Effect of activin A on tumor necrosis factor-α/intercellular adhesion molecule-1 pathway in endometrial stromal cells

Silvia Mangioni a, Paola Viganò b, Pasquale Florio c, Orietta Borghi b, Michele Vignalid, Felice Petraglia c, Anna Maria Di Blasio b,∗

a Department of Obstetrics, Gynecology and Neonatology, Clinica “L. Mangiagalli”, University of Milano, Milano, Italy
b Istituto Auxologico Italiano, Milano, Italy
c Department of Obstetrics and Gynecology, University of Siena, Siena, Italy
d Department of Obstetrics and Gynecology, Clinica “Macedonio-Melloni”, Milano, Italy

Received 12 July 2004; received in revised form 9 February 2005; accepted 16 March 2005

Abstract

Objective[s]: Activin A and inhibin A are growth factors expressed by human endometrium involved in the control of endometrial functions. In the present study we investigated the effects of activin A and inhibin A in modulating the tumor necrosis factor (TNF)-α/intercellular adhesion molecule (ICAM)-1 system in cultured human endometrial stromal cells.

Study design: Endometrial samples were obtained from 34 reproductive age women undergoing laparoscopy for benign ovarian cysts or infertility. Endometrial stromal cells were cultured and soluble ICAM-1 and TNF-α were measured in cell-free supernatants following treatment with or without activin A or inhibin A. Cell surface ICAM-1 was assayed by flow cytometry by staining endometrial cells with specific monoclonal antibodies.

Results: Activin A and inhibin A did not influence either the expression of cell surface ICAM-1 or soluble ICAM-1 shedding by cultured endometrial cells. On the other hand, TNF-α secretion significantly increased in presence of activin A but not of inhibin A. Cell surface ICAM-1 was assayed by flow cytometry by staining endometrial cells with specific monoclonal antibodies.

Conclusions: Since TNF-α modulates several endometrial processes such as menstruation, proliferation, apoptosis, implantation and decidualization, an effect of activin A in the physiological control of endometrium is further supported by the present data.

© 2005 Elsevier Ireland Ltd. All rights reserved.

Keywords: TNF-α; ICAM-1; Activin A; Endometrium

1. Introduction

The role of the cytokine network in endometrial function is currently well established given its involvement in the physiological processes of menstruation and implantation as well as in several inflammatory changes associated with pregnancy [1]. Among the cytokines, tumor necrosis factor-α (TNF-α) is known to modulate endometrial cellular proliferation, differentiation and immunoregulation [2]. The presence of TNF-α in human endometrium has been detected within both stromal/epithelial cells and leukocytes with variations during the menstrual cycle [3–5]. The concentrations of TNF-α in endometrial glands resulted low at the beginning of the cycle, rose sharply in the mid- to late-proliferative phase and stayed high during the secretory phase [6].

Proposed as direct targets of TNF-α action in the endometrium, there are the genes coding for adhesion molecules. More specifically, intercellular adhesion molecule (ICAM)-1 is expressed by both epithelial and stromal cells throughout the entire menstrual cycle and is actively engaged in the intercellular interactions between the immune system and endometrial cells [7]. Endometrial cells are also constitutively able to release the soluble form of ICAM-1 and higher concentrations of this soluble protein have been detected in women with endometriosis [8]. Soluble ICAM-1 interferes with several immune functions by inhibiting the binding of the membrane form of the molecule to its putative receptor on lymphoid cells [8].
Other secretory endometrial proteins are activins and inhibins [9]. Indeed, cultured endometrial stromal and epithelial cells express and secrete inhibin-related proteins, supporting the local production of these growth factors [10,11]. High concentrations of these proteins were also detected in the peritoneal fluid and in ovarian endometriotic cysts [12,13].

It is well established that inhibins and especially activin A are involved, as paracrine factors, in regulating the cytokine network in different systems [14–16]. Activin A significantly increases TNF-α production by choriodecidual and villous placental tissues [17]. However, studies that have specifically investigated the ability of inhibins and activins to modulate the TNF/ICAM-1 system in the human endometrium are lacking. Given the effects of inhibins and activin A on cytokine production and action [18,19], their presence in peritoneal fluid [11] and their involvement in embryonic invasion and in support to placental functions [15], the present study has investigated the potential ability of activin A and inhibin A to influence TNF-α/ICAM-1 system in human endometrial stromal cells.

2. Materials and methods

2.1. Collection of endometrial samples

Human endometrial samples were obtained from reproductive age women (n = 34) undergoing laparoscopy for unexplained infertility, pelvic pain or benign ovarian pathology. The criteria for inclusion were: (1) that the day of the last menstrual period was certain; (2) that the patients were normally cycling (length of the cycle, from 26 to 33 days) and did not receive hormones for at least 3 months before surgery; (3) that there was no evidence of either endometritis, endometriosis or previous autoimmune or neoplastic disorders. Based on the date of the last menstrual period and the histologic examination of the samples, 16 women were in the proliferative phase, 13 in the secretory phase and 5 in the ovulatory phase. All tissues of uterine endometrium were obtained at the time of laparoscopy using an endometrial biopsy curette after informed consent was given.

Specimens of endometrium were rinsed several times in PBS and then processed immediately. Approval for this study was granted by the local Human Institutional Investigation Committee.

2.2. Cell preparation and culture

In previous studies, we have successfully established and employed stromal cell monolayer from normal endometrial samples [20–23]. Diffuse and strong cytoplasmatic immunostaining for vimentin was demonstrated in nearly all (90%) cultured endometrial stromal cells even after the trypsin procedure to detach the cells from the plate. Phenotypic analysis by flow cytometry indicated that less than 2% of the cells expressed the CD-14 antigen, which represents the specific monocyte/macrophage marker. Briefly, tissue was gently minced into small pieces (1–2 mm3) and washed in fresh medium to remove mucus or debris. Thereafter, they were incubated for 2 h at 37 °C in a shaking water bath in 10 ml Ham’s F-10 (Sigma, Mi, Italy) containing 0.2% collagenase (Sigma, Mi, Italy). At the end of the incubation, single stromal cells were separated from large clumps of epithelium by a 10 min. period of differential sedimentation at unity gravity. The top 8 ml of medium, containing predominantly stromal cells, were then slowly removed and the cells were collected by centrifugation (200 × g). The stromal-enriched fraction was washed twice in Ham’s F-10 supplemented with 10% fetal calf serum (Sigma, Mi, Italy) and antibiotics and allowed to adhere selectively to 25 cm2 tissue culture dishes for 15 min. Thereafter, non-attached epithelial cells still present were removed and a purified stromal preparation was obtained on the surface of the culture dishes. Stromal cells were allowed to proliferate to subconfluence in Ham’s F-10 supplemented with 10% FCS and antibiotics at 37 °C in a 95% air and 5% CO2 incubator. Thereafter, cells were plated at a density of about 3 × 105/ml in Petri dishes (1 ml/dish, 35 mm in diameter) and treated for 48 h with and without different concentrations of TNF-α (0.1, 1 ng/ml), interleukin (IL)-1β (500 pg/ml), activin A (1, 10, 100 ng/ml) or inhibin A (1, 10, 100 ng/ml). A maximum of n = 8 Petri dishes has been prepared from a single endometrial biopsy and the different treatments were tested on cells from different biopsies. The culture medium was then collected from both unstimulated and stimulated conditions, centrifuged for 5 min and stored at −20 °C for the subsequent quantification of levels of TNF-α and ICAM-1 in the supernatants. Cells detached from the plates by a rapid trypsinization were used for flow cytometric analysis.

2.3. Flow cytometry for surface ICAM-1 expression

The presence of cell surface adhesion protein ICAM-1 was determined by staining endometrial cells, derived from different experimental conditions, with specific monoclonal antibodies. Immunofluorescent staining was performed using an unconjugated murine anti-ICAM-1 (anti-CD54; Immunotec, Marseille, France) followed by a secondary polyclonal goat anti-mouse IgG-fluorescein isothiocyanate as previously described [21]. A matched IgG isotype control obtained from murine myeloma was used to evaluate background fluorescence. Stained cells were examined by a flow cytometer (FACScan; Becton Dickinson & CO., Mountain View, CA, USA). Acquisition gates were set at large size cell area, with forward scatter >400 and side scatter >350. Gated cells (5000 events) were acquired and stored. Markers were set according to negative controls to quantify the percentage of positively stained cells. The
percentage of positive cells was calculated on histogram displaying log10 of fluorescence (in arbitrary units) versus number of cells.

2.4. Soluble ICAM-1 and TNF-α enzyme-linked immunosorbent assays (ELISAs)

The quantitative detection of sICAM-1 and TNF-α in cell-free undiluted supernatants was performed using commercially available enzyme-linked immunosorbent kits provided by Bender (MedSystems, Vienna, Austria). Standard unconditioned culture medium was also assayed in order to exclude the presence of ICAM-1 or TNF-α. The concentration of ICAM-1 was expressed as ng/ml and the interassay and intra-assay coefficients of variation were 8 and 4%, respectively. The concentration of TNF-α was expressed as pg/ml and the interassay and intra-assay coefficients of variation were 7.4 and 7%, respectively. Lower limits of detection were 0.5 ng/ml for ICAM-1 and 5 pg/ml for TNF-α.

2.5. Statistical analysis

TNF-α and ICAM-1 concentrations in the supernatants have been normalized for cell number in the Petri dishes. ICAM-1 expression has been expressed as percentage of positive cells. Difference between treatments were compared, as appropriate, by analysis of variance (ANOVA) and Fisher PLSD-test as post-test or the non-parametric Friedman’s test. Probability < 0.05 was considered as statistically significant.

3. Results

3.1. Effect of TNF-α on the release of soluble ICAM-1 by cultured endometrial cells

Before proceeding testing activin A/inhibin A effect on TNF-α/ICAM-1 system in the endometrium, we set up experiments aimed to verify the ability of TNF-α to stimulate ICAM-1 in endometrial cells. Indeed, while this stimulation has been well demonstrated in several other cell populations, it has not been evaluated in endometrium. Soluble ICAM-1 concentrations were measured in conditioned media by cultured endometrial stromal cells incubated for 48 h with different concentrations of TNF-α. In line with the effects previously reported in other cell types, TNF-α significantly stimulated soluble ICAM-1 production by endometrial stromal cells (Fig. 1).

3.2. Effects of activin A and inhibin A on the secretion of TNF-α by cultured endometrial cells

TNF-α concentrations were measured in conditioned media by cultured endometrial stromal cells incubated for 48 h with and without different doses of activin A, inhibin A and IL-1β. Activin A, but not inhibin A, had a significant stimulatory effect on TNF-α secretion by cultured endometrial cells. This effect was statistically significant only at a concentration of activin A of 100 ng/ml although the effect appeared to be dose-dependent (Fig. 2, upper panel). The rationale to evaluate whether activin A could also affect IL-1β-mediated effects was based on the assumption that both the inflammatory agents, IL-1β and TNF-α, are strongly expressed at endometrial level during the secretory phase of the cycle [6,24] and they act via the same transduction pathways to influence the transcription of inflammatory response genes [25]. As expected, after treatment with IL-1β, stromal cells secreted higher levels of TNF-α when compared to unstimulated cells. Activin A did not modify the IL-1β-mediated increase of TNF-α secretion (Fig. 2, lower panel).

3.3. Effects of activin A and inhibin A on expression and release of ICAM-1 by cultured endometrial cells

To evaluate whether ICAM-1 expression could be modulated by activin A or inhibin A, cultured endometrial stromal cells were treated with and without activin A or inhibin A in combination or not with IL-1β. ICAM-1 surface expression was assayed using immunofluorescence flow cytometry. Visual appraisal of the FACS profiles revealed a normal distribution of the molecule on the surface of the cells. As expected, endometrial stromal cells stimulated by IL-1β alone showed significantly higher levels of cell surface ICAM-1 when compared to unstimulated cells. In contrast, activin A or inhibin A, associated or not with IL-
1β, did not influence the expression of cell surface ICAM-1 (Table 1).

To evaluate soluble ICAM-1 secretion, cultured endometrial stromal cells were incubated with and without activin A, inhibin A and IL-1β for 48 h. After treatment with IL-1β, stromal cells secreted higher levels of soluble ICAM-1 when compared to unstimulated cells. In contrast, inhibin A alone or activin A with and without IL-1β, did not have any effect on soluble ICAM-1 secretion (Table 2).

4. Discussion

The present study has demonstrated that activin A significantly increased TNF-α secretion by cultured endometrial stromal cells. This effect was significant only using a high dose of the protein that, however, was within the order of magnitude frequently used to evaluate paracrine action of activin A in other tissues [17].

TNF-α is supposed to be a major participant of several events of endometrial physiology such as menstruation, proliferation, apoptosis, implantation and decidualization [1,26,27]. The effect of activin A on its secretion underlies the involvement of this molecule in these phenomena and further supports its ability to modulate even inflammatory and immunologic mechanisms.

Activin A exerts both pro- and anti-inflammatory actions by modulating the production of cytokines such as TNF-α, IL-1 and IL-6 [18,19]. Keelan et al. [17] have reported that activin A is able to modulate the production of IL-8, IL-6, TNF-α by gestational tissues with a biphasic response, being stimulatory at low concentrations and inhibitory at high doses. Finally, studies performed in sheeps have shown that systemic serum levels of activin A increase very rapidly in response to an inflammatory stimulus and its concentrations peak before those of the early-response proinflammatory mediator TNF-α [28].

The present data also showed that TNF-α, but not activin A, directly induced soluble ICAM-1 production by cultured endometrial cells. Intercellular adhesion molecule-1 is expressed by human endometrial cells throughout the entire menstrual cycle and regulates the intercellular interactions between the immune system and endometrium [7,8,21]. The TNF-α/ICAM-1 pathway is actively involved in the recruitment of lymphoid cells in several inflammatory sites and in the lymphocyte migration through endothelium [29]. The apparent lack of any modulation of activin A on ICAM-1 might be related to technical factors such as the limited time of treatment of endometrial cells. Alternatively, the activin A-mediated stimulation of TNF-α might be
Endometrial stromal cells 4 7.89
/C6
Endometrial stromal cells 8 73.46
/C6

... increase of TNF-α agents act via...

Firstly, experiments standardized in cell number and confluency status are difficult to set up without a subculture passage. For a similar technical problem we have chosen to use stromal rather than epithelial cells of the endometrium. Epithelial cells are the major source of endometrial TNF-α [3–6] but, as they do not survive subculture, standardisation of experimental conditions are much more complicated. Certainly, the herein proposed effect of activin A on TNF-α might be, under the present experimental conditions, underestimated. The increase in TNF-α secretion by endometrial stromal cells seen in the presence of activin A is very small (approximately an increase of 25%) compared to that seen with IL-1β (approximately 10-fold) and therefore this effect of activin A needs to be confirmed by further experiments. In addition, an experiment showing an abolition of the effect by the addition of a neutralising anti-activin A antibody would greatly strengthen our results.

In conclusion, the results of this study support the following observations: (1) similarly to what occurs in other tissue systems, the cytokine TNF-α modulates soluble ICAM-1 secretion in human endometrial stromal cells; (2) a high dose of the inhibin-related protein activin A is able to stimulate TNF-α secretion by endometrial stromal cells; (3) activin A does not have any effect on the adhesion molecule ICAM-1 in the same cell type.

The role of activin A as a modulator of local interactions between the immune and the endocrine systems is gaining particular importance [16]. While the TNF-α/ICAM-1 pathway does not seem to constitute a major target of activin A immunomodulatory action in the endometrium, further studies are needed to evaluate whether the molecule might affect other parameters underlying immune-mediated reactions in this tissue.

**References**


