Expression and regulation of fucosyltransferase 4 in human endometrium

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Abstract

It has been suggested that selectin ligands expressed by the endometrial epithelium are essential for the initial adhesion of the blastocyst to the luminal epithelium of human endometrium. One of the enzymes responsible for the production of selectin ligands is fucosyltransferase 4 (FUT4), a member of α1,3 fucosyltransferases. The aims of the present study were to characterize FUT4 mRNA and protein in human endometrium during the menstrual cycle and to investigate the hormonal regulation of FUT4 whose mRNA expression was quantified by real-time PCR in fresh endometrial tissue from cycling women and protein expression was analyzed by immunohistochemistry and Western blotting. Hormonal regulation of FUT4 transcription was investigated using an endometrial explant system. FUT4 mRNA was significantly upregulated in fresh tissues during early and mid-secretory phases when compared with other phases of the menstrual cycle. FUT4 protein was localized to glandular and luminal epithelium and the expression levels followed the same pattern as for FUT4 mRNA. Our data also show that, in proliferative explants, progesterone significantly increased FUT4 transcription and translation after 24 h in culture. The inductive effect of progesterone on FUT4 transcription was lost after 48 h of treatment. Estrogen did not have any significant effects. These data suggest that the upregulation of selectin ligands in the human endometrium at the time of implantation may be mediated, at least in part, by the regulation of FUT4 expression.


Introduction

Endometrium goes through a complex series of cyclic changes each month, under the influence of fluctuating levels of the ovarian steroids, estrogen and progesterone, in preparation for implantation. In the absence of pregnancy, the fall in serum levels of steroids results in the shedding of the functionalis layer of the endometrium. Uncovering the molecular basis of these complex changes is fundamental to understanding the mechanisms that direct the biological processes associated with the endometrium such as proliferation, embryo implantation, and menstruation. We have previously investigated the changing transcriptional profile of the human endometrium during the menstrual cycle to better understand the molecular complexities of endometrial biology (Ponnampalam et al. 2004). Data from this study showed that one of the genes that altered expression significantly during the menstrual cycle was fucosyltransferase 4 (FUT4).

FUT4 is a member of a family of glycosyltransferases called fucosyltransferases. The fucosyltransferase gene family encodes enzymes that transfer fucose in α1,2, α1,3/4, and α1,6 linkages on a large variety of glycans (Huang et al. 2000, Wagers & Kansas 2000, Withers & Hakomori 2000, de Vries et al. 2001). FUT4 belongs to a subfamily of α1→3/4 fucosyltransferases. Glycosylation is one of the main post-translational modifications that have been shown to be important for the function of numerous glycoproteins. In mammals, fucosylated glycans linked to proteins are involved in a wide range of mechanisms such as cell adhesion during development (Clarke & Watkins 1996, Wiederschain et al. 1998), inflammatory response, and leukocyte trafficking (Lowe 1997, Blander et al. 1999, Huang et al. 2000). Fucosyltransferases play a crucial role in the production of ABO blood group antigens and Lewis systems (Mollicone et al. 1995).

Our array and real-time PCR data show that FUT4 mRNA is upregulated during early and mid-secretory phases of the menstrual cycle (Ponnampalam et al. 2004). The aims of the current study were to further investigate cyclic changes of FUT4 mRNA in human endometrium during the menstrual cycle, to localize protein expression, and to examine the hormonal regulation of FUT4 mRNA and protein expression. Based on the array results, we hypothesized that FUT4 expression is regulated by progesterone in human endometrium.
Results

**FUT4 mRNA expression and protein localization during the menstrual cycle**

Temporal expression of *FUT4* mRNA during the menstrual cycle is shown in Fig. 1. *FUT4* mRNA is significantly upregulated during the early and mid-secretory stages of the menstrual cycle compared with the other menstrual phases, with maximal expression during the mid-secretory phase. These results confirm and extend previous observations (Ponnampalam et al. 2004) by significantly increasing the sample number and utilizing a histopathological rather than molecular classification of the menstrual cycle.

**FUT4 protein localization in the human endometrium**

Immunostaining for FUT4 was seen throughout the menstrual cycle in glandular and luminal epithelium. The intensity of the staining was weak in proliferative and menstrual endometrium and was strong during early and mid-secretory phases of the menstrual cycle. Replacing the FUT4 antibody with an equivalent amount of control goat IgG resulted in the complete absence of immunoreactivity (Fig. 2).

**Steroid hormone regulation of FUT4 transcription after 24 and 48 h**

There was a significant difference between 24-h treatment groups for proliferative and secretory explants (*P* = 0.0033 and *P* = 0.0062 respectively, Friedman test). Individual group comparisons showed that FUT4 transcription was significantly increased in proliferative explants treated with progesterone. Although there was a trend toward an increase in FUT4 expression in proliferative explants treated with estrogen and progesterone, as well as secretory explants treated with progesterone alone, or estrogen and progesterone, these did not reach significance (Fig 3A and B). This may be due to low sample numbers. After 48 h, the increase was lost in both proliferative and secretory endometrial explants treated with progesterone (Fig. 3C and D). *FUT4* mRNA levels did not change significantly in explants of proliferative or secretory endometria that were treated with estrogen alone for 24 or 48 h (Fig. 3).

**Steroid hormone regulation of FUT4 protein expression after 24 h**

The effects of steroid hormones on FUT4 translation were examined by Western blotting in proliferative endometria after 24 h of hormonal treatments. FUT4 protein expression was significantly stronger (*P* ≤ 0.05) in explants treated with progesterone alone, compared with the control (Fig. 4). There were no significant differences in FUT4 protein expression between other groups.

Discussion

This study demonstrates the cyclic changes and hormonal regulation of *FUT4* mRNA and the localization of FUT4 protein in human endometrium. Our data show that *FUT4* mRNA is significantly upregulated during the early and mid-secretory phases compared with other phases of the menstrual cycle, and that *FUT4* mRNA and protein expression is induced in progesterone-treated *in vitro* explants after 24 h in culture. The protein is localized to glandular and luminal epithelia.

The FUT4 gene family encodes enzymes that transfer fucose in α1,3 linkage on a variety of glycans. Fucosylated glycans linked to proteins are involved in many biological processes such as cell adhesion during development, inflammatory response, leukocyte trafficking, and fertilization (Javaud et al. 2003). It is likely that FUT4 regulates the expression of carbohydrate antigens that facilitate the initial attachment of blastocyst to endometrial epithelium.

Leukocyte rolling is a crucial step in leukocyte adhesion to the endothelium before moving through the endothelial layer into the tissue. Leukocyte rolling is mediated by a class of adhesion molecules known as selectins (Lasky 1992), which bind to sialylated and fucosylated oligosaccharide antigens such as Le x and sLe x. Regulated specific expression of the α1,3 fucosyltransferases responsible for fucosylation controls the expression of these antigens (Burne & Rabb 2002). Leukocyte homing was eliminated in mice that lack both FUT4 and FUT7 (Homeister et al. 2001). In addition, data from Weninger et al. (2000) showed that FUT4 is crucial for the selectin-mediated slow rolling of leukocytes on endothelial cells. FUT4 was the first leukocyte-associated enzyme linked to selectin ligand synthesis (Goelz et al. 1990, 1994, Burne & Rabb 2002), such as Le α, Le α and sLe α, although the activity for sLe α synthesis is very weak.
The similarities between leukocyte rolling on endothelial cells and the initial stages of blastocyst attachment to the endometrial epithelium in humans have been recognized in recent years (Genbacev et al. 2003, Dominguez et al. 2005). The L-selectin system has also been shown to be important in the initial contact of the trophoblast to the luminal epithelium of human endometrium. The human trophoblast expresses high levels of L-selectin after hatching and uses L-selectin to bind to uterine epithelial oligosaccharide ligands. When L-selectin is blocked by specific antibodies, human trophoblast adhesion to the epithelium is impaired in vitro (Genbacev et al. 2003). In the endometrial epithelium, the levels of glycosylation increase significantly during the implantation period under the influence of progesterone (Aplin et al. 1997), which gives rise to changes in the glycoprotein composition of the cell surface and secretions. Changes include the upregulation of oligosaccharide ligands that bind to L-selectin in the endometrial epithelium during the time of implantation. Studies show that sLeα is expressed in human endometrium throughout the menstrual cycle with a significant increase from early to mid-secretory phases (Ravn et al. 1992, Hey & Aplin 1996). Lai et al. (2005) showed that the expression of L-selectin ligands that bind to MECA-79 antibody (an antibody that recognizes a sulfate and carbohydrate epitope on a group of endothelial L-selectin ligands) significantly increases throughout the early and mid-secretory stages of the menstrual cycle. Therefore, it is possible that FUT4 participates in the production of these ligands. Interestingly, L-selectin-deficient mice are fertile. There are considerable differences in the processes of implantation between mice and humans, thus whether other selectins or integrin ligands compensate for this deficiency, or the role of L-selectin in implantation is restricted to humans, remain to be elucidated.

The Leα is another carbohydrate antigen that may facilitate the initial attachment of the trophoblast to the endometrium. It is present on the blastocyst surface (Fenderson et al. 1986, Kimber 1990) as well as on the luminal epithelium of the mouse endometrium (Kimber et al. 1988, Kimber & Lindenberg 1990, Zhu et al. 1995).

Figure 2 Representative micrographs of immunolocalization of FUT4 protein in human endometrium during the menstrual cycle. FUT4 protein was localized to glandular and the luminal epithelia of the endometrium. EP, early proliferative; ES, early secretory; MS, mid-secretory; LS, late secretory; M, menstrual; g, glandular epithelium; s, stroma; le, luminal epithelium; and -ve, negative control (goat IgG). Scale bar is 50 μm.

Figure 3 Effects of ovarian steroids on FUT4 mRNA in endometrial explant culture (A) after 24 h from proliferative phase samples (n=8) and (B) secretory phase samples (n=4), and (C) 48 h for proliferative phase samples (n=8) and (D) secretory phase samples (n=8). The y-axis shows the fold change of mRNA levels following different treatments compared with control, with all results corrected against expression of 18S rRNA. The x-axis shows the different treatment groups: C, control; E, 10 nM estrogen; P, 100 nM progesterone; and E+P, both 10 nM estrogen and 100 nM progesterone. Groups that do not share a letter in common are significantly different (P<0.05).
Injection of mAb to Le^y into the murine uterus on day 4 of pregnancy inhibits blastocyst implantation (Zhu et al. 1995). The expression of Le^y has been shown to be significantly upregulated during the early and mid-secretory phases of the menstrual cycle in humans (Ravn et al. 1992). Furthermore, Ghosh et al. (1998) reported that Le^y expression peaks during the mid-secretory period in rhesus monkeys. It was also shown that treatment with a low dose of the anti-progestin mifepristone significantly inhibited the expression of Le^y, suggesting that it is regulated by progesterone in rhesus monkeys. Since the final step in the production of Le^y is catalyzed by FUT4, it is likely that progesterone effects the expression of Le^y through its regulation of FUT4.

Apart from the glycans discussed above, several other hormonally modulated glycans are also upregulated during the time of implantation in endometrial epithelium. Hence, there is also a possibility that FUT4 might play a part in the regulation and maintenance of glycocalyx in the endometrium during implantation.

To our knowledge, this is the first study to report hormonal regulation of a member of the subfamily α1,3 fucosyltransferases. However, there are studies reporting the hormonal regulation of α1,2 fucosyltransferases. Data from Sidhu & Kimber (1999) showed that the expression of FUT1, a member of α1,2 fucosyltransferase family, is significantly upregulated around day 1 of pregnancy in mice and that estrogen treatment increases the expression of FUT1 in ovariecotized mice. FUT1 catalyzes the final step in the production of H-type-1 antigen. Evidence from mouse studies suggests that H-type-1 antigens may also be involved in the initial adhesion cascade of trophoblast to the luminal epithelium of the endometrium (Kimber et al. 1993). No studies have been performed to examine the cyclic changes in humans. Interestingly, H-type-1 antigen expression appears to be stimulated by estrogen in mice but progesterone in rats (Kimber et al. 1995, Sidhu & Kimber 1999), which suggests that there may be species differences in the regulation of these enzymes. DNA and protein sequence analysis of α1,2 fucosyltransferases from different species including humans showed that they contain hormone responsive elements with well-conserved nucleotide sequences within the promoter regions, giving direct hormonal responsiveness via nuclear steroid receptors (Medvedova et al. 2003). However, this study did not specify which steroid hormone-response elements were present, nor did they find any hormone-response elements for α1,3 fucosyltransferases.

In conclusion, this study has shown that the expression of FUT4, an enzyme responsible for the final catalyzing step in the production of many L-selectin ligands is upregulated in human endometrium during the period of implantation and is regulated by progesterone.

Materials and Methods

Tissue collection

Ethical approval for the study was obtained from Southern Health Human Research and Ethics Committee B. Endometrial biopsies were obtained from normal cycling women after informed consent. All endometrial samples used in the study were classified as normal by routine histopathology. Subjects ranged in age between 18 and 47 years and had not used hormonal contraception in the 3 months prior to tissue collection. A small portion of the tissue was sent to histopathology to evaluate the cycle stage. An experienced pathologist later reconfirmed the cycle stage of each tissue sample. For the evaluation of mRNA and protein profiles, the menstrual cycle stages of 64 samples were divided into seven groups by histopathology, based on well-established criteria (Noyes et al. 1950): early proliferative (EP, n=8), mid-proliferative (MP, n=11), late proliferative (LP, n=6), early secretory (ES, n=10), mid-secretory (MS, n=11), late secretory (LS, n=10), and menstrual (M, n=8). Endometrial tissues were either snap-frozen on dry ice immediately after collection and stored at −80 °C until RNA and protein extraction, or frozen in OCT for immunohistochemistry. All the immunohistochemistry work was performed on hysterectomy specimens. The RNA and protein work were performed on either curettes or hysterectomies.

The methods for the endometrial explant system were performed as described in Ponnampalam & Rogers (2006) with slight modifications. Endometrial biopsies were collected in ice-cold phenol red-free HEPES-buffered Dulbecco’s modified Eagle’s medium/Hams F-12 (DMEM/F-12; Invitrogen) with 1% antibiotic–antimycotic solution (final concentrations: 100 μg/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B; Invitrogen). A small portion of the tissue was sent to histopathology to evaluate the
cycle stage, which was recorded as either proliferative or secretory. Endometrial samples were cut into pieces of about 1 mm³ with a sterile surgical blade and placed in tissue culture inserts (6 explants/12 mm insert; Millipore, North Ryde, NSW, Australia). DMEM, free of serum and phenol red (supplemented with 1% antibiotic–antimycotic solution), was placed in the lower chamber (300 µl in 12 mm inserts). Medium was either hormone free, or supplemented with 10 nM estrogen (E), 100 nM progesterone (P), or a combination of both (E+P). At the end of the culture (after 24 or 48 h), explants were stored at −80 °C until RNA and protein extraction.

**RNA extraction and RT**

Total RNA was extracted using Trizol reagent (Invitrogen Life Technologies). Curettings were homogenized (800 µl Trizol + 200 µg glycogen) and incubated at room temperature for 5 min. After the addition of chloroform (0.2 × volume of Trizol), the samples were incubated for another 3 min at room temperature, centrifuged for 15 min at 12 000 g (4 °C), the aqueous phase RNA was separated from DNA/protein fraction, mixed with 400 ml isopropanol, and incubated at room temperature for 20 min. This was followed by centrifugation for 10 min at 12 000 g (4 °C). The RNA pellet was washed twice with 75% ethanol followed by centrifugation for 6 min at 10 000 g (4 °C). RNA was resuspended in RNase-free water. To eliminate potential genomic DNA contamination, RNA samples were treated with RNase-free DNase (Promega) according to the manufacturer’s instructions. After the DNase treatment, RNA was purified by storing overnight at −20 °C in two volumes of 100% ethanol and 0.1 volume of 3 M sodium acetate. RNA was centrifuged at 12 000 g for 40 min at 4 °C, the pellet was washed twice with 70% ethanol, and then the RNA was resuspended in RNase-free water. Quantification and estimation of purity were derived by measuring the absorbance of each RNA sample at 260 and 280 nm. The cutoff 260/280 ratio for the sample inclusion was 1.8–2.1. All RNA samples were treated with RNase-free DNase (Promega) according to the manufacturer’s instructions. RNA (0.5 µg) from each sample was used.

**Real-time quantitative RT-PCR (RT-QPCR)**

A Roche Light Cycler and an LC Fast Start DNA Master SYBR Green Kit were used to perform the real-time PCR according to the manufacturer’s instructions. Primer concentrations were 0.5 µmol/l. Each set of primers was optimized for annealing temperature and extension times. The primer sequences used and the protocols are shown in Table 1. Relative mRNA expression was determined by the measurement against a specific CDNA standard. 18S rRNA was used as a housekeeping gene to normalize all results.

**Immunohistochemistry**

Frozen full-thickness endometrial tissue samples were cut into 5 µm sections and fixed in cold acetone for 10 min. Endogenous peroxidase activity was blocked prior to immunohistochemistry by incubation in 0.3% H₂O₂ in methanol for 10 min. Tissue sections were then washed in PBS and blocked with protein blocking solution for 30 min (PBA; Immunon Shandon, Pittsburgh, PA, USA) to prevent non-specific binding, followed by overnight incubation at 4 °C with goat anti-human FUT4 antibody (1.3 µg/ml sc-20477 in 1% BSA in PBS; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Negative control was goat IgG at 1.3 µg/ml. The primary antibody step was followed by a biotinylated secondary antibody and streptavidin-peroxidase steps at room temperature for 15 min each, using reagents of the LSAB + Kit (Dako, Carpenteria, CA, USA). DAB (Sigma) was applied for 5 min at room temperature as a chromogen.

**Protein extraction**

Total protein was extracted from endometrial tissues/explants using Trizol reagent according to the manufacturer’s instructions. Following RNA/DNA removal, the protein pellet was washed and dissolved in 1% SDS. Protein quantification was performed by BCA Protein Assay Kit, following manufacturer’s instructions (Pierce Biotech, Rockford, IL, USA).

**Western blotting**

Twenty micrograms of protein from total endometrial tissues were subjected to SDS-PAGE. The protein samples were mixed with 2 × SDS loading buffer with or without 2-mercapto ethanol, and the samples were heated at 95 °C for 5 min (for reducing conditions) or warmed at 40 °C for 15 min (for non-reducing conditions). The SDS-PAGE was run on a 10% agarose gel at 100 V for 1 h. The proteins resolved in the gel were electrophoretically transferred overnight to nitrocellulose membrane (Bio-Rad). The transferred membrane was treated with blocking solution for 1 h (SuperBlock; Pierce Biotech). The membrane was then incubated with 1:1000 dilution of the goat anti-human FUT4 antibody (sc-20477; Santa Cruz Biotechnology) for 1 h. This was followed by washing and incubation with horse-radish

| Table 1 Primer sequences, Light Cycler conditions used, and amplicon sizes for fucosyltransferase 4 (FUT4) and 18S rRNA. |
|---|---|---|---|---|
| **Gene** | **Primer sequence** | **Annealing temperature (°C)** | **Extension time (s)** | **Amplicon size (bp)** | **Reference** |
| FUT4 | Sense: 5’-CAG CTG GTT CGA CCG GTC AAG CCG CGC T-3’ | 63 | 16 | 435 | Taniguchi et al. (2000) |
| | Antisense: 5’-CAG AAA AAC GTC AAT CCG GGA TGT TGT-3’ | | | | |
| 18S rRNA | Sense: 5’-CGG CTG CTA CCA CAT CCA AGG AA-3’ | 60 | 10 | 187 | Ponnampalam et al. 2004 |
| | Antisense: 5’-GCT GGA ATT ACC GCG GCT-3’ | | | | |
peroxidase (HRP)-conjugated rabbit anti-goat secondary antibody (1:50 000; Zymed Laboratories, South San Francisco, CA, USA) and finally developed using Supersignal West Dura Extended Duration Substrate (Pierce Biotech) according to the manufacturer’s instructions. The developed films were scanned and assessed by densitometry (Quantity One Software, Bio-Rad). Pre-stained SDS-PAGE standard protein markers (Bio-Rad) were used to calibrate the molecular mass. β-Actin was used as the loading control (1:4000 dilution; Sigma–Aldrich).

Statistical analysis
Statistical tests were performed using the SPSS 12 statistical analysis package (SPSS Inc., Chicago, IL, USA) and GraphPad Prism software (version 4.00; GraphPad Software, San Diego, CA, USA). For mRNA profile of FLT4 during the menstrual cycle, one-way ANOVA with Bonferroni correction was used. For hormonal regulation data, the fold change of the hormonal cycle, one-way ANOVA with Bonferroni correction was used.

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