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TGFB1 modulates *in vitro* secretory activity and viability of equine luteal cells

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ABSTRACT

In the present report we describe the involvement of transforming growth factor B1 (TGF) in functional regression and structural luteolysis in the mare. Firstly, TGF and its receptors activin-like kinase (ALK) 5 and TGF receptor 2 were identified in corpus luteum (CL) steroidogenic, endothelial and fibroblast-like cells. Also, TGF and ALK5 protein expression were shown to be increased in Mid-, and Late-CL ($p < 0.05$). Subsequently, using an *in vitro* model with Mid-CL cells, we studied the role of TGF on secretory activity and cell viability. Cell treatment with TGF decreased progesterone (P4) and prostaglandin (PG) E2 concentrations in culture media ($p < 0.05$), and downregulated mRNA and protein of StAR, CYP11A1, cPGES and mPGES1 ($p < 0.05$). Conversely, TGF augmented PGF2a concentration in culture media, through PTGS2 and PGFS gene expression activation ($p < 0.05$). When cells were incubated with PGF2a, both TGF and ALK5 were upregulated ($p < 0.05$). Additionally, treatment with the pharmacological inhibitor of ALK5, ALK4 and ALK7 - SB431542 (SB) attenuated PGF2a functional and structural luteolytic actions. Indeed, SB blocked: (i) PGF2a inhibitory effect on StAR, CYP11A1, 3BHSD and mPGES1; (ii) PGF2a auto-amplification signal via PTGS2 and PGFS expression ($p < 0.05$); (iii) the PGF2a-induced BAX and FASL expression ($p < 0.05$). Finally, TGF decreased cell viability ($p < 0.05$) and promoted caspase 3 activity ($p = 0.08$) and the expression of pro-apoptotic FASL and BAX ($p < 0.05$). Our results suggest that TGF supports functional regression and structural luteolysis, and also confirm the importance of ALK5, ALK4 and ALK7 activation during PGF2a mediated luteolysis in mares.

1. Introduction

The corpus luteum (CL) is a transient endocrine organ and an intricate set of interactions between pro- and anti-luteolytic forces during mid-CL stage determine its fate. In the absence of pregnancy, programmed functional regression and structural luteolysis must be activated for the resumption of a new oestrous cycle. Using an *in vitro* model with equine luteal cells, we have recently demonstrated the involvement of Nodal, a morphogen from transforming growth factor superfamily [1], in functional regression [2]. Presently, we characterise the supportive role of transforming growth factor B1 (TGF) ligand itself on functional regression and cell death and apoptosis of equine mid-CL cells. Additionally, we confirm that canonical activation of TGF and Nodal signalling pathway is required for the amplification of intraluteal prostaglandin (PG) F2a signal during luteolysis initiation.

The main role of the CL is progesterone (P4) secretion, and its synthesis is critically regulated by the mitochondrial steroidogenic acute regulatory protein (StAR). Furthermore, enzymes such as cytochrome P-450 side-chain cleavage enzyme (CYP11A1) and 3B-hydroxysteroid dehydrogenase (3BHSD) also control P4 production [3]. Previous studies demonstrated that downregulation of P4 can be mediated not only by PGF2a [4], but also by growth factors and cytokines [5,6], such as those from transforming growth factor superfamily [2,7]. Over the last few years we have been characterising the auto-, paracrine role of cytokines like tumour necrosis factor alpha (TNF), interferon gamma, or Fas-ligand (FASL) on either functional regression and structural luteolysis in the mare [5,6,8]. More recently, we reported that Nodal supports functional regression, by decreasing the secretion of P4 and PGE2 and promoting the expression of the eicosanoid PGF2a in equine luteal cells [2]. Indeed, the orchestration of luteolysis is a very complex process far from being fully understood.

The transforming growth factor superfamily is a large group of structurally related cytokines responsible for different biological processes, like regulation of cell behaviour in physiological and

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pathological contexts [9]. The superfamily includes two families: (i) TGF/Nodal/activin family, and (ii) bone morphogenetic protein/anti-Mullerian hormone/growth and differentiation factor family. Generally, TGF related cytokines, such as TGF itself or the morphogen Nodal, signal through cell surface receptors activin-like kinase (ALK), which then phosphorylate intracellular signalling molecules (Smad) to regulate gene transcription after translocation into the nucleus [10]. Indeed, these pathways have been linked to luteal function. TGF was previously associated with structural regression of bovine CL, mediating extracellular matrix remodelling and angioregression [11,12]. Additionally, in human granulosa cells TGF was found to downregulate StAR expression and P4 levels [13].

In the present work we hypothesise that: (i) TGF and its receptors are expressed in equine CL, supporting functional and structural luteolysis; and (ii) the downstream receptors ALK4, ALK5 and ALK7 are necessary for PGF2a mediated luteolysis. Thus, after confirming the rise in expression of TGF and ALK5 in Late CL, we found that *in vitro* treatment of Mid-CL cells with TGF decreased P4 and PGE2 secretion and the expression of their secretory enzymes. Conversely, TGF supported PGF2a production through PGF2a synthase (PGFS) and prostaglandin-endoperoxidase synthase 2 (PTGS2) activation. Regarding structural luteolysis, TGF decreased cell viability, promoted caspase 3 activity and upregulated FASL and BAX expression. Cell treatment with SB431542 (SB), the pharmacological inhibitor of ALK5, ALK4, and ALK7 (the last two are type I receptors of Nodal signalling) blocked the *in vitro* luteolytic actions of PGF2a. Not only SB perturbed PGF2a inhibitory effect on P4 secretory machinery and hampered its supportive role on PGFS and PTGS2, but also attenuated PGF2a supportive action on BAX and FASL protein expression.

2. Materials and methods

2.1. Collection of equine corpora lutea

Biological material was collected as previously described [2]. Briefly, luteal tissue from the ovary and venous blood from the jugular vein were collected *post mortem* at the local abattoir from randomly assigned mares. The animals were euthanized by stunning according to the European Legislation for welfare aspects of animal stunning and killing methods (EFSA, AHAW/04-027) and the Portuguese legislation (DL 98/96, Art. 1°), and also as approved by the Faculty of Veterinary Medicine Ethics Committee. After euthanasia, internal genitalia were collected and luteal structures were classified as following: early luteal phase CL, (presence of corpus hemorrahagicum, plasma P4 > 1 ng/ml, Early CL); mid luteal phase CL, (CL associated with follicles 15 to 20 mm in diameter and plasma P4 > 6 ng/ml, Mid-CL); and late luteal phase CL (CL structure associated with preovulatory follicle 30–35 mm in diameter and plasma P4 between 1 and 2 ng/ml, Late CL), and subsequently transported to the laboratory [6].

2.2. Isolation and culture of luteal cells

The isolation of equine luteal cells followed the methodology previously described [6]. After washing the CL with sterile phosphate buffer solution (PBS) 0.1 M (pH 7.4), luteal tissue was cleaned and connective tissue and blood clots removed. Subsequently, the tissue was minced into small pieces and enzymatically digested. Finally, cells were collected in Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 medium (D/F medium; 1:1 [v/v], D-8900, Sigma) containing 10% foetal bovine serum (FBS) (26140-079, Gibco, NY, USA) and gentamicin [20 μg/ml]. Cell viability was assessed with trypan blue (T8154, Sigma) and varied between 81 and 85% of living cells. Dispersed cells were cultured in 96-well, 24-well culture plates (142475, Nunc, Kamestrupvej, Denmark), or T25 culture flasks (136196, Nunc), supplemented with 10% FBS, amphotericin (250 μg/ml) and gentamicin (20 μg/ml), at 37 °C with 5% CO_2. The Mid-CL culture system consisted of approximately 70% large luteal cells and 20% small luteal cells and endothelial cells and fibroblasts accounted for 10% of the cells. Culture media and cells were stored at −80 °C after the experiments.

2.3. Luteal cell viability and proliferation and caspase 3 activity assessment

Viability was assessed in cells plated in 96-well plates (Corning, Corning, NY, USA), cultured at the concentration of 1.0×10^4/ml and incubated for 24 h at 37 °C in a humidified, 5% CO_2 in air atmosphere. The kit Cell Titter 96® Aqueous One Solution Cell Proliferation Assay (G3581, PR omega, Madison, WI, USA) was used according to the manufacturer's instructions and plates were read at 490 nm.

For caspase 3 activity, cells were cultured in T25 flasks and lysates were prepared following the manufacturer's instructions of the kit (CASP3C-1KT, Sigma). After sample incubation, plates were read at 405 nm.

2.4. Immunohistochemistry analysis

Immunohistochemistry studies were performed on 5 μm histological sections [5]. Primary rabbit polyclonal (RP) antibodies against TGF (Anti-mouse, 1:100, ab9758, Abcam, Cambridge, UK), ALK5 (anti-mouse, 1:50, ab31013, Abcam), and TGF receptor 2 (TGFR2) (anti-human, 1:50, ab61213, Abcam) were used. The primary antibody was detected after incubating samples with anti-mouse/rabbit IgG -Poly-HRP (each at 8 μg/mL) containing 10% (v/v) serum in TBS/0.09% ProClinTM 950 for additional 30 min. Staining was evident after 2–4 min incubation in 3,3-diaminobenzidine (DAB) peroxidase substrate solution. Slides were counterstained with haematoxylin prior to mounting and for negative control we replaced the primary antibody by RP IgG (ab27478; Abcam) or PBS. Immunostaining was defined as a characteristic brown staining visible with a light microscope (Olympus BX51, Tokyo, Japan). Finally, a total of 10 randomly assigned fields at different magnifications were photographed (DP11 Olympus, Tokyo, Japan).

2.5. Western blotting

Samples for western blotting analysis were collected in radio immunoprecipitation assay buffer (RIPA, 250 μL) (R0278; Sigma) supplemented with protease inhibitor (complete), pH 7.4. The phosphatase inhibitor (88662, ThermoFisher, Waltham, MA, USA) was added to samples used for SMAD3 phosphorylation (SMAD3P) analysis. Protein concentration was measured using the bicinchoninic acid assay (BCA-1, Sigma) and 20–60 μg of protein were loaded on acrylamide gel (161-0155; Bio-Rad, Hercules, CA, USA) [14]. Protein expression for TGF signalling components was analysed with the same antibodies as previously done for immunohistochemistry (TGF 1:500, ALK5 1:200, and TGFR2 1:500). As described before, the activation of TGF signalling pathway was confirmed with the quantification of SMAD3 phosphorylation (SMAD3P) (1:1000, rabbit polyclonal ab51451, Abcam) and SMAD3 (1:500, rabbit polyclonal ab73942, Abcam) levels of activity [2]. Regarding steroidogenic enzymes, we used RP antibody against StAR (1:1000, ab96637, Abcam); RP against 3BHSD (1:1000, ab80363, Abcam); and goat polyclonal antibody against CYP11A1 (1:300, sc-18043, Santa Cruz Biotechnology, CA, USA). The eicosanoids were quantified with RP against PGFS (1:400, ab84327, Abcam), RP against PTGS2 (1:200, sc-7951, Santa Cruz Biotechnology), RP cytosolic PGE_2 synthase (cPGES, 1:200, 160150, Cayman, MI, USA), or RP microsomal PGES-1 (mPGES1, 1:200, ab62050, Abcam). Results were normalised against mouse monoclonal antibody against B actin (1:10000; A5441, Sigma). All primary antibodies used for western blotting were incubated overnight at 4 °C, followed by the detection of target proteins with secondary polyclonal anti-rabbit alkaline phosphatase-conjugated (1:30000, A3812, Sigma), polyclonal anti-mouse alkaline phosphatase-conjugated (1:30000, A3562, Sigma) or polyclonal anti-goat alkaline