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Glucagon rDNA origin (GlucaGen®) and Recombinant LH (No. 12 in a series of articles to promote a better understanding of the use of genetic engineering)

1. Glucagon rDNA origin (GlucaGen®)

1.1. Glucagon

Glucagon is an endogenous 29 amino acid peptide produced by α-cells in the islets of Langerhans. It is derived from a 180 amino acid preproglucagon that is cleaved at dibasic sequences to release a couple of small peptides including glucagon. Physiologically, glucagon plays a significant role in the regulation of glucose metabolism. It is considered the primary hormone counter-regulatory to insulin. Glucagon induces hepatic glucose production and release by stimulating hepatic glycogenolysis when hypoglycemia occurs. Glucagon also exhibits extrahepatic effects which include increase in lipolysis, inotropic and chronotropic effects on the heart, and relaxation of smooth muscle of the gastrointestinal tract. An inhibitory effect on gastric and pancreatic secretions has also been shown.

Glucagon purified from beef and pork pancreas has been marketed for the treatment of hypoglycemia and as diagnostic aid when a hypotonic state of the gastrointestinal tract is desirable.

1.2. Glucagon for treatment of hypoglycemia

Insulin treatment without adequate carbohydrate intake may lead to hypoglycemia with loss of consciousness. Glucagon injection is a rapid and effective treatment of severe hypoglycemia when exogenous glucose is not readily available.

Unconsciousness caused by hypoglycemia is a dangerous potentially lethal condition which may occur occasionally in diabetes patients. Obviously the unconscious patient cannot swallow carbohydrate making parenteral treatment of hypoglycemia required. Glucagon may be injected and can therefore easily be administered by appropriately instructed persons.

1.3. Glucagon for use as a diagnostic aid

Glucagon has an inhibitory effect on the motility of gastrointestinal smooth muscle. The effect of glucagon in upper and lower gastrointestinal radiography, endoscopic examinations, abdominal CT-scanning and other diagnostic procedures has been documented.

Glucagon relaxes the smooth muscle of the gastrointestinal tract. Glucagon is therefore of value as pre-medication for diagnostic procedures such as certain radiologic examinations and endoscopic procedures where immobility of the gastrointestinal tract is critical. Glucagon injections are not associated with the common side-effects of anti-cholinergic compounds which are alternatively used for this indication.

1.4. Glucagon made by genetic engineering

The commercial product, GlucaGen-R (Table 1), was first marketed in 1992.

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Due to the risk of infectious particles of animal origin in the product prepared from pancreases, it is an advantage to produce glucagon from a safe recombinant micro-organism.

Saccharomyces cerevisiae is accepted as a non-pathogenic organism which is well suited for recombinant DNA production. Due to its capability of secreting specific peptides into the growth medium in relatively large amounts, and at the same time secreting very low levels of proteins and proteases, it was chosen as the host for glucagon expression.

Accordingly, a yeast strain was transformed with a plasmid harbouring the sequence coding for a truncated mating factor α_1 leader sequence joined with a synthetic gene encoding the 29 amino acids of glucagon. This recombinant strain produces and secretes significant amounts of glucagon.

Glucagon made by genetic engineering has a molecular structure and therapeutic effect identical to that of glucagon derived from animals, but presents no risk of transmission of known or unknown diseases such as bovine spongioform encephalitis because the recombinant glucagon is produced without use of materials derived from animals.

1.5. GlucaGen-R

The production of GlucaGen-R is based on an aerobe fed batch fermentation of the recombinant yeast strain with standard nutrients and a continuous controlled dosage of glucose.

Before removing the yeast by centrifugation, the fermentation broth is treated with alkali in order to dissolve precipitated glucagon, as glucagon has a low solubility at the pH level used during fermentation.

The glucagon is recovered and purified from the supernatant by a series of chromatographic and precipitation steps. The more than 98% pure Glucagon is finally stabilized by lyophilization.

1.6. Conclusion

The production of GlucaGen-R, free of any risk of animal infection and more pure than glucagon of pancreatic origin, is another example on how genetic engineering can be used for the production of safe proteins for therapeutic and diagnostic uses.

Table 1
Made by genetic engineering, no. 12

Product	Glucagon	Recombinant human luteinising hormone (r-hLH)
Principal trade names	GlucaGen	—
Principal uses	Hypoglycemia and diagnostic aid (inhibition of motility of smooth muscle)	Treatment of infertility
Manufacturers	Novo Nordisk AS, Denmark	Ares-Serono International, Switzerland
Donor organism	None (synthetic DNA)	Human gene
Host organism	*Saccharomyces cerevisiae*	Chinese Hamster Ovary (CHO) cells
Advantages	Not of animal origin, therefore no risk of infectious diseases	First available preparation containing LH alone; unlimited supply, not dependent upon urine collection; fully controlled closed manufacturing process, better batch-to-batch consistency; greater purity, absence of contaminant human proteins, suitable for subcutaneous injection; better local tolerance and safety profiles.

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2. Recombinant LH for infertility treatment

Luteinising hormone (LH), a glycoprotein produced in the anterior pituitary, plays an important role in normal reproductive function. It enhances follicular oestradiol production, provokes resumption of oocyte meiosis through the mid-cycle LH surge, stimulates follicular rupture and then maintains the corpus luteum, providing support for the early stages of pregnancy.

For most women undergoing assisted reproductive techniques (ART), administration of follicle-stimulating hormone (FSH) alone is sufficient to promote multiple follicular development before in vitro fertilisation/intracytoplasmic sperm injection–embryo transfer (IVF/ICSI-ET). In most cases FSH is also sufficient for stimulation of single follicular development in the case of anovulation. However, a small proportion of women whose infertility results from hypogonadotrophic hypogonadism require treatment with both FSH and exogenous LH to stimulate the oestradiol production essential for establishment of pregnancy.

For many years, the only source of LH for women undergoing ART was human menopausal gonadotrophins (hMG), which were extracted from the urine of postmenopausal women. Such preparations contain not only LH but also FSH, human chorionic gonadotrophin (hCG) and many other urinary proteins, which account for most of their total protein component. Their production also requires large volumes of raw material. Following the development of recombinant FSH for use in ART, Ares-Serono used the same recombinant DNA techniques to develop a consistent, reliable LH preparation free from the potential problems associated with human source material.

LH is a heterodimer glycoprotein, composed of two non-covalently linked subunits, designated α and β. The α-subunit, which is common to all gonadotrophins, consists of 92 amino acids and has two N-linked glycosylation sites. The β-subunit, which gives the hormone its biological specificity, consists of 121 amino acids and has a single N-linked glycosylation site. The gene for the α-subunit was isolated from a bacteriophage lambda library containing human foetal liver genomic DNA. The gene for the β-subunit was isolated from a pBR322 library containing human pituitary cDNA. However, the intact 5′ sequence for this gene could not be isolated. Consequently, a complete gene was constructed by insertion of a small portion of the hCG β-subunit fragment (the hCG and LH β-subunits are structurally similar). This contributed the ATG start codon and the first 12 amino acids of the secretory signal peptide for the β-subunit of the recombinant LH (r-hLH).

The selected expression vectors had a bacterial origin of replication, to allow maintenance in *Escherichia coli* K12. They also included the mouse metallothionein-I gene promoter for constitutive expression of the inserted sequence and the SV40 early polyadenylation signal. The dihydrofolate reductase-selectable marker on the α-subunit expression vector was used to isolate the transfectants in the presence of methotreate. These expression vectors were transfected into a standard Chinese Hamster Ovary (CHO) cell line for production of r-hLH (Table 2). Sequencing of cDNA and genomic 5′ and 3′ non-transcribed regions, restriction enzyme analysis, mRNA size analysis and fluorescence in situ hybridisation all confirmed the stable integration of the transfected sequences into the CHO cell genome and the integrity of the r-hLH subunit gene sequences.

Extensive physico-chemical analyses have demonstrated that the primary structures of both subunits of the r-hLH molecule produced in this system correspond to those of the native peptides. As in both urine- and pituitary-derived human LH, N-terminal heterogeneity is seen in both subunits of r-hLH, and truncated forms of the C-terminus of the r-hLH β-subunit are present. The glycosylation sites of the gonadotrophins are important to their biological activity, and these sites are conserved on r-hLH. The biological activity of r-hLH has been demonstrated in the rat seminal

Table 2
Summary of products published in the series MBGE

Product	Author/Manufacturer	Reference
Lipase	B. Diderichsen, Novo Nordisk, Denmark	MBGE No. 1, Biotech Forum Europe 8, 246–247, 1991.
Hepatitis B vaccine	P. Crooy, SmithKline Beecham Biologicals, Belgium	
Human insulin	E. Rasmussen, Novo Nordisk, Denmark	MBGE No. 2, Biotech Forum Europe 9, 144–145, 1992.
Human growth hormone	L. Fryklund, KabiPharmacia, Sweden	
Protein G	R. Hjorth, Pharmacia LKB Biotechnology, Sweden	MBGE No. 3, Biotech Forum Europe 9, 641–642, 1992.
Interferon alfa-2a	S. Ryser, F. Hoffmann-La Roche AG, Switzerland	
AIDS test	E. Baumann, F. Hoffmann-La Roche AG, Switzerland	MBGE No. 4, J. Biotechnol. 38, 193–197, 1995.
α-Amylase	B. Diderichsen, Novo Nordisk, Denmark	
Erythropoietin	C. Kionka, Boehringer Mannheim, Germany	MBGE No. 5, J. Biotechnol. 43, 73–77, 1995.
Interferon beta-1b	T. Petri, Schering AG, Germany	
Interferon gamma	E. Falkner and I. Maurer-Fogy, Bender & Co GesmbH/Boehringer Ingelheim Vienna, Austria	MBGE No. 6, J. Biotechnol. 46, 155–159, 1996.
Rabies vaccine	J. Terré, G. Chappuis, M. Lombard and P. Desmettre, Rhone Mérieux, France	
Tissue plasminogen activator (rt-PA)	W. Werz and R.G. Werner, Dr. Karl Thomae GmbH/Boehringer Ingelheim Pharma, Germany	MBGE No. 7, J. Biotechnol. 61, 157–161, 1998.
Granulocyte–macrophage colony-stimulating factor (GM-CSF)	R. Till, Novartis/Schering Plough International, Switzerland	
Human coagulation factor VII	U. Hedner and T. Lund-Hansen, Novo Nordisk A/S, Denmark	MBGE No. 8, J. Biotechnol. 61, 231–236, 1998.
Follicle stimulating hormone (FSH)	J.C. Heikoop and W. Olijve, N.V. Organon, Akzo Nobel, The Netherlands	
Alzheimer tau test	E. Vanmechelen and H. Vanderstichele, Innogenetics N.V., Belgium	MBGE No. 9, J. Biotechnol. 66, 229–233, 1998.
Detergent Cellulase	B. Jones and W. Quax, Genencor International, The Netherlands	
Chymosin	P.W.M. van Dijck, Royal Gist-brocades, The Netherlands	MBGE No. 10, J. Biotechnol. 67, 77–80, 1998.
Phytase	P.W.M. van Dijck, Royal Gist-brocades, The Netherlands	
Recombinant Glucocerebrosidase	H. Hoppe, Genzyme Corporation, USA	MBGE No. 11, J. Biotechnol. 76, 259–263, 2000.
Lyme Disease vaccine	P. Crooy and Y. Lobet, SB Biologicals, Belgium	
Glucagon	T. Olsen, B. Diderichsen, Novo Nordisk A/S, Denmark	MBGE No. 12, J. Biotechnol. 79,185–189, 2000.
Recombinant Luteinising Hormone	P. Fonjallaz, E. Loumaye, Ares-Serono International, Switzerland	

vesicle weight gain bioassay and also in a primate model of in vitro fertilisation. In both cases, biological responses to r-hLH and hMG were shown to be similar.

Clinical studies indicate that FSH therapy alone is highly effective in inducing follicular growth, with or without supression of endogenous LH with a gonadotrophin-releasing hormone (GnRH)agonist. In the majority of infertility patients, administration of additional LH would have no practical benefit. However, the small population of patients with hypogonadotrophic hypogonadism (< 6% of all patients) would be expected to benefit from exogenous LH administration. Two clinical studies were undertaken to investigate the efficacy and safety of r-hLH in such patients. Both studies found that daily treatment with r-hLH in addition to r-hFSH was well tolerated, and produced a dose-related effect on oestradiol production and related parameters such as endometrial thickness. A low daily dose of 3.4 μg (75 IU) of r-hLH produced sufficient steroidogenesis and follicular development in most patients. Other studies suggest that r-hLH at an intermediate dose (20 μg, 450 IU) may be useful in minimising the number of pre-ovulatory follicles in women undergoing ovulation induction, in order to reduce the risk of multiple pregnancy. Finally, a dose-finding study has shown that r-hLH at high doses (1 mg, 20 000 IU) is as effective as hCG in inducing final follicular maturation and early luteinisation in women undergoing IVF/ET, and is associated with significantly lower incidence of severe ovarian hyperstimulation syndrome (OHSS).

Recombinant hLH has been developed, is manufactured and will be marketed by Ares-Serono. It is the first pharmaceutical presentation of human luteinising hormone from any source that is free of other gonadotrophins. In contrast to hMG, which has been the mainstay of infertility treatment for many years, r-hLH is a well-characterised protein of high purity, which can be produced reliably and consistently in large quantities without the supply problems or risks of contamination associated with human-derived products. It therefore represents an important development in the treatment of infertility.

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The products published in this series so far are summarised in Table 2.