCG.

3. BIOLOGICAL PROPERTIES OF RECOMBINANT eCG EXPRESSED IN BACULOVIRUS-INSECT CELL SYSTEMS

This expression system allows a high level of production of recombinant protein but N-glycans processed in insect cells such as Sf9 cells are not complex and sialylated N-glycans such as those found in placental eCG. However, the in vivo biological activity of eCG strongly depends on the presence of sialylated complex saccharidic chains on protein. To overcome this problem, we expressed recombinant eLH/CG in Mimic^{TM} cells that had been claimed to produce biantennary, terminally sialylated N-glycans. Equine CG possesses extensive N- and O-glycosylation with 45% of its mass due to its carbohydrate moiety. In addition to the complex sialylated biantennary N-glycans [7], eCG possess twelve uncommon disialylated poly-(N-acetyllactosamine) O-glycans located at the C-terminus of the β-subunit [6, 9].

In Sf9 cells, N-glycans consist of paucimannose structures with or without fucose residues linked to the chitobiose core. However, some insect cell lines like *Estigmene acrea* Ea4 cells possess low levels of N-acetyl-β-glucosaminidase leading to N-glycans with non-reducing terminal N-acetylglucosamine residues and others like *Trichoplusia ni* (TN-5B1-4, High Five) cells may possess a low level of galactosyltransferase activity [25]. Although having a low N-glycosylation potential, Sf9 cells have the advantage of synthesizing no immunogenic glycans in contrast to *Trichoplusia ni* cells in which immunogenic α1-3-fucose linkages are found [26].

Recombinant eLH/CG and its subunits expressed in Sf9 cells and in Mimic^{TM} cells exhibited lower apparent molecular weights compared to mare plasma eCG and its subunits, highlighting the presence of shorter glycans in these cells. Nevertheless, the higher apparent molecular weights found for eLH/CG and its subunits expressed in Mimic^{TM} cells compared to Sf9 cells strongly suggest that glycosylation was indeed improved. The major differences in MW between natural and recombinant hormones seem to be essentially due to very short O-glycans on the CTP domain of eLH/CG produced in insect cells.

Both eLH/CG expressed in Sf9 and Mimic^{TM} cells exhibited full in vitro LH and FSH bioactivities confirming that recombinant hormones possess a conformation similar to that of natural eCG. Equine LH/CG expressed in Mimic^{TM} cells and Sf9 cells exhibit in vitro FSH/LH ratios that are not significantly different. By contrast, both recombinant eLH/CG exhibit no in vivo biological activity. Even recombinant eLH/CG produced in Mimic^{TM} cells was found to be inactive in the FSH in vivo bioassay of Steelman and Pohley, which does not require a hormone with a very long half-life as in the eCG in vivo bioassay of Cole and Erway. The absence of in vivo bioactivity

of recombinant eLH/CG might be the result of a rapid clearance of the hormone in the plasma as is the case for single-chain eLH/CG expressed in the milk of transgenic rabbits [19]. Sialic acids are mainly responsible for a prolonged half-life in plasma suggesting they are partially or totally lacking on eLH/CG produced in Sf9 cells and in Mimic^{TM} cells. Native eLH is known to bear sulfated N-glycans and sialylated O-glycans. Although displaying similar in vitro FSH/LH ratios, native eLH, which bears less sialic acids compared to eCG, exhibits a much shorter half life. The presence of sialylated O-glycans is also involved in the half-life since the elimination of the O-glycosylated CTP from hCG led to a drastic decrease of its half-life [27].

4. BIOCHEMICAL PROPERTIES OF RECOMBINANT eCG EXPRESSED IN BACULOVIRUS-INSECT CELL SYSTEMS

Natural and recombinant eCG α-subunits were found to be N-glycosylated since their hydrolysis with PNGase F led to proteins with lower apparent MW that were not recognized by Concanavalin A. Sf9 cells produce glycoprotein hormone with N-oligomannosidic structures, whereas Mimic^{TM} cells elaborate complex-type N-glycans bearing terminal galactose without any sialylation. Lectin analyses also show the presence of O-linked oligosaccharidic chains on recombinant subunits and eLH/CG secreted by Sf9 and Mimic^{TM} cells, with a GalNAc-Gal disaccharide structure (Legardinier et al, submitted) as previously shown for insect or recombinant glycoproteins expressed in Sf9 and other insect cell lines [28, 29].

5. CONCLUSIONS

Two strategies can be proposed to engineer N- and O-glycosylation; one by modifying the cell genome of insect cells and the other by introducing all needed glycosyltransferase genes in the baculovirus genome. Recently, a new transgenic insect cell line (SfSWT3) was described [30], that corresponds to the transformation of Mimic^{TM} cells (SfSWT1) with two additional mammalian genes encoding a sialic acid synthase and CMP-sialic acid synthase. It has been claimed to correctly sialylate a recombinant glycoprotein on its two antennary N-carbohydrate chains in the absence of fetal bovine serum and could thus be of great interest in the production of active recombinant gonadotropins.

Another approach will be to construct transgenic viruses containing the genes encoding eCG subunits together with all required glycosidase and glycosyltransferase genes.

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