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Original article

Differential expression of epidermal growth factor and transforming growth factor beta isoforms in dog endometrium during different periods of the estrus cycle

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Abstract

Both epidermal growth factor (EGF) and transforming growth factor (TGF) play an important physiological role in the processes of proliferation and differentiation of several different cell types. However, the expression profiles of these factors in domestic bitches endometrium are still poorly recognized. The aim of the present study was to identify and analyze the differential expression of these factors in various stages of the estrus cycle.

Endometrial tissue from proestrus (n=17), estrus (n=10), day 10 diestrus (n=15), day 35 diestrus (n=18) and anestrus (n=25) was collected soon after ovariohysterectomy. Total RNA was isolated from the endometrium by means of Chomczyński and Sacchi method, treated by DNase I, and reverse-transcribed into cDNA. Quantitative analysis of EGF, TGFβ1, TGFβ2, and TGFβ3 cDNA was performed by real-time quantitative polymerase chain reaction (RT-PCR).

EGF expression in canine endometrium was increased in the estrus stage as compared to proestrus ($P<0.05$), day 10 diestrus ($P<0.05$), day 35 diestrus ($P<0.01$) and anestrus ($P<0.001$). We also found the differences in EGF expression between day 10 and day 35 of estrus as well as between day 35 of estrus with anestrus ($P<0.05$, $P<0.01$, respectively). The TGFf1 transcript contents were also higher in estrus as compared to other stages ($P<0.01$). The TGFβ2 and TGFβ3 in the estrus stage was increased compared to proestrus, day 10 diestrus, day 35 diestrus and anestrus ($P<0.05$).

We proved that expression of EGF and TGFβ transcript isoforms is related to the phase of estrus in bitches and therefore may be regulated by specific hormone concentrations during these periods. Our results confirm the hypothesis that these growth factors play a role in the regulation of biochemical changes in the endometrial tissues during the estrus cycle.

Key words: EGF, TGFβ, endometrium, estrus cycle, dogs

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Introduction

The reproductive ability and specificity of the estrus cycle differentiates the canidae family from other species of mammals. The main difference is the length of the estrus cycle. It is also clear and simple to demonstrate the influence of several factors such as body condition, body weight (obesity), physiological or pathological features of ovary and endometrium on reproductive ability of donors, as well as developmental competence of COC's (Bukowska et al. 2008, 2010, Jaskowski et al. 2010). It is well known that the receptivity of endometrial tissues and their ability to host the embryos during implantation is dependent of several factors such as changes in physiological hormone concentrations, secretion or synthesis of selected proteins that induce the biochemical, metabolic, and chemical pathways in the epithelial cells, as well as specific expression of growth factors, which lead to proper regulation of the endometrium. The growth factors that may play an important role in these mechanisms include epidermal growth factor (EGF) and transforming growth factor beta (TGFβ) (Harada et al. 1997, Cai et al. 2003, Beceriklisoy et al. 2009, Zhang et al. 2010).

Epidermal growth factor (EGF), first described by Cohen (1962), is well known as a potent mitogenic factor for a variety of cultured cells of both ectodermal and mesodermal origin, and also plays a central function in the differentiation of specific cells *in vivo* (Carpenter and Cohen 1979). It has recently been demonstrated that EGF is expressed in several types of reproductive tissues, including the uterus during different stages of the estrus cycle in several species of mammals (Lim and Dey 2008). Furthermore, it has been hypothesized that this protein may play an important role in the regulation of physiological functions of the endometrium, including embryo attachment and apposition and preparation of the endometrial tissues for recognition of the embryo during implantation. However, there is not too much data regarding the role of EGF expression in the regulation of biochemical and morphological transformations of canine endometrium collected from donors at different stages of estrus cycle (Tamada et al. 2005, Kida et al. 2010). Kida et al (2010) investigated the expression of EGF, transforming growth factor-alpha (TGF-alpha), and epidermal growth factor receptor (EGF-R) in endometrial tissues in canine. They concluded that low expression of EGF as well as it's various localization may be involved in the aberrant growth of endometrial glands and may lead to cystic endometrial hyperplasia development.

Transforming growth factor (TGF) is a multifunctional peptide that controls proliferation, differentiation, and other important functions in many cell types, including normal and cancer cells. TGFβ acts synergistically with TGFα in inducing cell specific transformation. It also acts as a negative autocrine growth factor. Disregulation of TGFβ synthesis and activation of signaling pathways may result in cell apoptosis. Many cell types may synthesize TGFβ and almost all of them have specific receptors for this protein. TGFβ1, TGFβ2, and TGFβ3 activation pathways share the same receptor signaling systems. It has been shown that some of the TGF isoforms are expressed in endometrial tissues and may induce the specific mechanisms of endometrial cell differentiation and transformation (Doré et al. 1996, Antosik et al. 2010). Since TGF-alpha is actively synthesized by endometrial cells, it is the fact that TGF-beta may be also synthesized by embryos, where it plays, as it is suggested, the important role in causing biochemical changes in endometrium during implantation period (Cross et al. 1994). The physiological studies indicated that TGF-beta acts synergistically with TGF-alpha in inducing transformation. TGF-beta-1, TGF-beta-2, and TGF-beta-3 all function through the same receptor signaling systems. The C-terminal 112 amino acids of TGF-beta-3 share approximately 80% sequence identity with beta-1 and beta-2. Caron et al. (2009) showed that all of TGF-beta isoforms are expressed in endometrium during decidual basalis regression. The role of TGF-beta-1 in apoptosis of decidual cells was also shown. However, the function of other two TGF-beta isoforms (TGF-beta-2 and TGF-beta-3) in this process needs future investigations.

Therefore, the aim of the present study was to investigate the expression of EGF and TGFβ isoforms in bitch endometrium during different stages of the estrus cycle.

Materials and Methods

Groups of animals

In total, 85 mixed-breed bitches were used in this study. The females were monitored daily to determine the onset of proestrus/estrus and breeding. When signs of estrus were detected, the bitches were divided into five groups according to the estrus stage; (1) proestrus (n=17), estrus (n=10), day 10 diestrus (n=15) and day 35 of diestrus (n=18), as well as anestrus (n=25). The specific stage of the estrus cycle was determined from each animal's reproductive records such as vaginal cytology, and detection of ovarian status by laparotomy. The uterus and reproductive organs were removed by ovariohysterectomy under general anesthesia using ketamine hydrochloride, 3 mg/kg (Bioketan Vetoquinol Biowet) given i.v., following preanesthetic treatment with atropine sulphate 0,05 mg/kg, s.c.(Polfa S.A.) and xylazine hydrochloride 1-3 mg/kg i.m. (Xylavet 2%, Scanvet,). General anesthesia was maintained using an inhaled mixture of halothane, nitrous oxide and oxygen.

Table 1. Oligonucleotide sequences used for RT-PCR analysis.

Transcript	Sequence (5'-3' direction)	Gene accession no.	Product size (bp)
TGFβ1	GGAACTGGTGAAGCGGAAG GCGGGTGCTGTTGTAGAGG	NM001003309	142 bp
TGFβ2	TTCAGAGTCTTTCGTTTACAG CGCTGGGTTGGAGACGTTA	DQ525400	104 bp
TGFβ3	CTGTGCGTGAATGGCTCTTG ATGGATGTTTTCTAGGATGTCT	DQ525399	110 bp
EGF	CCCTTCTTAATTTTCTCCC ATACTCTCTCTTGCCTTGT	NM001003094	199 bp
GAPDH	CGCCATCAATGACCCCTTC ACTCAGCACCAGCATCACC	NM001003142	105 bp
β-actin	AGCCGTGTTCCCGTCCATC AGGATGCCCCGCTTGCTCT	NM001003349	111 bp
28S ribosomal protein S15	ATCCAATCGCGGCCCCTTC AAGTTTCCCGTTCTCAGCC	XM 532559	105 bp

Sample collection

The samples were isolated from the middle of the left horn from all investigated groups of animals, which were cut longitudinally. The endometrium was washed with PBS and separated from myometrium under the microscope. The collected endometrium samples were immediately used to isolate total RNA.

The experiments were approved by the Local Ethical Committee.

RNA extraction from tissues

Total RNA was isolated from the endometrium by means of Chomczyński and Sacchi method (Schlafke and Enders, 1967). Two (2) ml of TRIzol (Invitrogen, USA) were added to homogenized pieces of uterine tissue. The samples were homogenized using a Virtishear homogenizer (Virtis Company, Inc., Gardiner, NY, USA). They were then incubated at room temperature for 5 min, after which 1 ml chloroform was added to each sample, and incubation continued at room temperature for 3 min, which was followed by centrifugation at 4°C for 30 min at 5000 g. The aqueous phase was transferred into a fresh tube, 2.5 ml isopropyl alcohol was added, and the samples were then placed in a -80°C freezer overnight. The samples were subsequently centrifuged at 4°C for 30 min at 22 500 g. The supernatant was discarded and the pellet was washed with 3 ml of 75% ethanol and then air-dried for 5 min. Total RNA was resuspended in 500 µl diethyl pyrocarbonate (DEPC)-treated water and further purified by phenol:chloroform:isoamyl alcohol extraction, followed by ethanol precipitation. Samples were treated with DNase I (Invitrogen, USA) according to the manufacturer's protocol to eliminate possible DNA contamination. Total RNA was quantified with a spectrophotometer at an absorbance of 260 nm and purity was verified based on the ratio of 260:280 nm. For reverse transcription PCR (RT-PCR), 1.5 µg of total RNA was used.

RT-PCR analysis of cDNA expression of epidermal growth factor and transforming growth factor beta isoforms

RT-PCR was conducted in a LightCycler real-time PCR detection system Roche Diagnostics GmbH, (Mannheim, Germany) using SYBR® Green I as detection dye, and target cDNA was quantified using relative quantification method. For amplification, 2 µl of total (20 µl) cDNA solution was added to 18 µl of QuantiTect® SYBER® Green PCR Master Mix Qiagen GmbH (Hilden, Germany) and primers (Table 1). One RNA sample of each preparation was processed without RT-reaction to provide a negative control in subsequent PCR. The housekeeping genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin, were amplified as references for mRNA quantification. To quantify specific gene expression in endometrium, the levels of expression of specific endometrial mRNAs in each sample were calculated relative to GAPDH and β-actin. To ensure the integrity of these results, an additional housekeeping gene, 28S ribosomal protein S15 mitochondrial precursor (S15mt), (MRP-S15) was used as an internal standard to ensure that GAPDH and β-actin mRNA was not regulated in the groups of animals. This gene has been identified as an appropriate housekeeping gene for use in quantitative PCR studies. Expression of GAPDH, β-actin and 28S ribosomal protein S15 mRNA was measured in cDNA samples from

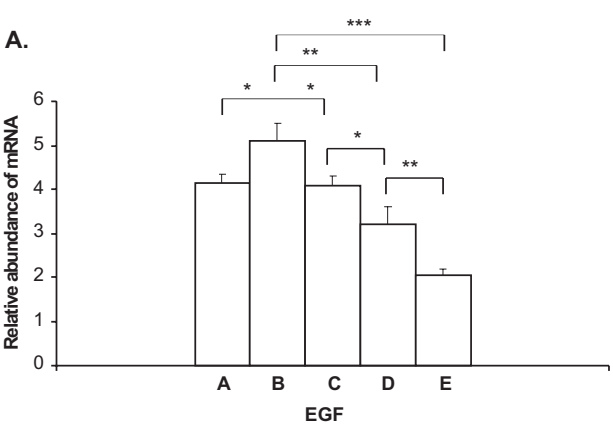
endometrium. The expression of GAPDH and β-actin did not vary when normalized against 28S ribosomal protein S15 (results not shown).

Statistical analyses

All results are given as means ± SEM. Since data were not normally distributed, the non-parametric Mann-Whitney *U*-test was chosen for comparison of the relative abundance of mRNA expression between the groups of bitches in various stages of estrus cycle. All calculations were carried out with the SPSS software (Version 14 for Windows; SPSS Inc., Chicago, IL, USA). A p-value of P<0.05 was considered statistically significant.

Results

After using RT-PCR analysis we investigated the expression pattern of EGF and TGF β transcripts isoforms in bitches endometrium at different stages of estrus. We demonstrated an increased level of EGF transcript in bitches endometrium at estrus as compared to other cycle stages. The results were statistically significant and dislpay the differences between estrus as and other stages such as; proestrus (P<0.05), day 10 diestrus (P<0.05), day 35 diestrus (P<0.01) and anestrus (P<0.001). When comparing differences in EGF expression between day 10, day 35 of estrus, the level of significance at P<0.05 was noted. We also found an increased expression of this gene between day 35 of estrus and anestrus (P<0.01). Similar results were obtained when the TGF β transcript isoform levels was compared. We found higher expression of TGF β1, β2 and β3 in bitch endometrium isolated from estrus donors as compared to proestrus (P<0.01 for TGF β1, P<0.05 for TGF β2, P<0.05 for TGF β3), day 10 of diestrus (P<0.01 for TGF β1, P<0.05 for TGF β2, P<0.05 for TGF β3), day 35 of diestrus (P<0.01 for TGF β1, P<0.05 for TGF β2, P<0.05 for TGF β3) and anestrus (P<0.01 for TGF β1, P<0.05 for TGF β2, P<0.05 for TGF β3). When day 10 and 35 of estrus and anestrus in relation to TGF β1, TGF β2 and TGF β3, was compared, no statistical differences were found.



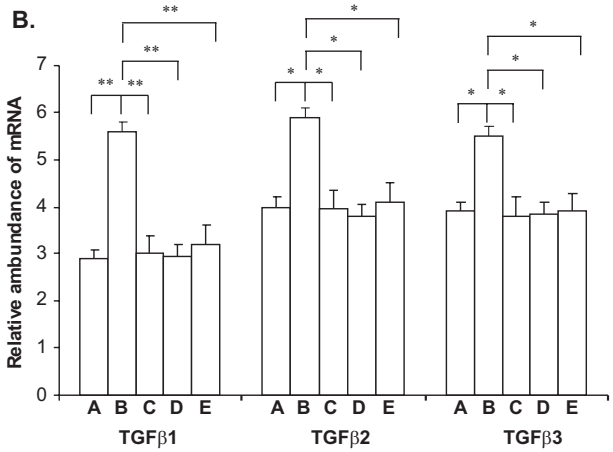


Fig. 1. Expression of EGF, TGFβ1, TGFβ2 and TGFβ3 transcripts in endometrial tissues in different estrus stages of bitches.
Proestrus (A), estrus (B), day 10 diestrus (C), day 35 diestrus (D) and anestrus (E). (Fig. 1A), TGFβ1, TGFβ2 and TGFβ3 (Fig. 1B).
Each sample was assayed in triplicate from three independent tissue collections from thirty animals. Results are presented as mean ±SEM with the level of significance, * P<0.05, ** P<0.01, *** P<0.001.

Discussion

The proliferation and differentiation of endometrial cells during estrus are under control of the steroids hormones, 17β-estradiol (E2) and progesterone (P4). This environment is necessary and crucial for early embryo development in post-implantation stages (Spencer and Bazer 2002, Spencer et al. 2007). The activation of the EGF-EGFR system is one of the important factors influencing the mitogenic effects of the endometrial cells (Wollenhaupt et al. 1999, 2002, 2004). Our results may support this hypothesis that the specific hormone secretion may influenced the regulation of EGF expression, or activation of the EGF-EGFR system in endometrial tissues during the estrus cycle.

The concentration of E2 is increased around day 20 of the estrus cycle in pigs, so it has suggested that this hormone may play an important role in the regulation of the EGF-EGFR system in endometrial tissues. Watson et al. (1996) suggested that P4 is important for increased sensitivity of the endometrial cells to estrogen, and concluded that both of these steroids may lead to a complex response by endometrial cells via activation of the EGF-EGFR system. In fact, in pigs there is a relatively lower expression of EGF mRNA at day 20 of estrus, which contrasts experiments based on human and mouse endometrium (Gonzalez et al.

1984). Our results seem to be similar to the data obtained from experiments based on human and mouse models, where EGF expression was increased during the exposition to endogenous steroid stimulation during the estrus cycle. Moreover, in similar studies, Kida et al. (2010) investigated the expression pattern and immunohistochemical localization of EGF, EGFr and TGF alpha in bitch endometrium during estrus cycle and pyometra. However, contrary to our results, they found an increased expression of EGF mRNA in bitches endometrium at day 35 of diestrus as compared to other stages. These differences may be explained by different number of investigated subjects (in our study, n=85), compared to those obtained by Kida (n=21). Furthermore, in the study of Kida et al. (2010) they used as the experimental model the endometrium isolated from bitches with pyometra. Therefore, the increased activity of immune system in bitches with pyometra may explain the influence of immune cytokine activation on the growth factors expression. Since TGF belongs to the large cytokine family this potential association could be explained by up-or down-regulation of growth factors expression in bitches' endometrium with pyometra (Cross et al. 1994, Engel et al. 2005).

There is increasing evidence that TGF/EGF-EGFR systems modulate several processes involved in the steroids-induced uterine growth, blastocyst implantation and decidualization, as well as having a stimulatory effect on embryonic growth and development (Nelson et al. 1992, Tamada et al. 1997). Research based on the porcine model has revealed that both of these growth factors may be secreted into the lumen of the reproductive tract and may supplement embryonic production of these factors (Rappolee et al. 1988, Vaughan et al. 1992). It has also been suggested that the primary role of these growth factors is related to embryonic development.

Activation of the EGF-EGFR system is regulated by a series of biochemical pathways, including specific kinase phosphorylation mechanisms. Analysis of EGF-dependent autophosphorylation in porcine endometrial cells has revealed that the activity of EGFR protein kinase is independent from plasma steroid hormone concentration. However, the activation of transcription, translation and signal transduction pathways of EGF-related kinases significantly influenced the local growth and differentiation of endometrial cells (Wollenhaupt et al. 2004).

Tamada et al. (2005) investigated the expression of TGF-alpha and EGF in bitch endometrium throughout the estrus cycle. These results partially confirmed our observations by demonstrating an increased expression of TGF-alpha and EGFR in luminal and glandular epithelia at proestrus and estrus. Decreased expression, as well as immunostaining of these proteins, was observed in diestrus and anestrus. Both of these experiments demonstrate that the TGF and EGF-EGFR systems play an important role in the process of growth regulation, as well as differentiation and regression of endometrial epithelial cells during the estrus cycle in bitches.

There is little published data regarding the role of the estrus cycle in the regulation of TGF/EGF-EGFR expression in bitch endometrial tissues. Many authors have used the porcine model for such experiments (Kennedy et al. 1994, Chabot et al. 2004). Therefore, our results supply some new information on the understanding of mechanisms of influence of TGF and EGF systems on endometrial tissue growth, as well as tissue differentiation in bitches during various estrus cycle stages.

Conclusions

In this study we clearly demonstrated the differential expression of TGF and EGF in bitch endometrial tissues during different phases of the estrus cycle. However, the molecular mechanisms that regulate expression or activation of these growth factors are still poorly understood. Therefore, the aim of future studies would include an investigation of the influence that selected factors – such as specific hormonal plasma concentrations, several abnormalities around the reproductive organs including endometrial pathology or pregnancy – may have on the regulation of expression of these growth factors in endometrial tissues.

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