Detection of transforming growth factor-α and epidermal growth factor receptor mRNA and immunohistochemical localization of the corresponding proteins in the canine uterus during the estrous cycle

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Summary. Uterine expression of the epidermal growth factor (EGF) family of growth factors has not been studied in the dog. The present study looks at the presence of mRNA transcripts and immunohistochemical localization for transforming growth factor-α (TGF-α), which is the potent EGF family member, and for EGF receptor (EGF-R) in the canine uterus during the estrous cycle. The reverse transcriptase-polymerase chain reaction together with sequencing of the products confirmed the presence of their mRNA transcripts in the endometrium throughout the estrous cycle. Immunohistochemical analysis found clear positive staining for TGF-α and EGF-R in the luminal and glandular epithelia at proestrus and estrus. Immunoreactivity decreased at the early stage of diestrus. In the mid stage of diestrus, clear staining for TGF-α was again found in the glands of the luminal region, and staining for EGF-R was observed in all glands. Very little staining was seen at anestrus for either TGF-α or EGF-R. These results suggest that TGF-α expressed in the uterus may be involved in regulating growth, differentiation and regression in the endometrial epithelial cells during the estrous cycle in the dog.

Key words: TGF-α, EGF receptor, Uterus, Estrous cycle, Dog

Introduction

Uterine expression of the epidermal growth factor (EGF) family and the receptor EGF-R has been shown in several mammals (DiAugustine et al., 1988; Gardner et al., 1989; Huet-Hudson et al., 1990; Tamada et al., 1991, 1997, 2000, 2002; Das et al., 1994a,b, 1995, 1997; Paria et al., 1994; Slowey et al., 1994; Wang et al., 1994; Boomsma et al., 1997; Wollenhaupt et al., 1997; Flores et al., 1998; Reese et al., 1998; Zhang et al., 1998; Gerstenberg et al., 1999; Song et al., 2000; Yue et al., 2000; Klonisch et al., 2001; Sivridis and Giatromanolaki, 2004). Evidence for involvement of EGF family members in the implantation process has also been reported (Paria et al., 1991; Johnson and Chatterjee, 1993; Tamada et al., 1994, 1997, 1999, 2001; Song et al., 2000; Paria et al., 2001). The EGF family includes EGF, transforming growth factor-α (TGF-α), amphiregulin, heparin-binding EGF-like growth factor (HB-EGF), betacellulin, epiregulin, heregulins/neu differentiation factors, Schwannoma-derived growth factor and vaccinia virus growth factor. These ligands bind to the receptor subtypes of the ErbB gene family, which is comprised of four receptor tyrosine kinases: ErbB1 (EGF-R), ErbB2, ErbB3, and ErbB4. EGF-family members can interact either with EGF-R or other ErbB members via homodimerization or heterodimerization (Lim et al., 1997).

Temporally and spatially regulated proliferation and differentiation of endometrial cells by progesterone and estrogen is generally believed to be essential for implantation (Weitlauf, 1994). Uterine expression of some EGF-family members and of EGF-R is regulated by progesterone and/or estrogen (DiAugustine et al., 1988; Gardner et al., 1989; Huet-Hudson et al., 1990; Nelson et al., 1992; Das et al., 1994a, 1995; Paria et al., 1994; Wang et al., 1994; Zhang et al., 1998; Gerstenberg et al., 1999), and EGF and TGF-α are involved in steroid-induced growth-promoting action in mouse endometrial cells (Nelson et al., 1991, 1992; Komatsu et al., 2003). Furthermore, EGF enhances apoptotic susceptibility in human endometrial epithelial cells in vitro (Tanaka et al., 1999; Tanaka and Umesaki, 2000).
The physiological roles of the family members in the endometrium are not fully understood, however. Of domestic animals, the dog has an unusual breeding cycle. The bitch is monoestrous, with several months of anestrus between active reproductive phases. Barrau et al. (1975) reported that the canine endometrium shows remarkable histological and functional changes during the estrous cycle. Investigation of uterine expression of the EGF-family members in the dog should therefore provide valuable information about their relations to physiological factors and function. Expression of the EGF-family members and EGF-R in the canine uterus has not been studied. The present study looks at the presence of mRNA transcripts and immunohistochemical localization of TGF-α, the potent EGF family member, and of EGF-R in the canine uterus during the estrous cycle.

Materials and methods

Animals

Purebred, 2–10-year-old beagle bitches were used, having normal estrous cycles. They were housed and used for the experiments in accordance with NIH guidelines, with the regulations of the local Institutional Animal Care and Use Committee, and accepted veterinary medical practice. All dogs were examined for vulvar swelling, vaginal smear and estrous behavior as reported previously (Tani et al., 1997; Inaba et al., 1998), and were classified into six groups (n=3-4 per group). In summary, the bitches were monitored daily for visual and cytologic signs of proestrus, which is characterized by vulvar enlargement and vaginal bleeding. At the observed onset of proestrus, the bitches were tested for sexual receptivity daily with the presence of a male dog until the end of the proestrous/estrous period. A bitch was considered to be in estrus when showing tail deflection, allowing the male to mount. The groups were: proestrus, estrus, days 3-5 of diestrus, days 10-11 of diestrus, days 33-45 of diestrus and anestrus. The bitches were euthanized using i.v. sodium pentobarbital. The uterine horn was excised, frozen in liquid nitrogen and stored at -90°C for RNA extraction.

RT-PCR

Total RNA was extracted from the endometrium using acid guanidium thiocyanate-phenol-chloroform (Chomczynski and Sacchi, 1987). RNAs were quantified by UV absorption measurements and were stored at -80°C. Isolated RNA was reverse transcribed into cDNA using the Takara RNA PCR Kit (AMV) Ver.2 (Takara, Ohtsu, Japan) according to the manufacturer’s instructions. The starting material was 1 µg of total RNA, and the reverse transcription was primed with 2.5 mM random 9-mer oligonucleotides. To ascertain that cDNA was not contaminated with genomic DNA, the reverse transcription procedure for each sample was also performed in the absence of the reverse transcriptase. The reaction mixture, including random 9-mer oligonucleotides, was preincubated for 10 min at 30°C prior to cDNA synthesis. The reverse transcription reactions were carried out at 45°C for 30 min and then heated to 99°C for 5 min to terminate the reaction. They were then cooled to 5°C for 5 min using a MiniCycler (MJ Research, Watertown, MA, USA). The reaction mixture was stored at -20°C. After the reverse transcription reaction, PCR amplification was performed with 200 nM primers. The primer pair for TGF-α was designed based on the cDNA sequence of hamster TGF-α (Ikematsu et al., 1997): sense primer 5’-GGCCAAGCCTGAATCAGCAA, antisense primer 5’-GGAGGGCGCTTGCGTCTCG with an expected size of 374 basepairs (bp). This primer pair covers the region encoding the part of mature TGF-α peptide. For canine EGF-R, the primer pair reported by Matsuyama et al. (2001) was used: sense primer 5’-TACAGCTTGGGTGCCACTG, antisense primer 5’-GGCCAGCCTGAATCCCAA with an expected size of 380 bp. The region covered by this primer pair has no homology with the sequences of ErbB2, ErbB3 and ErbB4 of the other species. This primer pair covers the region (exons 7-11) encoding the extracellular domains (subdomains II and III). The PCR conditions for TGF-α were: 3 min at 94°C for denaturation, followed by 36 cycles of 1 min at 94°C, 1 min at 66°C, and 2 min at 72°C. The PCR conditions for EGF-R were: 3 min at 94°C for denaturation, followed by 31 cycles of 1 min at 94°C, 1 min at 62 °C, and 2 min at 72°C. After the PCR reaction the products were immediately cooled down to 4°C, and 10 µl of the products were electrophoresed through a 1.5% agarose gel containing 0.5 µg/ml ethidium bromide. The resulting bands were examined by a UV transilluminator.

Sequencing of RT-PCR products

The bands of the PCR products for TGF-α and EGF-R were dissected from the gel on a UV transilluminator, and cDNAs were extracted using a QIAEX II Gel Extraction Kit (QIAGEN, Hilden, Germany). Sequences of the extracted cDNA for TGF-α were generated in both directions using an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Branchburg, NJ, USA). Sequencing reactions were analyzed on an ABI Prism 310 Genetic Analyzer (Perkin Elmer, Norwalk, CT, USA). The results were compared using the Genetyx-Mac Version 8.0 software (Software Development, Tokyo, Japan). The purified cDNA for EGF-R was subcloned into the pCR2.1 plasmid vectors using a TA Cloning Kit (Invitrogen, Carlsbad, CA, USA), and transformed into the INVαF chemically competent Escherichia coli. The cDNAs in the bacteria were isolated from the bacterial
solutions originating from 3 colonies using a QIAGEN Plasmid Mini Kit (QIAGEN). Sequences of the cDNA were determined as described above using an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit.

Immunohistochemistry

Immunohistochemical staining of TGF-α and EGF-R was performed as described previously (Tamada et al., 1990, 2002; Kapur et al., 1992). In summary, the fixed uterine pieces were paraffin embedded, and sectioned at 7 µm. Sections were deparaffinized and hydrated in PBS for 20 min. The sections were then incubated in blocking solution (10% normal goat serum) for 10 min prior to incubation with primary antibodies for 24 h at 4°C. For TGF-α and EGF-R staining, a mouse monoclonal antibody (IgG2a) raised against recombinant human TGF-α, and a rabbit affinity purified polyclonal antibody (IgG) raised against the peptide of intracellular domain (DVVDAYLIPQ; aminoacids 1005-1016) of the human EGF-R were purchased from Oncogene Science Inc (Uniondale, NY, USA) and were used at concentrations of 5 µg/ml and 20 µg/ml, respectively. Immunostaining was performed using Zymed Histostain-SP kits for mouse or rabbit primary antibody (Zymed Laboratories, San Francisco, CA, USA). The kits use the biotinylated secondary antibodies obtained by immunizing the goats with mouse or rabbit IgG, a horseradish peroxidase-streptavidin conjugate, and a substrate chromogen mixture (Hsu and Raine, 1984). Blocking of endogenous peroxidase activity was achieved by 45-sec incubation in 0.23% periodic acid in PBS following the secondary antibody incubation (Kelly et al., 1987). A red reaction product produced by 3 amino-9 ethylcarbazole (AEC) indicated the sites of immunostaining. The stainings were repeated at least 3 times for each block, and some sections were lightly stained with hematoxylin. The specificity of the anti-human TGF-α antibody and antiserum against the peptide of human EGF-R was tested by immunoblotting with recombinant human TGF-α (Oncogene) and EGF-R peptide (Oncogene) respectively. The concentrations of these antigens was 80 µg/ml for TGF-α and 180 µg/ml for EGF-R. As a control for the immunoreactive procedure, contiguous sections were stained according to the described protocol but incubation in the primary antibody was omitted. Also, non-immune rabbit serum was used as primary antiserum. No positive structures or cells were found in these sections.

Results

Detection of mRNA transcript by RT-PCR and sequence determination

With primers designed to amplify TGF-α or EGF-R, one distinct band was observed in RNA samples extracted from the uterine endometrium of the bitches at proestrus, estrus, day 3 of diestrus and anestrus (Fig. 1). PCR products had approximately the expected sizes for both genes (374 and 380 bp for TGF-α and EGF-R, respectively) in all samples studied. RNA samples obtained from the endometrium on days 10-11 and days 33-45 of diestrus also showed one distinct band by RT-PCR. The PCR products of samples in which the reverse transcription was not performed showed no bands, indicating that cDNA was not contaminated with genomic DNA. Sequence analysis of the PCR product for TGF-α revealed that the size of the product was 377 bp; the sequence had 90.2, 84.6, 87.3 and 84.4% homology with that of the human (Derynck et al., 1984), rat (Blasband et al., 1990), hamster (Chiang et al., 1991) and mouse (Berkowitz et al., 1996) respectively, as shown in Fig. 2. Analysis for EGF-R showed that the size of the product was 380 bp; the product had the same sequence as that reported by Matsuyama et al. (2001).

Immunohistochemistry of TGF-α and EGF-R in the canine uterus

Figs. 3 and 4 show representative photomicrographs of immunohistochemical staining of TGF-α and EGF-R in canine uteri during the estrous cycle. After incubation with anti-TGF-α or anti-EGF-R antibodies preincubated with excess antigenic peptides, uterine sections showed no staining. The staining pattern for TGF-α or EGF-R was basically similar in all sections examined for the same stage of the estrous cycle. Distinct staining of TGF-α and EGF-R was found in the luminal and glandular epithelial cells of the endometrium at the stages of proestrus and estrus (Figs. 3A, B, 4A, B). Slight staining was found in the myometrium (Figs. 3A, 4A). Immunoreactivity in the epithelial cells decreased in the subsequent early stages of diestrus (Figs. 3C, D, 4C, D) and increased again at the mid stage of diestrus (Figs. 3E and 4E). Clear staining for TGF-α was observed in the glands of the luminal region (Fig. 3E), and staining for EGF-R was found in all glands (Fig. 4E). At anestrus, immunoreactivity was scarcely seen for either TGF-α or EGF-R (Figs. 3F, 4F).
Discussion

No published report has yet examined the expression of TGF-α and EGF-R in the canine uterus. Here, using canine endometrium obtained at differing stages of the estrous cycle, one distinct band was found in the RT-PCR for both TGF-α and EGF-R (Fig. 1). The sequence of the products for TGF-α had more than 84% homology with the corresponding sequence from any species examined (Fig. 2), and the PCR product for EGF-R had the same sequence as that reported by Matsuyama et al. (2001). These results indicate that TGF-α and EGF-R mRNAs are expressed in the canine endometrium throughout the estrous cycle.

In previous reports (Tani et al., 1997; Inaba et al., 1998) using the same procedure for timing the estrous cycle and the same bitch colony as here, the plasma estradiol-17β level increased during proestrus and decreased during estrus, and the progesterone concentration increased during estrus, being maintained at a high level during the first 2-3 weeks after estrus. In relation to hormonal influence on the endometrium, Barrau et al. (1975) reported that glandular epithelial cells in the canine endometrium show two principal phases of growth and differentiation during the estrous cycle. Phase one (from the end of anestrus through proestrus), which correlates with the rising level of circulating estrogen, is characterized by the growth of the crypts and differentiation of the glandular epithelial cells. The second phase (from the middle of estrus through the early stage of diestrus), which correlates with a rise in the level of circulating progesterone, is characterized by hypertrophy and hyperplasia of the glandular epithelium. During the third week of diestrus, the uterus begins to involute. To clarify the relation between these histological changes and the TGF-α-EGF-R system, immunohistochemical staining was employed in the present study.

In the uteri of mice (Tamada et al., 1991; Paria et al., 1994), rats (Johnson and Chatterjee, 1993; Tamada et al., 1997), rabbits (Klonisch et al., 2001), goats (Flores et al., 1998) and sheep (Tamada et al., 2002), distinct immunostaining of TGF-α was observed in the luminal and glandular epithelia. Correspondingly, it was found in the endometrial epithelial cells in the dog. Localization of EGF-R was seen in the stroma, deciduum and myometrium in the mouse (Das et al., 1994a) and in the epithelial and stromal cells in the rat (Boomsma et al., 1996a).
1997) and sheep (Tamada et al., 2002); it was mainly found in the epithelial cells in the rat (Johnson and Chatterjee, 1993, Tamada et al., 1997), rabbit (Klonisch et al., 2001), rhesus monkey (Yue et al., 2000), baboon (Slowey et al., 1994) and goat (Tamada et al., 2000). EGF-R localization in the uterus is likely to vary with species. Here, since EGF-R was observed in the epithelial cells, TGF-α produced in the epithelia may function in autocrine fashion in the dog, as in the rat, rabbit, rhesus monkey, baboon and goat.

At proestrus, when the blood estrogen concentration increases and endometrial epithelial cells grow and differentiate, TGF-α and EGF-R were localized in the endometrial luminal and glandular epithelia in the dog. Correspondingly, estrogen induces expression of TGF-α mRNA in mouse uterine epithelial cells (Nelson et al., 1992). Furthermore, uterine expression of EGF-R is stimulated by estrogen in the mouse (Das et al., 1994a) and rat (Gardner et al., 1989), and in the pig the concentration of EGF-R in the endometrium is higher at estrus than at other stages (Wollenhaupt et al., 1997). These findings support the hypothesis that TGF-α acts as a mediator of estrogen action in stimulating epithelial growth and/or differentiation, as suggested by Nelson et

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**Fig. 3.** Immunohistochemical localization of TGF-α in the canine uterus during the estrous cycle. Clear positive staining was observed in the glandular (ge) and luminal (le) epithelium at estrus (A and B). Slight staining was seen in the myometrium (m) and stroma (s). Similar staining was seen at proestrus. The immunoreactivity decreased at days 3 (C) and 10 (D) of diestrus. At day 45 of diestrus (E), clear positive staining was found in the glandular epithelium of the luminal region (gelr), but not in the glandular epithelium of the deep region (gedr). Slight staining was observed at anestrus (F). Bars : 100 µm.
al. (1992). In the present study, localization of TGF-α and EGF-R in the epithelial cells was also found at estrus when the blood progesterone concentration begins to increase. In the mouse, progesterone treatments induce accumulation of proTGF-α in uterine epithelial cells (Paria et al., 1994), and progesterone causes a rapid transient upregulation of uterine EGF-R expression (Das et al., 1994a). It is possible that in the dog progesterone also enhances endometrial epithelial expression of TGF-α and EGF-R. However, since the blood estrogen concentration, which increases during proestrus, is relatively high at estrus (Tani et al., 1997), this estrogen level might maintain the increased expression of TGF-α and EGF-R in the epithelial cells at estrus. In any case, the fact that TGF-α and EGF-R are localized in the endometrial epithelial cells at proestrus and estrus suggest that these factors are involved in the growth and differentiation of epithelial cells.

At the early stage of diestrus the intensity of immunohistochemical staining for TGF-α and EGF-R in the epithelial cells decreased, but it then increased at the mid stage of diestrus. Specifically, at day 33-45 of diestrus when the uterus involutes, clear staining was observed in the glands of the luminal region for TGF-α and in all glands for EGF-R. EGF enhances apoptotic susceptibility of the endometrial epithelium, especially

Fig. 4. Immunohistochemical localization of EGF-R in the canine uterus during the estrous cycle. Clear positive staining was observed in the glandular (ge) and luminal (le) epithelium at proestrus (A and B). Slight staining was seen in the stroma (s) and myometrium (m). Similar staining was seen at estrus. Immunoreactivity was very weak at days 5 (C) and 10 (D) of diestrus. At day 33 of diestrus (E), clear positive staining was found in the glandular epithelium. At anestrus (F), little staining was observed. Bars: 100 µm.
in the secretory epithelium that is under the influence of progesterone (Tanaka et al., 1999; Tanaka and Umesaki, 2000). Together, these observations suggest that TGF-α may have an important role in atrophic changes of the glands in the mid stage of diestrus. Localization of TGF-α in the glands of the luminal region may be related to its local action. Barrau et al. (1975) show that the epithelia lining the lumina and crypts display marked atrophic changes at this stage.

The other EGF-family members, i.e., EGF itself, HB-EGF, amphiregulin, betacellulin, epiregulin and neu differentiation factor are all expressed in the uterus (DiAugustine et al., 1988; Huet-Hudson et al., 1990; Das et al., 1994b, 1995, 1997; Slowey et al., 1994; Wang et al., 1994; Boomsma et al., 1997; Flores et al., 1998; Reese et al., 1998; Zhang et al., 1998; Gerstenberg et al., 1999; Song et al., 2000; Tamada et al., 2000; Yue et al., 2000); ovarian steroids regulate the uterine expression of EGF (DiAugustine et al., 1988; Huet-Hudson et al., 1990; Gerstenberg et al., 1999), HB-EGF (Wang et al., 1994; Zhang et al., 1998) and amphiregulin (Das et al., 1995). The role of these EGF-family members in regulating canine endometrial changes is yet to be resolved.

In conclusion, the present results show the endometrial expression of TGF-α and EGF-R during the estrous cycle in the dog, and suggest that the ligand-receptor system of TGF-α is involved in the growth, differentiation and regression of the endometrial epithelial cells.

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