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TNF-α EXPRESSION OF IN-VITRO CULTURED HUMAN ENDOMETRIAL STROMAL AND EPITHELIAL CELLS

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Abstract: The *human endometrium* is a *complex tissue composed* of various cell types, whose interaction determine its main functions in relation to embryo adhesion and implantation. During each menstrual cycle, the human endometrium undergoes a series of well controlled changes in anticipation to the arrival of the blastocyst that are still not completely understood. Previous analyses have shown the presence of TNF-α and its receptors within both the stromal and epithelial cells of the human endometrium. TNF-alpha has a specific menstrual cycle-dependent expression, which is the highest during the secretory phase. This cytokine has a local role in a variety of endometrial functions in order to support embryo implantation, including cell differentiation and tissue remodelling.

In the present study, we examined the secretion of TNF-α by primary human endometrial stromal (ESC) and epithelial (EEC) cells cultured in (1) *RPMI*1640 media, supplemented with *10% FBS* only and (2) *RPMI*1640 media, supplemented with *10% FBS*, *progesterone (P4) (1 μmol/L) and* 17β-*estradiol* (E2) *(30 nmol/L)*. Endometrial epithelial and stromal cells were obtained from mid-secretory stage endometrial samples from 28 healthy women and cultured in vitro *in 3D Matrigel for 48 hours. The presence of* TNF-α in the culture media was measured using enzyme-linked immunosorbent assay (ELISA).

Cell culture measurements revealed that epithelial cells secrete higher quantities of TNF-α (2.2 pg/ml ± 0.7 pg/ml), compared to stromal cells (0.5 pg/ml ± 0.2 pg/ml). Moreover, co-culture of epithelial and stromal cells showed the highest expression of

TNF-alpha (16.5 pg/ml ± 3.2 pg/ml). Additionally, P4 and E2 supplementation raised the TNF-alpha level in the culture media.

These findings suggest that co-culture of these primary cell types has a synergetic effect on the enhancement of TNF-α secretion. Its higher expression in the co-culture proves that the combination of both cell types is far more relevant to *in vivo* conditions, compared to primary ESC or EEC lines cultured separately. Furthermore, culturing ESC and EEC together in the context of hormone presence and 3D culture conditions establishes an adequate architectural and functional *in vitro* model for studying of certain aspects of endometrial receptivity and implantation in humans.

INTRODUCTION

It is generally assumed that approximately two-thirds of implantation failures are due to bare endometrial receptivity or to defects in the crucial embryo-endometrium interaction (Edwards, 1994; Simon et al., 1998). However we have no reliable tools for the routine exploration of endometrial receptivity, because of the high complexity of this tissue. To ensure embryo survival, implantation and successful pregnancy the endometrium undergoes cyclic remodeling that is ultimately controlled by several autocrine and paracrine factors such as hormones, cytokines, interleukins and growth factors (Cavagna and Mantese, 2003; Giudice, 1994; Chaouat et al., 1995; Chard, 1995; Lessey and Castelbaum, 1995; Tabibzadeh et al., 1995).

TNF-α was at first described as a cytokine secreted by activated macrophages that induced the necrosis of tumours (Carswell *et al*., 1975). Additionally it has been later detected in the ovaries (Roby et al., 1990), oviduct (Hunt, 1993), in maternal sera and in the medium of preimplantation blastocysts (Witkin et al., 1991; Zolti et al., 1991). Furthermore, all types of endometrial cells (fibroblasts, immunocompetent cells, glandular epithelial and vascular cells) produce TNF-α and it is generally accepted that it is expressed in a cyclic pattern by the regulation of the ovarian steroids (Hunt, 1992; Tabibzadeh, 1995, von Wolff, 1999).

The role of TNF- α in implantation is not completely understood, but it is known that implantation is a physiological inflammatory process. It is characterized by an influx of cytokines and growth factors at the feto-maternal surface (Carson, 2000). For instance TNF-α regulates in a dose dependent manner the presence of the barrier transmembrane mucin MUC1 (Dharmaraj et al., 2010) that is expressed throughout the receptive phase and the local loss of MUC1 at the implantation site, crucial for the successful embryo implantation (Hoffman et al., 1998).

These findings suggest that the expression of TNF-α may serve as a possible marker of collective endometrial function in physiologically relevant 3D model (Antoni et al., 2015). The purpose of this study was to demonstrate that the mixed culture of epithelial and stromal cells could imitate the natural *in vivo* conditions more accurately, than single cell type cultures. The development of an adequate model for in-vitro testing could help in gaining better understanding of embryo-endometrium interaction.

MATERIALS AND METHODS

Tissue collection and cell separation

Written informed consent was obtained from all patients. Endometrial samples were collected from 28 healthy fertile woman aged 23–39 years in mid-secretory phase, by biopsy. Endometrial samples of each patient were minced into small pieces of 1 mm, and then subjected to mild collagenase digestion. Endometrial epithelial cell (EEC) and endometrial stromal cell (ESC) were isolated as previously described (Mercader, 2006). EECs and ESCs of each woman were cultured separately until confluence in 3.5 ml 24-well tissue culture plates (VWR 734-2325).

Cell cultures

The endometrial stromal (ESC) and epithelial (EEC) cells were single cell type cultured and co-cultured in a 1:1 ratio in RPMI1640 media, supplemented with sodium pyruvate (10 mmol/L), penicillin–streptomycin-amphotericin B-mixture (100 U/mL) and 10% FBS *in vitro* in 3D Matrigel (BD Biosciences) for 48 hours. In second examination P4 (1 μmol/L) and E2 (30 nmol/L), corresponding to the physiological levels of these hormones in the endometrium during the mid-luteal phase were also supplemented in the culture media (Downie, 1974). The cell culture supernatants were collected and were subjected to enzyme-linked immunosorbent assay for quantitative detection (ELISA).

ELISA

TNF-α ELISA was performed using a Human TNF-alpha High Sensitivity Kit (Ebioscience BMS223HS) with monoclonal anti-human-TNF-alpha antibody as a capture antibody, a polyclonal anti-human-TNF-α antibody for detection with an Biotin-Conjugate anti-human TNF-α polyclonal antibody and streptavidin-HRP as a substrate.The optical density was measured at 450 nm and TNF-α concentrations were extrapolated from a standard curve using seven human TNF-α standard dilutions. The sensitivity limit of the assay was 0.13 pg/ml, with intra- and inter-assay coefficients of variation <10%.

Statistical Analysis

In this experiment results are expressed as mean ± standard deviation. Statistical analysis was performed by using statistical program SPSS (vers. 21.0) followed by Student t-test to find out whether any significant mean difference exist between the studied groups. A *p*-value of less than 0.05 was considered significant.

RESULTS AND DISCUSSION

Firstly, the secretion of TNF- α from the two cell types was measured. When a single cell type was grown, the EEC culture secreted much more TNF-α (2.2 pg/ml ± 0.7 pg/ml), than ESC (0.5 pg/ml ± 0.2 pg/ml) (Fig. 1). Similar results were obtained from earlier experiments where TNF-α was only detected in the supernatants from epithelial cells, but not in the supematants from stromal cells prepared from endometrial tissue (Laird, 1996). Furthermore, it was previously shown that the endometrial epithelial cells are the major source of the TNF- α in human endometrium (Hunt, 1992). Nevertheless endometrial stromal cells also produce TNF- α.

There are previous evidence of paracrine control over the cytokine expression in the endometrium and the autocrine role of TNF- α itself (von Wolff et al., 2000; Tabibzadeh et al., 1999). Due to this, we investigated the TNF- α secretion from epithelial and stromal cells co-culture. The amount of TNF-α was remarkably higher in the mixed culture (16.5 pg/ml ± 3.2 pg/ml), suggesting synergetic induction of TNF-α secretion (Fig. 1). Therefore, the collaborative action of EEC and ESC illustrates the importance of co-culturing when investigating endometrial receptivity.

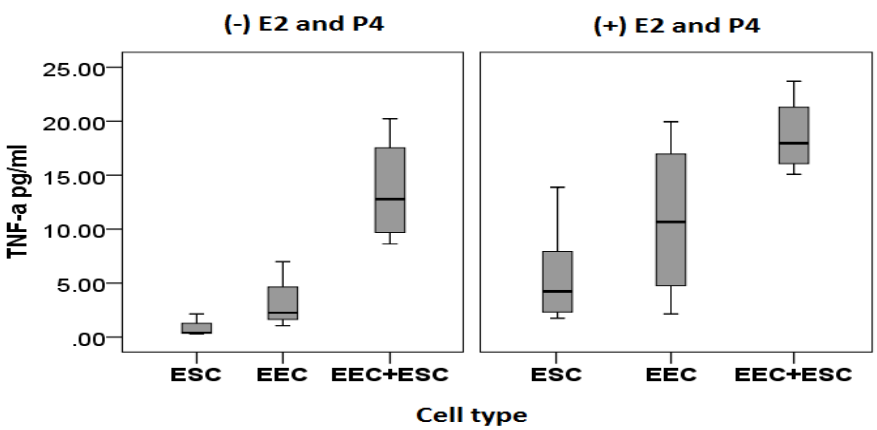

Fig. 1. Tumour necrosis factor α (TNF- α) production by human endometrial cells in culture media with / without E2 and P4. ESC – endometrial stromal cells, EEC – endometrial epithelial cells, EEC+ESC – mixture of these two cell types.

Earlier reports indicate cycle-dependent release of TNF-α by endometrium suggesting regulation by steroid hormones (Roby, 1994). In our study, the impact of P4 and E2 on the TNF- α secretion was also investigated. Adding hormones to the culture media led to increased secretion of TNF-α (Fig. 1). This result corroborates with previous reports showing a precise E2 and P4 regulation of TNF-α expression by epithelial and stromal endometrial cells in mouse (Roby, 1994). However several studies on TNF- α expression in human endometrium remain controversial, which can in part be explained by the use of different methodological procedures. According to von Wolff and colleagues who quantified TNF-α mRNA (1999), progesterone down-regulate TNF- α expression in the endometrium. On the other hand Hunt et al. (1999) observed cytochemically an increase in TNF-α mRNA during the proliferative phases and during mid-to-late secretory phases. Other authors that performed *in situ* hybridization conclude that the second phase of the menstrual cycle is associated with an accumulation of TNF-α and TNF-α mRNA (Philippeaux and Piguet, 1993) even in the absence of a substantial mobilization of macrophages.

Although initially associated with destructive inflammatory processes, TNF-α is now known to have pleiotrophic effects on cellular growth and to modulate many other cellular functions (Tabibdzadeh et al., 1999; Osteen et al., 1994). It is possible that TNF-α may play an important role in the cyclic changes occurring in the human endometrial tissue (Cao et al., 2006). In addition, TNF-α has also been shown to be expressed by the embryo (Hunt et al., 1992; Ben-Yair et al. 1997; Kawamura et al. 2007) and uterine cells at the implantation site (Chou et al., 2015).

CONCLUSIONS

These findings suggest that co-culture of ESC and EEC has a synergetic effect on the enhancement of TNF-α secretion. Its higher expression in the co-culture proves that the combination of both cell types is far more relevant to *in vivo* conditions, compared to primary ESC or EEC lines cultured separately. Furthermore, culturing ESC and EEC together in the context of hormone presence and 3D culture conditions establishes an adequate architectural and functional in vitro model for studying of certain aspects of endometrial receptivity and implantation in humans.

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