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The role of toll-like receptors 2 and 4 in the pathogenesis of feline pyometra

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ABSTRACT

Pyometra is the most common uterine disease in queens. To protect itself from infection, the female reproductive tract possesses several immune mechanisms that are based on germline-encoded pattern recognition receptors (toll-like receptors [TLRs]). The aim of our study was to examine endometrial immunolocalization of *TLR2/4*, study the influence of lipopolysaccharide (LPS) and tumor necrosis factor (TNF) α on messenger RNA expression of both receptors in pyometric queens, and compare these patterns between estrous cycling queens and those hormonally treated with medroxyprogesterone acetate (MPA). Thirty-six queens, ranging in age from 7 months to 11 years, were allocated into seven groups (anestrus, estrus, mid-diestrus and late diestrus, short-term and long-term hormonally treated queens, and pyometric queens). At the messenger RNA level, the real-time polymerase chain reaction was applied, whereas at the TLR2/4 protein level, the expression was tested by immunohistochemistry. In queens at estrus, gene expression of *TLR2* was upregulated after stimulation of endometrial explants by TNF ($P < 0.001$) and by TNF together with the LPS ($P < 0.01$). Moreover, gene expression of *TLR2* was significantly upregulated after stimulation by TNF ($P < 0.001$) and LPS ($P < 0.01$) explants derived from queens that had been long-term hormonally treated with MPA. Endometrial gene expression of *TLR4* was significantly upregulated after incubation of explants with TNF ($P < 0.001$) in queens at estrus and with LPS ($P < 0.05$) in queens short-term hormonally treated with MPA. Immunolocalization reported that TLR2/4 receptors are mainly localized in the surface and glandular epithelia. These data show that short-term and especially long-term administration of progesterone derivatives impairs TLRs in the endometrial epithelium, presumably enabling pathogens to break through this first natural barrier and thereby increase the risk of pyometra development.

1. Introduction

Hormonal contraception designed to control reproductive activity by preventing heat may frequently cause pyometra. Changes in the uterine microenvironment and decreased contractility assist bacterial invasion [1]. The uterine lumen is usually free from pathogenic bacteria; however, the lower parts of the genitourinary tract are readily colonized by opportunistic bacteria. Closure of the uterine cervix normally protects the uterine lumen against bacteria invading from the vagina. However, in females in heat and in periparturient animals, the uterine cervix is open, facilitating bacterial invasion into the endometrium. During pyometra, the most common bacterium isolated from the feline uterus is *Escherichia coli*, followed by

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Streptococcus spp., *Klebsiella* spp., *Staphylococcus aureus*, *Pasteurella* spp., *Proteus* spp., and *Pseudomonas* spp. [2]. To protect the female reproductive tract against infection, several innate immune mechanisms have developed. The first line of protection against pathogens is a mucous membrane [3]. The mucosal epithelial cells are in constant contact with microbial flora within the female reproductive tract and recognize various components of bacterial, fungal, and viral pathogens commonly known as pathogen-associated molecular patterns through pattern recognition receptors, which include the toll-like receptors (TLRs) family [4]. Nine types of TLRs (TLR 1–9) have been described in feline tissues [5,6]. Bacterial cell wall components binding to the respective TLRs initiate an early nonspecific immune response. Toll-like receptor 2 recognizes lipoteichoic acid from gram-positive bacteria and lipoproteins/lipopeptides from gram-negative and gram-positive bacteria [7], whereas TLR4 connects with lipopolysaccharide (LPS) from gram-negative bacteria and heat shock proteins [8]. Activation of TLR4 by LPS causes increased secretion of cytokines such as tumor necrosis factor (TNF) α and chemokines [9]. It has been proved that TNF can be synthesized not only by immune-competent cells such as macrophages and monocytes but also by epithelial cells in the feline endometrium under the influence of LPS [10].

We hypothesized that in cats receiving progestagens as a hormonal contraceptive, the resulting alterations in TLR2 and TLR4 expression are involved in the pathogenesis of endometritis and accompany the pyometra. Therefore, the aim of our study was to examine endometrial immunolocalization of TLR2/4 and study gene expression of both receptors after stimulation with TNF and LPS in pyometric queens or queens hormonally treated with medroxyprogesterone acetate (MPA) and in reproductively healthy queens throughout the estrous cycle.

2. Materials and methods

2.1. Animals

All procedures were approved by the Local Animal Care and Use Committee in Olsztyn, Poland (No. 60/2010/DTN).

Thirty-six queens aged between 7 months and 11 years (average, 2.67 years) were ovariohysterectomized. To assign each animal to the appropriate group (anestrus, estrus, mid-diestrus and late diestrus, short-term and long-term hormonally treated queens, and pyometric queens), information was obtained from macroscopic observations of the ovaries as previously described [11], from circulating levels of progesterone and interviews with owners (if possible). A short description of the animals is included in Table 1.

2.2. Tissue collection

After ovariohysterectomy, the reproductive organs were washed with sterile saline to remove blood, placed into fresh saline at 4 °C, and transported to the laboratory. Both uterine horns of each uterus (n = 26) were slit longitudinally, and the endometrium was separated from the myometrium under a binocular microscope (Olympus SZX7, Tokyo, Japan).

2.3. Stimulation

The endometrium collected from each uterus (n = 26) was divided into 16 explants (four explants of the 16 served as controls, another four explants served for treatment with TNF, four more explants served for LPS treatment, and the last four explants served for treatment with TNF together with LPS). The explants, each weighing 30–50 mg, were washed in fresh saline and placed into a 24-well plate (one fragment per well), containing 1-mL DMEM medium supplemented with 0.5% BSA (Sigma–Aldrich) and 20 μg/mL of gentamicin (Invitrogen, San Diego, CA, USA). After 1 hour of preincubation in an incubator with a shaker at 37.5 °C, the medium was replaced by a fresh medium. Explants were stimulated for 4 hours at 37.5 °C at doses selected in a previous experiment [10] as follows: TNF (Sigma–Aldrich) 1 ng/mL, LPS purified from *E coli* O55:B5 (Sigma–Aldrich) 50 ng/mL, and TNF together with LPS (same concentrations). As a control, the nonstimulated explants were incubated under the same conditions as the treated ones. After 4 hours of incubation, all the explants

Table 1
Descriptions of test animals used in the experiments.

Group	Day	Description	Concentration of P4	n		
				Tissue culture	Cell culture	Immunohistochemistry
Estrus	1–7	Follicles were ≥2 mm in diameter. Queens were housed individually or in pairs, with or without contact with an intact male. No pharmacologic treatment was performed to provoke ovulation in the animals.	<1.5 ng/mL	5	5	3
Mid-diestrus	15–20	Reddish CL 3–4 mm in diameter	>20 ng/mL	3	5	3
Late diestrus	30–35	Pale CL	<5 ng/mL	3		3
Anestrus		Smooth surface of the ovaries	<1 ng/mL	5		3
Short-term hormonally treated group		Animals had been orally treated with MPA (5 mg/animal/wk) for 1 mo		3		3
Long-term hormonally treated group		Animals had been orally treated with MPA (5 mg/animal/wk) for 4 to 12 mo		3		3
Pyometra		Inflamed uteri with purulent fluid		4		3

The beginning of estrus was considered as Day 1.
Abbreviations: MPA, medroxyprogesterone acetate; P4, progesterone.