Insulin-Like and Fibroblast Growth Factors and Their Receptors Are Differentially Expressed in the Oviducts of the Common Marmoset Monkey (Callithrix jacchus) during the Ovulatory Cycle

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ABSTRACT

It is suggested that growth factors support the process of maturation and differentiation in the mammalian oviduct. Fibroblast growth factors (FGF) and insulin-like growth factors (IGF) are possible mediators on these processes. The present study describes for the first time the expression of FGF-1 and -2 and their receptors as well as IGF-1 and -2 and the corresponding IGF receptor type 1 in the oviduct of the New World monkey Callithrix jacchus. Because of the limited RNA yields from oviducts, reverse transcription-polymerase chain reaction (RT-PCR) was performed to estimate expression levels. Expression patterns were found to be similar for all examined growth factors and receptors: the highest mRNA contents were obtained at the late proliferative and early to midsecretory phases, compared with lower levels during the early proliferative phase. Elevated amounts of these RNAs were correlated with high serum estradiol but not with progesterone concentrations. Each PCR product showed a high degree of homology (> 92%) to the known human sequences.

Immunohistochemical analysis indicated greater specific staining for FGF-1 and -2 and IGF-1 before ovulation on the luminal epithelial surface of marmoset oviducts in comparison to the other cycle phases. Differences in staining intensity were not observed between the ampulla and isthmus. In summary, the marmoset oviduct expresses all components of functional FGF and IGF systems, thus suggesting auto-/paracrine effects of these growth factors within the primate oviduct, possibly under the control of estrogentic hormones.

INTRODUCTION

Growth factors are expressed in a wide variety of tissues, including those of the female reproductive tract, and are considered to play an important role in cellular proliferation and differentiation. Fibroblast growth factor (FGF)-1 and -2 and their receptors (FGFR), as well as insulin-like growth factor (IGF)-1, IGF-2, and IGF receptor (IGFR)-1 and -2, have been detected in the ovary [1-7]; and it is suggested that they are involved in the development of the ovarian follicle and corpus luteum, where they may influence angiogenesis and granulosa cell proliferation in the developing follicle [8-10] as well as increase ovarian androgen production [6]. Additionally, these growth factors and their related receptors are reported to be expressed in uterine tissue [11-14]. FGF-1 and -2, IGF-1, and IGF-1 immunoreactivity was observed predominantly in glandular epithelial cells [11, 15], and it is proposed that they are involved in the cyclic growth of the endometrium.

The mammalian oviduct is the site of fertilization and is the site at which essential early embryonic events occur, including the first cell divisions and the onset of mRNA transcription. The oviduct has a ciliated and secretory epithelium, cells increasing their height and activity (ciliation and secretion) under the influence of estrogen [16, 17], possibly acting via local growth factors. Several reports indicate that the oviduct could be an essential source of growth factors: in human fallopian tubes, the presence of mRNA and specific immunoreactivity for IGF-1, IGF-2, and IGFR-1 [18, 19] as well as epidermal growth factor (EGF), transforming growth factor α (TGFα), and EGF receptor (EGFR) [20-23] have been reported. Recently FGF-1, FGF-2, and FGF-2 were identified in bovine oviducts [24, 25], and IGF-1 and -2 and the corresponding receptor IGFR-1 were identified in the ovine oviduct [26]. Both FGF bind to the same receptor types, though FGF-1 may be 30- to 100-fold less potent than FGF-2 [27, 28]. However, it has been shown [29] that the mitogenicity of FGF-1 is greatly enhanced by heparin; thus the two growth factors are assigned equal potency in cell activation.

To our knowledge, no such published data about the FGF system in the primate oviduct are available. IGF-1 is known to play a major role in the cross-talk between gonadotropins and sex steroids within the reproductive tract, acting mainly via IGFR-1 [30].

This study was done to analyze the expression at the mRNA and protein levels of FGF-1, FGF-2, IGF-1, and IGF-2 as well as of their corresponding receptors in marmoset oviducts during the ovulatory cycle. Results were compared with plasma estradiol and progesterone concentrations.

MATERIALS AND METHODS

Animals and Sample Collection

Oviducts from 13 normally cycling female common marmoset monkeys (Callithrix jacchus), as well as from an early-pregnant monkey and from an ovarian-dysfunctional (follicle cysts) marmoset monkey, were used for this study (15 monkeys total). The animals were housed in pairs as described previously [31]. Animals were bled twice weekly to determine the plasma progesterone concentration and to monitor ovarian cyclicity. Ovarian cycles were regulated by injection of prostaglandin (PG) F₂ 0.8 μg Estrumate; Pitman-Moore, GmbH, Burgwedel, Germany) on Day 12 of the luteal phase to induce luteolysis and the onset of preovulatory follicle development [32].

Ovulation in the marmoset monkey occurs, on average, 10.7 days after FGF₂-induced luteolysis [32]. The day of ovulation was defined as the day preceding a rise in plasma progesterone concentration above 10 ng/ml [33]. The proliferative phase has a mean length of 10 days, and the
interrupted secretory phase has a mean length of 12 days. Normal cycle length for marmoset monkeys in this colony is on average 28 days.

For mRNA analysis, oviducts were collected under halothane anesthesia: 1) prior to the LH rise on Day 7 of the proliferative phase (n = 3); 2) on Day 8 of the proliferative phase (22 h after exogenous hCG [75 IU; Vemir Veterinär Chemie GmbH, Kempen, Germany]) administration as described previously [34–35]) but prior to ovulation (n = 4); 3) during the secretory phase on Day 5 (early secretory phase) (n = 3); and 4) during the secretory phase on Day 9 (midsecretory phase) (n = 3).

Additionally, one monkey showing cystic ovaries and permanently elevated progesterone and a further animal at Day 44 of pregnancy were included in the study. The excised oviducts were immediately transferred to liquid nitrogen and subsequently stored at −80°C until examined.

Tissue samples to be used for immunohistochemistry from each cycle stage (2 animals, respectively) were mounted on Tissue Tek (Sakura Finetek Europe BN, Zoeter Woude, Netherlands) and then transferred to liquid nitrogen and stored at −80°C until analysis.

**Enzyme Immunoassay (EIA) for Progesterone and Estradiol**

For estradiol measurements, 50 μl plasma was extracted with 1 ml tert.-butylmethyl-ether (Riedel-de-Haen, Seelze, Germany), and the aqueous phase was frozen out at −60°C. The organic supernatant was recovered and evaporated, and the residue was dissolved in 150 μl assay buffer (40 mM NaH₂PO₄ [Merck, Darmstadt, Germany], 0.15 mM NaCl [Merck; pH 7.2], 0.1% BSA [A-7888; Sigma Chemical Co., St. Louis, MO]). Then 50 μl was analyzed by EIA using a 6-keto-17β-estradiol-6-carboxymethyloloxime-BSA antibody as described previously [36]. The intra- and interassay variations were 6.3% and 9.5%, respectively.

Progesterone in plasma was determined by a direct, nonextraction EIA using an antiserum raised in sheep against progesterone-11α-hemisuccinate-BSA and alkaline phosphatase linked to progesterone-11-glucuronide as enzyme conjugate. This assay has been previously described for the marmoset monkey [37].

**RNA Isolation**

Total RNA was extracted from whole monkey oviducts according to the method of Chomczynski and Sacchi [38] using Trizol reagent (Gibco BRL, Gaithersburg, MD). The yield of total RNA was spectrophotometrically determined at 260 nm. Quality and quantity of oviductal RNA was verified after denaturing electrophoresis on a 1% (w/v) formaldehyde-containing agarose gel followed by ethidium bromide staining.

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**

Four micrograms total RNA was reverse transcribed to obtain cDNA using Superscript II reverse transcriptase (Gibco BRL) at 45°C for 30 min, followed by 90°C for 2 min. Reaction mixtures contained 50 mM Tris/HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, 0.588 mM dNTPs, 2.5 mM random hexamers, and 140 U of reverse transcriptase in a final volume of 60 μl.

The following commercially synthesized primers (Pharmacia, Freiburg, Germany) were used to amplify specific monkey transcripts:

![Figure 1: Specific RT-PCR products for FGF-1 (317 bp), FGF-2 (288 bp), FGFR (471 bp), and ubiquitin (189 + 417 bp) separated by agarose gel electrophoresis.](image-url)
**GROWTH FACTORS AND THEIR RECEPTORS IN MARMOSET OVIDUCTS**

**FIG. 2.** Specific RT-PCR products for IGF-1 (210 bp), IGF-2 (215 bp), IGFIR-1 (314 bp), and ubiquitin (189 + 417 bp) separated by agarose gel electrophoresis. (1) Early and (2) late proliferative phase; (3) early and (4) mid secretory phase; (5) animal with ovarian dysfunction; (6) pregnant animal.

**FGF-1** [24]
- **Forward** 5' GCT GAA GGA GAA ACC AC 3'
- **Reverse** 5' GTT TTC CTC CAA CCT TCC CA 3'
- **Size** 318 bp

**FGF-2** [24]
- **Forward** 5' GGA CGG GGG CTT CTT CCT 3'
- **Reverse** 5' CCC AGT TCG TTT CAG TGC C 3'
- **Size** 289 bp

**FGFR** [39]
- **Forward** 5' GAR ATG GAG RTG ATG AAG MTG ATY GG 3'
- **Reverse** 5' CCC RAA RGA CCA SAC RTC ACT CTG 3'
- **Size** 471 bp

**Ubiquitin** [24]
- **Forward** 5' ATG CAG ATC TTT GTG AAG AC 3'
- **Reverse** 5' CTT CTG GAT GTT GTA TC 3'
- **Size** 189 bp

**IGF-1** [40]
- **Forward** 5' GGC CGA CTT GGC GGG CTT GA 3'
- **Reverse** 5' GGA CCC GAG ACC CTC TGC GGC 3'
- **Size** 210 bp

**IGF-2** (corresponding to bases 619–833 of the human sequence: EMBL no.: J03242)
- **Forward** 5' TAT CCT GCT GAC CCC AG 3'
- **Reverse** 5' ACA TCC TCC TCG GAC TGG GC 3'
- **Size** 215 bp

**IGFR-1** (corresponding to bases 2593–2906 of the human sequence: EMBL no.: X04434)
- **Forward** 5' TTA AAA TGC CCA GAA CCT GAG 3'
- **Reverse** 5' ATT AYA ACC AAG CCT CCC AC 3'
- **Size** 314 bp

The predicted size of each RT-PCR product is shown in parentheses. The PCR reactions were performed as described previously [24]. Individual amplification programs were applied for FGF-1 and FGF-2 (35 cycles at 94°C and 60°C, 1 min each), FGF-2 and IGF-1 (30 cycles at 94°C and 60°C, 1 min each), and ubiquitin (22 cycles at 94°C, 55°C and 72°C, 45 sec each). Five microliters of each PCR product was run on 1.5% agarose gels containing 1 xg/ml ethidium bromide. As a negative control, water instead of RNA was used for the RT-PCR, and all reactions were performed three times for each RNA preparation. Specificity of RT-PCR products was checked by subcloning into the pCR-Script SK(+) cloning vector (Stratagene, La Jolla, CA), followed by double-stranded DNA sequencing (Sequiserve, Vaterstetten, Germany).

As a control for quantification, sense cRNA transcripts were synthesized from the cloned inserts using the appropriate T3 or T7 RNA polymerase (Stratagene) according to the manufacturer's instructions. Such generated cRNAs and a mass ladder (Gibco BRL) were subjected to a 1% agarose gel electrophoresis followed by ethidium bromide staining. Resultant band intensities were scanned by a video documentation system (Pharmacia) and analyzed with the Image Master 1D program (Pharmacia). To estimate cRNA concentrations, signal intensities were compared with the known concentrations of the marker. Calibrated amounts of the specific sense cRNAs were then used in each RT-PCR performed with monkey total RNA.

**Immunohistochemistry**

Frozen cryostat sections (7 μm) from the marmoset monkey isthmus and ampulla were mounted together on gelatine-coated slides. The sections were treated as described previously [24] and then analyzed for the presence of FGF-1 and FGF-2 (both polyclonal antibodies, 1:5000), IGF-1 (polyclonal; 1:1000), and heparan sulfate (Clone 7E12, 1:500; Boehringer, Mannheim, Germany). The cross-reactivities of the antibodies to other growth factors and of FGF-1 versus FGF-2 and vice versa were below 0.1%. Immunoreactivity was visualized using the alkaline phosphatase technique (APAAP complex; Dianova, Hamburg, Germany) and the conjugated avidin-biotin method (LSAB kit; Dakopatts, Hamburg, Germany). To show specificity of the staining reactions, antibodies that had been preabsorbed with excess antigen were used. As further controls for specificity, in some sections the primary antibody was omitted or replaced by preimmune IgG. Sections were counterstained with hematoxylin, mounted in glycerin jelly, and examined using a Zeiss (Carl Zeiss, Thornwood, NY) microscope. For morphological investigations, sections were stained with hematoxylin.
TABLE 1. Homology relative to the human of the partial PCR-derived cDNA sequences for various growth factors and receptors.

<table>
<thead>
<tr>
<th>Factors</th>
<th>Homology (%) to human cDNA (EMBL No.)a</th>
<th>Homology (%) to human cDNA (EMBL No.)b</th>
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<tbody>
<tr>
<td>Ubiquitin (Z49056)</td>
<td>84.6 (M26880)</td>
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<td>FGF-1 (Z49053)</td>
<td>91.5 (X65778)</td>
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<td>FGF-2 (Z49054)</td>
<td>96.5 (M27968)</td>
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<td>IGF-2 (AJ001297)</td>
<td>94.9 (J03242)</td>
<td></td>
</tr>
<tr>
<td>IGF-1 (AJ001298)</td>
<td>94.6 (X04434)</td>
<td></td>
</tr>
</tbody>
</table>

a The EMBL access numbers of the Callithrix jacchus cDNA sequences are in parentheses.
b Percentage homology between the marmoset monkey cDNAs and the known genes (EMBL access number in parentheses).

RESULTS

 Estradiol and Progesterone Blood Concentrations and Quality of the RNA

Plasma concentrations of estradiol and progesterone were measured in six marmoset monkeys during three successive prostaglandin-primed cycles. These six animals showed the same hormone profiles through this controlled cycle: as expected, PGF₂α rapidly terminated the luteal phase as indicated by a decline of progesterone levels and ovulation within the next 11 days (data not shown). For each animal, plasma concentrations of these hormones were determined at the time of halothane anesthesia and allocated to the appropriate cycle stage. Physiological hormone levels were within the previously described ranges [33, 41]: 1) progesterone exceeded 10 ng/ml during the secretory phase (up to a maximum of 160 ng/ml) and 2) estradiol concentrations were found to be below 300 pg/ml during the secretory phase after a short increase before ovulation.

RT-PCR Data

As a control for the quantity and quality of the RNA, total RNA was first subjected to denaturing agarose gel electrophoresis. Because of the limited RNA yields (< 50 µg), an additional estimation of RNA quantity was obtained through estimation of expression of the mRNA for the housekeeping gene ubiquitin: two ubiquitin-specific RT-PCR products (189 + 417 base pairs [bp]) were found to be stably expressed during the ovulatory cycle in the Callicithrix jacchus oviduct (Figs. 1 and 2). These ubiquitin products were used to control for the efficiency of the RT-PCR between samples. In a separate analysis, ubiquitin transcript intensity was shown to be equivalent for all RNA samples by Northern blot hybridization analysis (data not shown).

RT-PCR analysis followed by sequence determination of the products verified that FGF-1, FGF-2, IGF-1, and IGF-2 and their related receptors, FGFR and IGFR-1, were specifically expressed in the oviduct of the marmoset monkey (Figs. 1 and 2). Each cloned cDNA shared a high homology with the known human gene (> 92%) (Table 2). Only the sequence homology between the marmoset ubiquitin cDNA and the human gene was lower (> 85%) owing to an unusual codon usage.

The relative signal intensities for PCR products specific for all growth factors and receptors and for all animals were assessed after correction based on the ubiquitin mRNA signals. Individual mRNAs were then additionally evaluated for their approximate content with the use of known amounts of sense cRNA.

For all examined growth factors and receptors, the expression levels during the early proliferative phase (numbered 1) appeared lower when compared with those of the late proliferative samples (2) and early secretory-stage samples (3 and 4) (Figs. 1 and 2).

On the basis of the sense cRNA standards, the following mean mRNA levels were measured after ovulation in the monkey oviduct: 50 pg ubiquitin mRNA, 30 fg FGF-1 mRNA, 300 fg FGF-2 mRNA, 25 pg FGFR mRNA, 30 pg IGF-1 mRNA, 25 pg IGF-2 mRNA, and 30 pg mRNA encoding IGFR-1 per microgram total RNA, respectively.

Additionally, the expression pattern of both oviducts from one individual was examined separately during the late proliferative and early secretory phases of the cycle (n = 2). Oviducts of the late proliferative stage with two ipsilateral follicles showed an increased mRNA expression for FGF-1, FGF-2, IGF-1, and IGFR-1 in comparison to the opposite oviduct with only one follicle (Fig. 3). In contrast, during the early secretory phase, no obvious difference in signal strength was visible, although one ovary contained three corpora lutea and the opposite ovary none. Oviducts obtained from one early-pregnant animal (sample 6), as well as one marmoset with ovarian dysfunction (sample 5), showed expression of all growth factor system components examined (Figs. 1 and 2).

Immunohistochemistry

Immunohistochemical observations indicated that marmoset oviductal cells contained immunoreactive FGF-1, FGF-2, and IGF-1. Staining for FGF-1 and -2 as well as for IGF-1 was detected specifically on the epithelial cell
FIG. 4. Immunohistochemical localization of IGF-1 (B) and (C), heparan sulfate (D), FGF-1 (E), and FGF-2 (F) in the marmoset oviduct (Day 8 of the proliferative phase, 22 h after exogenous hCG administration). Positive staining is shown by a black chromogen. Negative control (A) with rabbit immunoglobulin. The arrow shows blood capillaries. Original magnification: A, B, D-F) x375; C) x625 (reproduced at 72%).

layer (Fig. 4). Additionally, some positive immunostaining for these antigens was found in the nuclei of oviduct epithelial cells. For FGF-2 only, there was also strong immunostaining in the basal lamina and in capillary endothelial cells. The intensity of immunostaining differed between the proliferative and secretory phases, with highest signal intensities before ovulation (after hCG) in comparison with the other ovulatory cycle stages. No such differences in immunostaining intensity were found between the ampulla and isthmus, or between oviducts from the same animal.

Heparan sulfate was localized to the epithelial cell surface at the oviductal lumen, as well as in capillary endothelial cells, and showed no obvious cycle dependency (Fig. 4).

DISCUSSION

In this study the marmoset oviduct was identified as an important source of growth factors. Using RT-PCR, transcripts for FGF-1, FGF-2, IGF-1, and IGF-2 were identified, local biosynthesis of these factors being supported by specific immunostaining with an epithelial localization, suggesting a secretion of the mature proteins. These results thus are in agreement with reports describing mRNAs encoding IGF-1, IGF-2, and IGFR-1 in human fallopian tubes [19] or IGF-1 transcripts in oviducts of the rat, mouse, and cow [40, 42, 43]. In the cow there was increased expression of IGF-1 after ovulation, and in the rat oviduct, highest IGF-1 transcript levels were present during proestrus, similar to what we observe in the marmoset oviduct. Previously, we showed [24] that in bovine oviducts, only FGF-1 expression indicated cycle-dependant variation, with highest mRNA contents during the postovulatory stage; contents of mRNA for FGF-2 and FGFR remained unchanged. These results differ from those described here for the marmoset, where the highest mRNA contents of both FGFs and their receptors could be detected during the late proliferative and early secretory stages. This is similar to the expression pattern of another growth factor system, the EGF/TGFα family [20–23]. Increase in the amounts of EGF, EGF receptor, and IGF-1 occurred in the oviducts in association with a rise in estradiol, but not in progesterone levels, that has been described for humans and rats in vivo and in vitro in oviductal cells [21, 22, 43, 44]. Since serum estradiol and progesterone profiles during the human and marmoset cycles appear similar, expression of the growth factors might also be regulated in a comparable fashion in the marmoset. The observations made in the present study on the pregnant animal and on the animal with ovarian dysfunction would support this view. In the latter, expression of the growth factors appeared to be similar in
content to that at ovulation, during which the estradiol concentration in plasma is maximal—whereas in the pregnant animal, the expression data for the investigated growth factor systems were similar to those from the late secretory phase.

One would thus expect there to be a distinctly different expression pattern between the two oviducts within one animal just before ovulation, possibly corresponding with the individual ovarian follicle status.

The successful immunostaining for FGF-1 and -2 and IGF-1 in the marmoset oviducts indicates that these growth factors are located at the luminal surface of the epithelium, suggesting that the proteins may be secreted into the lumen around ovulation. This agrees with a recent report that immunoreactive FGF-1 and FGF-2 proteins were measured in bovine oviduct flushings around ovulation [24]. FGF-2 was additionally found on the basal membrane and in endothelial cells. Thus, the possibility of additional import of FGF-2 from the circulation cannot be excluded.

Within the human oviduct, staining for EGF, TGFα, and EGFPR [23], as well as for IGF-1 and IGFIR-1 [18], is cycle dependent, with higher signals before ovulation. This agrees with the results obtained in the present study showing higher immunostaining signal corresponding with higher expression levels before ovulation.

The appearance of heparan-like structures on the epithelial surface indicates that the heparin-binding FGF-1 and -2 could be easily fixed on the oviducal surface. This matrix is known to serve as a reservoir for these growth factors and thus may serve as a modulator of their action, possibly mediated by the FGFPR receptors (four human receptor types are known). The present observations suggest that functional growth factor systems for IGF and FGF could influence early embryonic development within the marmoset oviduct. Secondly, an auto-/paracrine mechanism between the oviduct cells themselves might additionally be involved in epithelial physiology linked to support of the embryo. In this regard, recent studies have demonstrated that FGF-2 can promote the in vitro formation of mesoderm embryo. In this regard, recent studies have demonstrated the influence of pituitary and gonad development and function. Am J Med 1995; 98:585-589.

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