Cytokine 67 (2014) 85–91

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.journals.elsevier.com/cytokine

Ovarian steroid-dependent tumor necrosis factor-α production and its action on the equine endometrium *in vitro*

Anna Z. Szóstek [a], Marek Adamowski [a], António M. Galvão [a,b], Graça M. Ferreira-Dias [b], Dariusz J. Skarzynski [a,*]

[a] Department of Reproductive Immunology and Pathology, Institute of Animal Reproduction and Food Research, Olsztyn, Poland
[b] C.I.I.S.A., Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal

ARTICLE INFO

Article history:
Received 18 July 2013
Received in revised form 6 February 2014
Accepted 12 February 2014
Available online 15 March 2014

Keywords:
Tumor necrosis factor-α
Prostaglandin
Ovarian steroids
Endometrium
Mare

ABSTRACT

Tumor necrosis factor-α (TNF) is a cytokine that plays important roles in functions of the endometrium. The aims of this study were to determine whether (i) ovarian steroids modulate TNF production by endometrial cells (Experiment 1); (ii) TNF effects on prostaglandin (PG) production in cultured equine endometrial cells and tissue (Experiment 2). Epithelial and stromal cells were isolated from equine endometrium (Days 2–5 of the estrous cycle; $n = 20$) and treated after passage 1. In Experiment 1, epithelial and stromal cells were exposed to progesterone (P_4; 10^{-7} M), 17-β estradiol (E_2; 10^{-9} M) or P_4+E_2 (10^{-7}/10^{-9} M) for 24 h. Then, TNF mRNA transcription was determined using Real-time PCR. Additionally, TNF protein production was investigated in response to ovarian steroids for 24 h using Enzyme-Linked Immunosorbent Spot (EliSpot). In Experiment 2, epithelial and stromal cells and endometrial explants (mid-luteal phase of the estrous cycle; $n = 5$) were exposed in vitro to TNF (10 ng/ml) and to oxytocin (OT; positive control; 10^{-7} M) for 24 h. The concentrations of PGE_2 and $PGF_{2\alpha}$ were determined using a direct enzyme immunoassay (EIA) method. The transcription of prostaglandin-endoperoxide synthase-2 (*PTGS-2*), prostaglandin E_2 synthase (*PGES*) and $PGF_{2\alpha}$ synthase (*PGFS*) mRNAs in the endometrial explants was determined using Real-time PCR. Results showed that TNF is produced by two types of equine endometrial cells and its production is up-regulated by ovarian steroids ($P < 0.05$) in stromal cells and by P_4 ($P < 0.05$) and E_2 ($P < 0.01$) in epithelial cells. Epithelial and stromal cells can also produce PG in response to TNF. In endometrial explants, TNF stimulated PGE_2 production to a large extent and $PGF_{2\alpha}$ secretion to a lesser extent. These actions are mediated by up-regulation of *PG synthases* mRNA transcription. The study indicates that TNF production is closely related to ovarian steroid actions and that the interaction between TNF and PG regulates physiologic processes in the equine endometrium.

1. Introduction

Tumor necrosis factor-α (TNF) is a non-glycosylated protein with a wide spectrum of bioactivities; most cells show at least some responsiveness to TNF. In general, this cytokine displays a functional duality, being involved in regeneration as well as destruction of tissues [1]. The distinctive effects of TNF are due to two types of TNF receptor which have different intracellular signaling pathways [2]. Tumor necrosis factor-α receptor type I (TNFRI) contains an intracellular death domain which is necessary for signaling pathways associated with apoptosis. This type of receptor is constitutively expressed in most tissues and seems to be a key mediator of TNF signaling [2]. In turn, TNFRII is strongly regulated and predominantly expressed in immune cells and its plays a major role in the lymphoid system. Signaling via TNFRII induce apoptosis but also support survival promoting tissue repair and angiogenesis [2].

Tumor necrosis factor-α is a cytokine that plays important autoparacrine roles in female reproduction [3–6]. It is well known that TNF is produced mainly by macrophages [7] and endothelial cells [8]. However, the ability of endometrial cells to produce TNF has been shown in several species [9–11]. Okuda et al. [9] revealed that TNF is co-located mainly in bovine luminal and glandular epithelial and endothelial cells in the estrous cycle. Furthermore, TNF was detected in stromal cell lysates and in conditioned cultured media [9]. In addition, it was confirmed that TNF is expressed to a larger extent in epithelial cells and, to a lesser extent, in stromal and lymphoid cells in human endometrium [10,11].

* Corresponding author. Tel./fax: +48 89 5393130.
E-mail address: d.skarzynski@pan.olsztyn.pl (D.J. Skarzynski).

http://dx.doi.org/10.1016/j.cyto.2014.02.005
1043-4666/

The action of TNF-α induced via prostaglandin (PG) stimulation in the corpus luteum (CL), oviduct and endometrium in many domestic species [3–6]. Prostaglandins play crucial roles in the regulation of several reproductive processes such as ovulation, luteolysis, uterine vascularization and maintenance of pregnancy [12]. These molecules are capable of a very broad spectrum of effects modulating a multitude of biological processes. In the PG production cascade, the prostaglandin-endoperoxide synthase (PTGS) enzyme converts arachidonic acid (AA) into prostaglandin (PG)H_2 [13]. There are two isoforms of PTGS, designated PTGS-1, which is constitutively expressed, and PTGS-2 which is the inducible isoform. Prostaglandin $F_{2\alpha}$ synthase (PGFS) and PGE_2 synthases (PGES) are downstream enzymes which catalyze the conversion of PGH_2 into $PGF_{2\alpha}$ or PGE_2, respectively.

Although Grünig and Antczak [14] have previously demonstrated that TNF is expressed in gravid endometrium and trophoblast in the mare, the knowledge about the role of TNF in the equine endometrium is still insufficient. We have recently shown the immunolocalization of TNF in equine endometrial epithelial luminal and glandular cells as well as in stromal cells [15]. In above-mentioned study TNF protein expression was up-regulated in the mid-luteal and follicular phases of the estrous cycle suggesting sex steroids-dependent mechanisms regulating TNF expression in the equine endometrium [15].

Therefore, the present work attempts to explain whether: (i) ovarian steroids modulate TNF production, and (ii) TNF influences PG secretion and *PG synthases* mRNA transcription.

2. Materials and methods

2.1. Animals and endometrial tissue collection

Uteri ($n = 20$) were collected *post-mortem* from cyclic mares at a local abattoir. All procedures for animal handling and tissue collection were approved by the Local Animal Care and Use Committee in Olsztyn, Poland (Agreement No. 51/2011). The mares were healthy as declared by official governmental veterinary inspection. The material was collected within 5 min of an animal's death. Immediately before death, peripheral blood samples were collected into heparinized tubes (Monovettes®-Sarstedt, Numbrecht, Germany) for P_4 and 17-β E_2 analysis. The phases of the estrous cycle were identified based on P_4 and E_2 analysis of blood serum and macroscopic observation of the ovaries as described before [16].

A small piece of each endometrium was fixed in buffered 4% paraformaldehyde for histological analysis [17], and for characterization according to the classification system developed by Kenney [18] and extended by Kenney and Doig [19]. Only cells derived from endometria that were class I (no degenerative changes) according to the Kenney [18] classification were assigned to this study.

For tissue and cell culture, the entire uterus was collected within 5 min of the animal's death, placed in sterile, incomplete (Ca^{2+}- and Mg^{2+}- free) Hank's balanced salt solution (HBSS) supplemented with gentamicin (20 μg/mL; Sigma–Aldrich, #G1272) and 0.1% bovine serum albumin (BSA; Sigma–Aldrich, #A9056), kept on ice and transported quickly to the laboratory.

2.2. Epithelial and stromal cell isolation and culture

A total of 15 uteri on days 2–5 of the estrous cycle were used. Equine epithelial and stromal cells were isolated following the methodology described previously [20]. The endometrial cells were cultured at 38.5 °C in a humidified atmosphere of 5% CO_2 in air. The culture medium was Dulbecco's modified Eagle's medium/Nutrient Ham's F-12 (DMEM/Ham's F-12; Sigma–Aldrich; D8900) supplemented with 10% fetal calf serum (FCS; Sigma–Aldrich, Madison, USA; #C6278) and 1% antibiotic and antimycotic solution (Sigma–Aldrich; #A5955); it was changed every 2–3 days. After reaching 90–95% confluence (Day 5 or 7 for epithelial or stromal cells, respectively), the cells were trypsinized as described previously [20]. Next, depending on the experiment, epithelial cells were seeded at a density of 5×10^5 viable cells/mL and stromal cells at a density of 2×10^5 viable cells/mL in 24- or 96-well plates. The viability of epithelial and stromal cells was 80% and 90%, respectively. The homogeneity of cell culture was evaluated using immunofluorescent staining for specific markers of epithelial cells (cytokeratin) and stromal cells (vimentin) as described previously [20].

2.3. Tissue culture

A total of 5 uteri at the mid-luteal phase of the estrous cycle were used. This phase of the estrous cycle was chosen based on a previous study [15] which showed that TNF, TNFRI and TNFRII expression was up-regulated at the mid-luteal phase of the estrous cycle. Endometrial explants were minced into small pieces, then ~50 mg amounts were washed three times in PBS containing gentamicin (20 μg/μL) and placed into culture tubes, each of which contained 1 mL Dulbecco's Modified Eagle's medium without phenol red (Sigma–Aldrich; D#2960) with 0.1% BSA and antibiotic/antimycotic solution as described above.

Tissue explants were preincubated on a shaker inside a tissue culture incubator at 38.0 °C with 5% CO_2 in air for 6 h, then the medium was replaced with fresh DMEM supplemented with 0.1% BSA and antibiotics and antimycotic. After this, endometrial tissue was further incubated for 24 h with TNF (10 ng/ml) and OT (10^{-7} M; positive control). Finally, viability of endometrial explant cells was confirmed using Alamar Blue according to the manufacturer's instructions (Invitrogen; Burlington; Ontario, Canada; #DAL1025).

2.4. Experimental procedure

2.4.1. Experiment 1. In vitro production of TNF by endometrial cells

2.4.1.1. Experiment 1.1. Effect of ovarian steroids on TNF mRNA transcription in equine endometrial cells. Stromal ($n = 5$) and epithelial ($n = 5$) cells derived from passage 1 were placed in a 24-well plate in DMEM/Ham's F-12 supplemented with 10% FCS and antibiotic and antimycotic solution. When the cells reached 90% confluence, the medium was replaced with fresh DMEM without phenol red, supplemented with 0.1% BSA and antibiotics and antimycotic. The most effective dose and the optimal treatment time for action of ovarian steroids were established in a preliminary study (data not shown). The epithelial and stromal cells were stimulated with P_4 (10^{-7} M), E_2 (10^{-9} M) or P_4+E_2 (10^{-7}/10^{-9} M) for 24 h. Next, the culture medium was removed and to each well 250 μl of Fenozol was added in order to determine TNF mRNA transcription using Real-time PCR.

2.4.1.2. Experiment 1.2. Effect of ovarian steroids on TNF production by equine endometrial cells. To determine TNF production by endometrial cells, the equine ELISpot system was used (R&D Systems, Minneapolis, USA; #EL1814) following the manufacturer's instructions. Stromal ($n = 5$) and epithelial ($n = 5$) cells derived from passage 1 were seeded at a density of 2×10^4 per well in a MultiScreen sterile 96-well plate with a PVDF membrane (Millipore) using fresh DMEM without phenol red supplemented with 0.1% BSA and antibiotics and antimycotic solution. The density of cell seeding was established in a preliminary experiment. Then, cells were incubated with vehicle, P_4 (10^{-7} M), E_2 (10^{-9} M) or P_4+E_2 (10^{-7}/10^{-9} M) for 24 h. The following controls were used: