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The expression of epidermal growth factor receptors and their ligands (epidermal growth factor, neuregulin, amphiregulin) in the bitch uterus during the estrus cycle

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ABSTRACT

In order to study the possible role of EGFR receptors in the bitch reproductive process, we have analyzed the expression pattern and localization of EGFR receptors and some of their ligands epidermal growth factor (EGF), neuregulin (NRG), amphiregulin (AREG), in the uterus during the estrus cycle using immunohistochemistry. The immunostaining for receptors and ligands of EGFR/ligand system was confined to membrane and cytoplasm of the target cells. Variations were observed, not only at the different stages of the estrous cycle, but also in the different tissue compartments of the uterus. However, it was detected that the immunostainings for NRG and AREG in the different cells do not show important differences at stages of the estrus cycle. In the luminal epithelium, strong immunostaining for ErbB1/HER1, ErbB2/HER2, ErbB4/HER4 and EGF was found at estrus. In the glandular epithelium, strong immunostaining for ErbB4/HER4 was observed at diestrus, while strong immunostaining for EGF was detected in both of estrus and diestrus. ErbB3/HER3 immunoreactivity in the stromal cells was higher at diestrus and anestrus, while ErbB4/HER4 immunoreactivity was lower at anestrus. In the myometrium, the highest levels of immunoreactivity of ErbB2/HER2 were found at estrus, while ErbB3/HER3 immunoreactivity was higher at anestrus. EGF immunoreactivity was lower at anestrus compared to other stage of cycle. Altered EGFR/ligand system expression during the estrus cycle suggests this growth factor system is a potent regulator of proliferation and differentiation events during preparation for implantation of bitch uterus.

1. Introduction

In all mammalian species, several changes in hormone concentrations occur, and the synthesis of specialized proteins in epithelial cells, including cytokines, chemokines, adhesion molecules, growth factors and their receptors, are effective for creating a favorable uterine environment for the implantation of an embryo after fertilization. Growth factors and their specific cell surface receptors may be inducible by estrogen and progesterone and may serve as para-, auto- or intracrine mediators for estrogen- and/or progesterone-stimulated proliferation, growth and differentiation in the uterus. Many growth factors and growth factor receptor systems, including epidermal growth factor (EGF), insulin-like growth factor-I (IGF-I), hepatocyte

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growth factor (HGF), and keratinocyte growth factor (KGF), have been detected within the uterus and are involved in normal cyclical changes (Gardner et al., 1989; Das et al., 1994).

EGFR is a member of a family of closely related growth factor receptor tyrosine kinases, including EGFR (ErbB-1; HER1 in humans), ErbB-2 (c-neu; HER2 in humans), ErbB-3 (HER3 in humans) and ErbB-4 (HER4 in humans) (Ejskjær et al., 2005; Sağsöz and Ketani, 2010). Similar to other growth factor receptors, molecules of the ErbB/HER family phosphorylate other molecules by forming dimers when induced by ligands and thereby activate certain signal pathways. These signal pathways depend not only on the type and number of receptors expressed by the cell but also on the type and number of ligands (Niikura et al., 1996; Ejskjær et al., 2005). Interactions between ligands and receptors occur via juxta-, para- and autocrine mechanisms (Singh and Harris, 2005; Sağsöz and Ketani, 2010). Currently, seven ligands for EGFR have been identified: EGF; transforming growth factor α (TGFα); heparin-binding EGF-like growth factor (HB-EGF); amphiregulin (AREG); betacellulin (BTC); epiregulin (EREG) and epigen (Schneider and Wolf, 2008). In mammals, these ligands and four receptors comprise the EGFR/ligand system. EGF, TGFα, and AREG bind to EGFR. HB-EGF, EREG and BTC bind equally to EGFR and ErbB4. ErbB2 does not have a known ligand, but recent structural studies suggest that ErbB2 is most likely regulated by ligand (Schneider and Wolf, 2008). ErbB3 and ErbB4 are also the targets of a group of structurally related cell–cell signaling proteins known as neuregulins (NRG) or heregulins. NRG-1 (neuregulin/heregulin/NDF) and NRG-2 bind to ErbB3 and ErbB4, and NRG-3 and NRG-4 bind to ErbB4 but not ErbB3 (Falls, 2003).

Previous research performed in humans and mammals has mainly focused on the expression of the EGFR/ligand system in the implantation of the embryo following fertilization (Das et al., 1994, 1997; Tamada et al., 2000; Klonisch et al., 2001). These studies have shown that all of the EGFR are expressed at specific sites in the uterus during the peri-implantation period (Das et al., 1994), and ErbB1 and ErbB4 are also expressed in blastocysts (Paria et al., 1999). Furthermore, at least five EGFR ligands (AREG, BTC, EREG, HBEGF and TGFA) are expressed in the uterus during implantation (Das et al., 1994). Das et al. (1995) have shown that AREG is expressed throughout the uterine epithelium beginning on day 4 of pregnancy. Furthermore, Brown et al. (2004) reported that NRG-1 appears in the mouse uterus during embryo-uterine attachment and requires the presence of an implantation-competent blastocyst. Several members of the EGFR/ligand system are expressed by the luminal, glandular epithelial, stromal and myometrial smooth muscle cells of the uterus during the menstrual cycle of humans (Imai et al., 1995; Niikura et al., 1996; Srinivasan et al., 1999; Chobotova et al., 2002; Ejskjær et al., 2005; Gui et al., 2008), rhesus monkeys (Yue et al., 2000) and baboons (Slowey et al., 1994), and the estrus cycle of rats (Gardner et al., 1989), goats (Tamada et al., 2000), bitches (Tamada et al., 2005; Kida et al., 2010; Ozyurtlu et al., 2010), cats (Boomsma et al., 1997) and cows (Sağsöz et al., 2012). Although EGFRs and their ligands form a complex signaling pathway that participates in a variety of malignancies and endometrial cell differentiations and embryo implantation, the physiological role of the EGFR/ligand system in the uterus is not yet fully understood in some mammalian species, including female domestic dogs (bitches).

The well-established role of the EGFR/ligand system as a regulator of cell proliferation, differentiation and migration in various cell types led us to hypothesize that this growth factor receptor/ligand system might also play a critical role in the survival, proliferation, differentiation and migration of uterine cells, preceding successful implantation, during the estrus cycle in the bitch. Data available for EGFRs and their ligands (EGF, NRG, AREG) in the bitch uterus during the estrus cycle are limited, which makes it difficult to demonstrate the functions of the EGFR/ligand system in the canine uterus. Therefore, the aim of the present study was to identify the localization of EGFRs, including ErbB-1/HER1, ErbB-2/c-neu/HER2, ErbB-3/HER3 and ErbB-4/HER4, and their ligands (EGF, NRG and AREG) in the bitch uterus during the estrus cycle.

2. Materials and methods

2.1. Animals

Twenty-three healthy 1–8-year-old adult dogs, which were referred to the Dicle University Faculty of Veterinary Medicine clinic for ovariohysterectomy, were used in the present study. An anamnesis was obtained for each dog with regard to age, breed, body weight, litter number and most recent proestrus bleeding. The dogs included in the study were 4 West Highland white terriers, 3 Cairn terriers, 3 German shorthaired pointers, 2 German shepherd dogs, 2 Anatolian shepherd dogs and 9 crossbreeds. Prior to the operation, all dogs were examined for vulvar swelling and estrus behavior and vaginal smears were obtained as previously reported (Tamada et al., 2005; Sağsöz et al., 2013). The dogs were then divided into four groups ($n = 4$–9 per group). An ovariohysterectomy was performed following the administration of a subcutaneous dose of 0.05 mg/kg of atropine sulfate (Vetaş Atropin 0.2%, Vetaş, Turkey) and an intramuscular dose of 2–4 mg/kg of xylazine hydrochloride (Alfazyne 2%, Egevet, Turkey) as pre-anesthetics. Under general anesthesia, the dogs were induced with an intramuscular dose of 5–10 mg/kg of ketamine hydrochloride (Ketasol %10, İnterhas, Turkey). General anesthesia was maintained with ketamine hydrochloride. The ovariohysterectomy operations were performed by I. Küçükaslan using standard techniques, and the postoperative health of the animals was monitored daily.

2.2. Sample collection

After the operations, both ovaries (right and left) and the uterus (right and left cornu) were dissected into small pieces (average 3 cm^3) for histological and immunohistochemical examinations. The tissues were fixed in a 10% neutral formalin solution for 24 h, transferred to 70% ethanol, dehydrated with a gradient series of ethanol (80, 96 and 100%), cleared in methyl benzoate and finally embedded in paraplast. Five