Oral and pulmonary delivery of FSH–Fc fusion proteins via neonatal Fc receptor-mediated transcytosis

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BACKGROUND: The α and β subunits of FSH were fused to the Fc domain of IgG1 either in a single chain or a heterodimer format. These molecules were absorbed through the epithelium in lung and intestine by neonatal Fc receptor (FcRn)-mediated transcytosis. METHODS AND RESULTS: Single chain and heterodimer FSH–Fc were made recombinantly in Chinese hamster ovary cells. Treatment of rats with a single s.c. dose of single chain or heterodimer FSH–Fc resulted in greater stimulation of ovarian weight (20.8 ± 3.9 and 26.9 ± 6.1 mg respectively) compared to those receiving vehicle (12.1 ± 1.0 mg) or an equimolar dose of recombinant human FSH (14.3 ± 1.7 mg). Both FSH–Fc fusion proteins were absorbed after oral dosing of newborn rats with long terminal half-lives of ~60 h, and pulmonary delivery in four cynomolgus monkeys produced maximum serum concentrations between 69 and 131 ng/ml with long terminal half-lives between 55 and 210 h. Serum inhibin levels increased after pulmonary dosing with single chain FSH–Fc (1.3- and 1.4-fold) and heterodimer FSH–Fc (5.9- and 7.1-fold) and remained elevated for >12 days after treatment with heterodimer FSH–Fc. CONCLUSIONS: We have shown that FSH–Fc fusion proteins have increased stability in blood and improved bioactivity in vivo, and that heterodimer FSH–Fc is more active in rats and monkeys than single chain FSH–Fc. These data suggest that Fc fusion proteins offer the potential for oral and pulmonary delivery of FSH.

Key words: FcRn/FSH/lung/pulmonary delivery

Introduction

Recombinant human (r)FSH is a common therapy in the treatment of both male and female infertility. Induction of folliculogenesis in women requires daily s.c. or i.m. administration of FSH for ~8–10 days, while daily injections are required for up to several months to induce spermatogenesis in hypogonadotropic males (Schaison et al., 1993; Schoot et al., 1994). Longer-acting FSH preparations would therefore have obvious advantages in the tolerability of infertility treatments. A novel and less invasive means of delivering FSH without the use of needles would also be desirable but remains problematic for a variety of reasons including the relatively large size of the protein, its heterodimeric nature (i.e. non-covalently linked α and β subunits; Pierce and Parsons, 1981) and the natural barrier represented by epithelial cells. An alternate approach to the delivery of FSH and other therapeutic proteins would therefore be the utilization of a natural transport system that exists within epithelial cells.

One candidate transport pathway involves the neonatal Fc receptor (FcRn) that was first described in newborn rodents as being responsible for the transport of IgG from maternal milk into the neonatal bloodstream (Simister et al., 1997). Human FcRn was first described in the placenta where it transports IgG from mother to fetus (Story et al., 1994). Although rodent FcRn levels decrease in epithelial tissues after weaning, human FcRn was recently detected in epithelial cells of adult human lung and intestine (Israel et al., 1997), and has been shown to transport IgG across human epithelial cells in vitro (Dickinson et al., 1999). Various proteins have been fused to the Fc domain of IgG1 and shown to retain biological activity (Ashkenazi et al., 1993; Chamow and Ashkenazi, 1996; Economides et al., 2003; Bitonti et al., 2004). Conjugation to Fc also increases the serum half-life of the fusion partner (Kim et al., 1998) due to the ability to bind FcRn which is the major regulator of IgG half-life (Kim et al., 1994; Junghans and Anderson, 1996; Simister et al., 1997). We therefore propose that FcRn may be used to transport therapeutic proteins, including FSH, across epithelial barriers and concomitantly increase the circulating half-life of the molecule.

FSH is a non-covalently linked dimeric protein consisting of α and β subunits (Pierce and Parsons, 1981). Subunit assembly is essential for bioactivity of FSH (Jia and Hseuh, 1986) as well as for the stability of the β subunit (Keene et al., 1989). However, it has been shown that a single chain
fusion of the α and β subunits of FSH is fully active (Sugahara et al., 1996) and has an increased serum half-life with the carboxy-terminal peptide of hCG (Bouloux et al., 2001; Duijkers et al., 2002; Klein et al., 2003).

In this study we have investigated the effect of linking the Fc domain of immunoglobulin G1 (IgG1) to FSH, either as a single chain or with the α and β subunits linked to separate arms of the Fc fragment as a heterodimer FSH–Fc molecule. These fusion proteins were used to examine carrier-mediated transport via FcRn across intestinal epithelia in neonatal rats and lung epithelia in cynomolgus monkeys.

Materials and methods

Construction of single chain and heterodimer FSH–Fc fusion molecules

For single chain FSH–Fc constructs, FSHβ was isolated with its native signal sequence from a human pituitary mRNA library (Clontech, USA) using the following primers: 5'-CTAGCTTCAG-GCCACCATGAAGACACTCCAGTTTCC-3' and 5'-TCCATTTCC-GGGTTCTTTCATTTCACCAAAGGAG-3'.

FSHα was isolated from the same human pituitary mRNA library but without the signal sequence using the following primers: 5'-TACCTTCGGGGCTCTGATGTCAGATT-3' and 5'-GCATGGGATATTTCATTTTGATATAAAACATTAC-3'.

The area of each primer predicted to anneal to the corresponding template is underlined. The two FSH subunits were ligated to form a contiguous FSHβ–FSHα fusion without a 3' termination codon. The Fc fragment of human IgG1 (hinge, CH2 and CH3 domains; amino acids 221–447, EU numbering) was isolated from a human leukocyte cDNA library (Clontech) using the following primers: 5'-AAAGATTCCTCGGCGCGCGCTGCGGTCGACAACTAC-3' and 5'-TACATTTGATATTTCATTTTTCAGGGACAGGG-3'.

The area of each primer predicted to anneal to the Fc region of IgG1 is underlined. The 5' primer also contains sequence shown in bold that will create an eight amino acid linker sequence (GGGGSGGGGSGGGGS) between the carboxy terminus of FSHα and the amino terminus of the Fc. The sequence of each primer corresponding to this linker sequence is shown in bold. The adenovirus major late promoter in pED.dC was replaced by a cytomegalovirus (CMV) promoter using standard PCR techniques. The mammalian expression vector pcDNA6 (Invitrogen, USA) that contains a CMV promoter was the template for this PCR reaction. The FSHα and Fc PCR fragments were then cloned into this CMV–pED.dC vector (Figure 1b). For FSHβ–Fc, FSHβ was isolated from a human pituitary mRNA library (Clontech) using the following primers: 5'-GGCTAGCTGGAGGDGGCGGTCCGCGGG-TGHAGGAGGACGAAAACACTACCACGTGCC-3' and 5'-GGTAAAAGACACGTGGCTCACTCAGTGTGATGATGATGTACACCCGG-GAGACAGGG-3'.

The sequence of each FSHβ and Fc primer predicted to anneal to the corresponding template is underlined. A 15 amino acid linker sequence (GGGGSGGGGSGGGGS) was generated between the carboxy terminus of FSHβ and the amino terminus of Fc. The sequence of each primer corresponding to this linker sequence is shown in bold. The 3' termination codon of the Fc region was replaced by a bacterial 6Histidine (6His) tag sequence was created on the Fc fragment. The sequence of the Fc primer corresponding to the 6His tag is shown in italics. The FSHβ and Fc–6His fragments were then cloned into the mammalian expression vector pcDNA6 (Invitrogen) that contains a CMV promoter and 6Histidine gene for selection purposes (Figure 1b). Heterodimer FSH–Fc protein generated from co-transfection of FSHα–Fc and FSHβ–Fc constructs will be ~94.5 kDa in size.

Expression and purification of single chain and heterodimer FSH–Fc

Single chain FSH–Fc was transfected into CHO DG44 cells lacking a dihydrofolate reductase gene (obtained from Dr L. Chasin, Columbia University, NY, USA; Urlaub et al., 1986) using standard Superfect transfection protocols (Qiagen, USA). After 48 h, transfected cells were selected in minimum essential medium (MEM) α without ribonucleosides and deoxyribonucleosides containing 5% dialysed fetal bovine serum (FBS). To obtain higher protein expression levels, cells were treated with methotrexate at concentrations ~200 mmol/l. For protein production, cells were seeded into roller bottles in Dulbecco’s modified Eagle’s medium (DMEM): F-12, 10% FBS and incubated for 3 days before changing the medium to DMEM: F-12 + 5 µg/ml human insulin (Invitrogen). Conditioned medium was collected daily for 10 days, then filtered through 0.2 µm filters, and stored at 4°C until purification. Single chain FSH–Fc was purified from culture medium using protein A affinity chromatography. After medium containing single chain FSH–Fc was loaded, protein A columns were washed with 5–10 column volumes of PBS (10 mmol/l phosphate pH 7.4, 2.7 mmol/l KCl and 137 mmol/l NaCl) and bound protein eluted with 0.1 mol/l glycine, pH 3.0. Eluted single chain FSH–Fc was then dialysed into PBS and stored at ~80°C in aliquots containing 10% glycerol. Single chain FSH–Fc was run on Tris–glycine gels (Invitrogen) and stained with GelCode Blue (Pierce Chemical Company, USA) under reducing and non-reducing conditions to determine the purity of the protein. Single chain FSH–Fc was ~90% pure following a single protein A chromatography step (Figure 1d).
Figure 1. Schematic diagram of DNA constructs created to make single chain FSH–Fc (a), and heterodimer FSH–Fc proteins (b). Schematic diagram of single chain and heterodimer FSH–Fc fusion proteins (c). SDS–PAGE gel of single chain FSH–Fc and heterodimer FSH–Fc run under reducing and non-reducing conditions (d). Lane 1: single chain FSH–Fc reduced; lane 2: heterodimer FSH–Fc reduced; lane 3: single chain FSH–Fc non-reduced; and lane 4: heterodimer FSH–Fc non-reduced.
Heterodimer FSH–Fc was expressed by co-transfection of FSHα–Fc and FSHβ–Fc–6His expression vectors in CHO DG44 (Urlaub et al., 1986) cells using standard Superfect transfection methods. Forty-eight hours after transfection, cells were incubated in MEMα without ribonucleosides and deoxyribonucleosides containing 5% dialysed FBS and 10 μg/ml basicidin (Invitrogen) to select only for cells containing both FSHα–Fc and FSHβ–Fc expression vectors. To obtain higher expression levels, cells were treated with methotrexate ≤ 50 mM/l. For protein production, cells were seeded into roller bottles and culture medium containing secreted protein collected in the same way as for single chain FSH–Fc. Because FSHα–Fc and FSHβ–Fc are co-transfected, culture medium contains not only the desired heterodimer FSH–Fc, but also FSHα–Fc homodimer and FSHβ–Fc homodimer that require separation. An initial purification using protein A affinity chromatography was performed in the same way as described above for single chain FSH–Fc. Proteins were further purified using nickel affinity chromatography. FSHβ–Fc homodimers and heterodimer FSH–Fc bound to nickel affinity columns due to the presence of the 6His tag on FSHβ–Fc. Heterodimer FSH–Fc was separated from FSHβ–Fc homodimer by elution with an imidazole gradient (0–500 mM/l). The imidazole concentration was then increased to 1 mol/l to remove any remaining protein. Heterodimer FSH–Fc eluted at ~30 to 90 mM/l imidazole. Heterodimer FSH–Fc was run on Tris–glycine gels (Invitrogen) and stained with GelCode Blue (Pierce Chemical Company) under reducing and non-reducing conditions to determine the purity of the protein. Heterodimer FSH–Fc was ~90% pure following protein A and nickel affinity chromatography steps (Figure 1d).

In vivo bioactivity: ovarian weight gain assay

In vivo activity assays were carried out according to the protocol of Steelman and Pohley (1953) with minor modifications. Briefly, 21 day old female rats (10 rats per group) were given a single s.c. dose (1 nmol/kg) of recombinant (r)FSH (Follistim; Organon, The Netherlands), single chain FSH–Fc or heterodimer FSH–Fc in PBS. Seventy-two hours after dosing, ovarian weight was measured in each rat. Statistics were analysed using SigmaStat version 2.0 (RockWare Inc., USA).

Pharmacokinetic studies after oral dosing in neonatal rats

Ten day old neonatal rats (four rats/group) were dosed orally with 0.3 mg/kg single chain FSH–Fc or heterodimer FSH–Fc in PBS containing 5 mg/ml soybean trypsin inhibitor. At various times after dosing, blood was collected by cardiac puncture, allowed to clot, then serum prepared and stored at ~20°C. A sandwich enzyme-linked immunosorbent assay (ELISA) was developed using an FSH coating antibody (Fitzgerald Industries, USA) and a horseradish peroxidase-conjugated Fc detection antibody (Pierce Chemical Company, USA). The standard curve for the ELISA (200–0.78 ng/ml) was created with the same lot of single chain FSH–Fc or heterodimer FSH–Fc used to dose the rats. Samples were analysed in triplicate. Pharmacokinetic parameters were estimated using WinNonlin version 4.1 (Pharsight, USA).

Role of FcRn in oral delivery of single chain FSH–Fc and heterodimer FSH–Fc in neonatal rats

Single chain FSH–Fc and heterodimer FSH–Fc were iodinated with ¹²⁵Iodine iodide (Perkin Elmer, USA) using iodobeads (Pierce Chemical Company) according to manufacturer’s protocols. Free iodine was separated from iodinated protein on a PD-10 desalting column. Ten day old rats were dosed orally with iodinated single chain or heterodimer FSH–Fc (25 pmol) or a mixture of iodinated single chain or heterodimer FSH–Fc (25 pmol) and a 300-fold molar excess of unlabelled human IgG (7.5 mmol; ICN, USA) in PBS with 5 mg/ml soybean trypsin inhibitor. Three hours after dosing, blood was collected by cardiac puncture and serum prepared. A 100 μl aliquot of serum was incubated with protein A Tris–acrylamide beads (Pierce Chemical Company) at 4°C for 1 h. Protein A beads were then washed twice with PBS and eluted with sodium dodecyl sulphate (SDS) sample buffer containing 20% β-mercaptoethanol. Samples were boiled and analysed on 4–20% Tris–glycine gels, dried and quantification was performed on a Storm Phosphorimager (Molecular Dynamics, USA).

Oral bioactivity: testis weight gain assay

Oral bioactivity in male rats was determined according to a published protocol (Meachem et al., 1996) that demonstrated an increase in Sertoli cell number, resulting in an increase in testis weight, in neonatal rats treated with FSH daily for 10–20 days. Briefly, 2 day old male rats (10 rats per group) were orally dosed with 1 nmol/kg human rFSH (Follistim; Organon), single chain FSH–Fc or heterodimer FSH–Fc in PBS with 5 mg/ml soybean trypsin inhibitor. Rats were dosed daily for 14 days before measuring testis weight in each rat. Statistics were analysed using SigmaStat version 2.0 (RockWare, Inc.).

Pharmacokinetic/pharmacodynamic studies in cynomolgus monkeys

All studies with cynomolgus monkeys were conducted using approved protocols, following NIH guidelines for the care and use of research animals. Prior to pulmonary administration, animals were anesthetized with a combination of ketamine and valium and intubated with endotracheal tubes. Aerosols of single chain FSH–Fc (in PBS, pH 7.4) or heterodimer FSH–Fc (in PBS, pH 7.4 with 0.1% HSA) were created with an Aeroneb Pro™ nebulizer (Aerogen, USA) and administered to cynomolgus monkeys (deposited dose ~45 μg/kg) through the endotracheal tubes. A Bird Mark 7 A respirator (Bird Products, USA) regulated the depth (20–40% vital capacity) and rate of respiration (28–30 breaths per minute) of each monkey such that the delivery of single chain FSH–Fc or heterodimer FSH–Fc was targeted to the central airways where epithelial expression of FcRn is predominantly located (Bitonti et al., 2004). Aerosol particle size was ~4–5 μm. Blood samples were collected at various times after pulmonary dosing and serum prepared. Single chain FSH–Fc and heterodimer FSH–Fc levels were quantified using commercially available FSH ELISA kits (DRG International, USA) according to manufacturer’s directions. The standard curve for each assay (60 ng/ml to 2.5 ng/ml, limit of detection ~10 ng/ml) was created with the same lot of single chain FSH–Fc or heterodimer FSH–Fc used to dose the monkeys. Pharmacokinetic parameters were estimated using WinNonlin version 4.1 (Pharsight). Serum samples obtained after pulmonary dosing of single chain FSH–Fc or heterodimer FSH–Fc were also used to determine inhibin levels using commercially available ELISA kits (Diagnostic Systems Laboratories, USA) following manufacturer’s instructions.

Results

Protein characteristics

Figure 1c shows a schematic diagram of single chain FSH–Fc and heterodimer FSH–Fc fusion proteins. Single chain FSH–Fc is a fusion molecule of FSHα, FSHβ and the Fc portion of a human IgG₁ molecule including the hinge, CH2.
and CH3 domains. Thus an Fc dimer of single chain FSH–Fc contains two FSHα and two FSHβ subunits. In contrast, heterodimer FSH–Fc was made in such a way that a single FSHα subunit and a single FSHβ subunit were added to separate arms of the Fc dimer. Because of the extra FSH subunits in the single chain FSH–Fc molecule, this protein is larger than heterodimer FSH–Fc when the proteins are run under either reducing (∼75 compared to 50 kDa respectively) or non-reducing (∼150 compared to 100 kDa) conditions on SDS–polyacrylamide gel electrophoresis gels (Figure 1d). After purification, both single chain and heterodimer FSH–Fc are ∼90% pure, and do not appear to contain Fc dimer (∼26 or 52 kDa under reducing or non-reducing conditions respectively) or free FSH subunits (∼30 kDa) on SDS–PAGE gels (Figure 1d).

**In vivo bioactivity: ovarian weight gain assay**

We compared the activity of single chain FSH–Fc and heterodimer FSH–Fc fusion proteins to that of recombinant human FSH in 21 day old female rats. Rats were given a single s.c. dose of 1 nmol/kg of either rFSH, single chain FSH–Fc or heterodimer FSH–Fc. Seventy-two hours after dosing, the right ovary from each animal was weighed (Figure 2). Ovarian weight was significantly increased in female rats treated with a single dose of rFSH compared to vehicle (14.3 ± 1.7 mg compared to 12.1 ± 1.0 mg, P = 0.003). Single chain FSH–Fc and heterodimer FSH–Fc produced a greater increase in ovarian weight compared to vehicle- and FSH-treated groups (20.8 ± 3.9 mg and 26.9 ± 6.1 mg respectively; P < 0.001). Heterodimer FSH–Fc was significantly more active than single chain FSH–Fc in this experiment (P = 0.016).

**Pharmacokinetic studies after oral dosing in neonatal rats**

Neonatal rats express high levels of FcRn in the small intestine during the first 3 weeks of life and therefore can be used to study oral uptake of FcRn binding molecules such as Fc-fusion proteins. Ten day old rats were dosed orally with single chain FSH–Fc or heterodimer FSH–Fc (0.3 mg/kg) and serum levels of protein determined with an FSH/Fc sandwich ELISA (Figure 3). Concentrations of single chain FSH–Fc and heterodimer FSH–Fc in neonatal rat serum reached maximum levels of 2.4 and 3.8 μg/ml respectively. Both proteins had long terminal half-lives of 60 h for single chain FSH–Fc and 69 h for heterodimer FSH–Fc.

![Figure 2](https://academic.oup.com/humrep/article-abstract/20/7/1805/2356655)

**Figure 2.** Ovarian weight in 21 day old female rats treated with a single s.c. dose of 1 nmol/kg recombinant FSH, single chain FSH–Fc or heterodimer FSH–Fc. Ovarian weight was measured 72 h after dosing. Data are presented as average ovarian weight ± SD. n = 10/group.

![Figure 3](https://academic.oup.com/humrep/article-abstract/20/7/1805/2356655)

**Figure 3.** Oral delivery of single chain and heterodimer FSH–Fc. Neonatal rats were given a single oral dose (0.3 mg/kg) of single chain or heterodimer FSH–Fc. Blood was collected at various times after administration, and serum levels of each protein measured with an FSH/Fc sandwich ELISA. n = 4/time-point.

![Figure 4](https://academic.oup.com/humrep/article-abstract/20/7/1805/2356655)

**Figure 4.** Effect of excess IgG on oral uptake of single chain FSH–Fc and heterodimer FSH–Fc. Neonatal rats were dosed with 25 pmol [125I]single chain FSH–Fc in the presence or absence of 300-fold molar excess (7.5 nmol) of human IgG. Protein A pulldowns from serum prepared were run under reducing conditions on a 4–20% Tris–glycine gel. Lane 1: 50 000 cpm single chain FSH–Fc; lane 2: serum [125I]single chain FSH–Fc; lane 3: serum [125I]single chain FSH–Fc in the presence of excess IgG; lane 4: 50 000 cpm heterodimer FSH–Fc; lane 5: serum [125I]heterodimer FSH–Fc; lane 6: serum [125I]heterodimer FSH–Fc in the presence of excess IgG.
Role of FcRn in oral delivery of FSH–Fc single chain and FSH–Fc Heterodimer in neonatal rats

To show that oral delivery of single chain FSH–Fc and heterodimer FSH–Fc is due to FcRn binding and transcytosis, 10 day old rats were orally dosed with ¹²⁵I-labelled single chain or heterodimer FSH–Fc, or a mixture of ¹²⁵I-labelled single chain or heterodimer FSH–Fc with a 300-fold excess of unlabelled human IgG. Both single chain and heterodimer FSH–Fc were readily detected in the serum and were intact. Oral delivery of both single chain FSH–Fc and heterodimer FSH–Fc was greatly reduced in the presence of excess IgG (83% reduction and 53% reduction respectively as determined by phosphorimage analysis; Figure 4). Since IgG is a natural ligand for FcRn, this suggests that single chain and heterodimer FSH–Fc bind specifically to, and are transported by, FcRn.

Oral bioactivity: testis weight gain assay

Two day old male rats were orally dosed with either rFSH, single chain FSH–Fc or heterodimer FSH–Fc at a dose of 1 nmol/kg/day. After 14 days of treatment, the right testis of each animal was weighed. rFSH did not produce an increase in testis weight compared to the vehicle-treated group (49.1 ± 8.1 compared to 55.4 ± 8.1 mg respectively; Figure 5a). In contrast, single chain FSH–Fc (Figure 5a and b) and heterodimer FSH–Fc (Figure 5b) treatments resulted in significant increases in testis weight compared to vehicle-treated animals (113.0 ± 19.8 and 139.6 ± 11.9 mg compared to 58.6 ± 10.4 mg respectively; \( P < 0.001 \)). Heterodimer FSH–Fc was significantly more active than single chain FSH–Fc in this experiment (\( P = 0.003 \)) which is consistent with the results of the s.c. dosing experiment in female rats.

Pharmacokinetic/pharmacodynamic studies in cynomolgus monkeys

We have previously shown expression of FcRn in cynomolgus monkey and human lung, and that an erythropoietin–Fc fusion protein is absorbed and retains activity after pulmonary administration (Spiekermann et al., 2002; Bitonti et al., 2004). We therefore tested whether FSH–Fc fusion proteins could be absorbed through the lung in...
a non-human primate and retain biological activity. Two cynomolgus monkeys were treated with a deposited dose of 45 μg/kg of either single chain FSH–Fc or heterodimer FSH–Fc, resulting in maximum serum concentrations of 92 and 94 ng/ml or 69 and 131 ng/ml respectively (Figure 6a and b). The terminal half-lives of single chain FSH–Fc and heterodimer FSH–Fc were long in both cases (55 and 210 h or 182 and 219 h respectively). To demonstrate biological activity of single chain FSH–Fc and heterodimer FSH–Fc after absorption through the lungs, inhibin levels were measured in the same serum samples analysed for protein levels (Figure 7a and b). In each of the two monkeys treated with single chain FSH–Fc, inhibin levels increased to a maximum concentration of 1 and 1.6 ng/ml corresponding to a 1.3- and 1.4-fold stimulation above baseline. In each of the two monkeys treated with heterodimer FSH–Fc, inhibin reached maximal serum concentrations of 2.7 and 7.4 ng/ml corresponding to a 5.9- and 7.1-fold stimulation over baseline levels. Similar to the results of the two rat models of FSH activity shown above, heterodimer FSH–Fc was more active in monkeys after pulmonary dosing compared to single chain FSH–Fc.

Discussion

FcRn is an endogenous receptor expressed in neonatal rodent epithelial cells of the small intestine, and is responsible for the transport of antibodies from maternal milk into the neonatal bloodstream (Simister et al., 1997). We have previously used this model to demonstrate pulmonary transport of another Fc fusion protein, erythropoietin–Fc (Bitonti et al., 2004). The results of the studies reported here demonstrate that single chain and heterodimer FSH–Fc fusion proteins of large molecular weight (128 and 94.5 kDa respectively) can be delivered systemically at high concentrations after oral dosing in neonatal rats. The terminal half-life of single chain and heterodimer FSH–Fc in this model (60–69 h) is significantly longer than that of rFSH (11.4 h; de Leeuw et al., 1996). We have also shown that the transport of single chain and heterodimer FSH–Fc fusion proteins is mediated by FcRn transcytosis since an excess of the endogenous ligand for FcRn (IgG) reduced the transport of FSH–Fc fusion proteins after oral dosing in this model.

Single chain and heterodimer FSH–Fc are active in both ovarian and testis weight gain assays in rats. This is somewhat surprising since it has been shown that the carboxy terminal of the α subunit of FSH is critical for binding to the FSH receptor (Chen et al., 1992; Arnold et al., 1998). In addition, the recent publication of the crystal structure of human FSH complexed with its receptor clearly shows that the carboxy terminus of FSHα is in close contact with the FSH receptor (Fan and Hendrickson, 2005), and comparison of this crystal structure with that of FSH alone (Fox et al., 2001) indicates that the carboxy terminus of FSHα undergoes a conformational change when bound to the FSH receptor, causing the carboxy terminus of FSHα to become more rigid in structure. Since the carboxy terminus of FSHα is critical for receptor binding, it would seem unlikely that FSH would retain biological activity if the FSHα subunit is tethered at the carboxy terminus by Fc. In both single chain and heterodimer FSH–Fc fusion proteins, a linker sequence (of eight or 15 amino acids respectively) was used to connect the carboxy terminus of FSHα to the Fc moiety. It is possible that these linker sequences provide enough flexibility for FSH to bind to its receptor, thus retaining in vivo FSH activity for both fusion proteins.

Single chain and heterodimer FSH–Fc both retain bioactivity after oral dosing in male neonatal rats, leading to an increase in testis weight in treated animals compared to rFSH and vehicle controls. Since FSH binding in the testis is restricted to the Sertoli cell (Simoni et al., 1997), and since it has been shown that daily treatment of newborn male rats with FSH for 10–20 days results in an increase in Sertoli cell number resulting in an increase in testis weight (Meachem et al., 1996), it seems likely that single chain and heterodimer FSH–Fc treatment results in an increase in Sertoli cell number that is similarly reflected by an increase in testis weight. Although single chain and heterodimer FSH–Fc are active after oral dosing in neonatal rats, rFSH is not. Since we also show that transport of FSH–Fc fusion proteins is...
specifically mediated by FcRn binding and transport through the Fc moiety, it is likely that rFSH is not transported effectively after oral administration, thus explaining the lack of effect on testis weight.

Bioactivity of single chain and heterodimer FSH–Fc after s.c. dosing was also demonstrated in 21 day old female rats using the Steelman–Pohley assay (Steelman and Pohley, 1953). FSH binds to specific receptors on granulosa cells of the ovary and is responsible for the selection and growth of ovarian follicles that under specific conditions results in an increase in ovarian weight. Rats treated with rFSH or either FSH–Fc fusion protein had increased ovarian weights compared to vehicle-treated controls, likely due to an increased number and size of ovarian follicles. However, the stimulation in ovarian weight was greater for the FSH–Fc fusion proteins compared to rFSH. Since it is unlikely that the FSH–Fc fusion proteins will inherently have more activity than rFSH, the increased activity is probably due to the long terminal half-life (60–69h) of these molecules in rodent circulation.

Ovarian and testis weight gain assays in rats indicated that both single chain and heterodimer FSH–Fc are significantly more active than rFSH. However, it is apparent that heterodimer FSH–Fc is also significantly more active than single chain FSH–Fc. Pulmonary delivery of single chain and heterodimer FSH–Fc in cynomolgus monkeys also supports this observation in that inhibin levels are increased to a higher level in response to heterodimer FSH–Fc compared to single chain FSH–Fc. Since single chain and heterodimer FSH–Fc have similar terminal half-lives in both rodents and monkeys, it is likely that the structure of heterodimer FSH–Fc may present the FSHα and FSHβ subunits in a more favourable conformation for bioactivity than if the FSHα and FSHβ are fused directly together with Fc to form single chain FSH–Fc.

rFSH is a common therapy in the treatment of both male and female infertility that requires daily injections for varying lengths of time. For example, treatment of hypogonadotropic men currently requires daily injections of FSH for up to several months in order to induce spermatogenesis (Schaison et al., 1993; Schoot et al., 1994). The half-life of heterodimer FSH–Fc in cynomolgus monkeys in this study is 182–219h. This is significantly longer than the half-life of rFSH of ~24h in humans (le Cotonnec et al., 1994) and in non-human primates (Porchet et al., 1993; Weinbauer et al., 1994). Thus an obvious advantage of using heterodimer FSH–Fc in infertility treatments is the potential for a greatly reduced dosing frequency. In addition, pulmonary or oral delivery of FSH–Fc fusion proteins using endogenous FcRn expressed in epithelial cells of the lung and intestine could significantly improve tolerability of current infertility treatments.

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