Activin A increases invasiveness of endometrial cells in an *in vitro* model of human peritoneum†

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The aim of this study was to investigate whether activin A has an effect on the attachment and/or invasion of endometrial cells in a modeled peritoneum in *vitro*. Cultured endometrial stromal cells (ESCs) and endometrial epithelial cells (EECs) were treated with activin A (6.25–50 ng/ml) and with activin A (25 ng/ml) with and without inhibin A or follistatin. Fluorescent labeled cells were added to confluent peritoneal mesothelial cells (PMCs) and to a monolayer of confluent PMCs grown in a Matrigel™ invasion assay. The rate of endometrial cell attachment and invasion through PMCs was assessed. The expression of cell adhesion proteins N- and E-cadherin was evaluated with real-time RT–PCR. Activin A (25 ng/ml) promoted invasion of the endometrial cells through the modeled peritoneum (>2-fold versus control) and this effect was partially reversed by inhibin A and follistatin. Activin A had no effect on the rate of attachment of the endometrial cells to the PMCs or in the rate of proliferation. In addition, activin A induced a decreased mRNA expression of E-cadherin in cultured EECs. In conclusion, activin A increases invasion of EECs and ESCs into modeled peritoneum. In EECs, this effect may be related to down-regulation of E-cadherin expression. Further studies are warranted to evaluate the role of activin-A in the genesis of the endometriotic lesion.

Keywords: activin A; inhibin A; follistatin; endometriosis; cadherins

Introduction

Various hypotheses have been promulgated to explain the genesis of the endometriotic lesion. For endometriosis arising on peritoneal surfaces, the most widely accepted is Sampson’s theory proposing endometrial tissue transplantation resulting from retrograde Fallopian tube flow during menses. This leads to adhesion and invasion of the peritoneum by endometrial cells (Sampson, 1927). Many crucial questions concerning the initial interaction of endometrial cells with peritoneal mesothelial cells (PMCs) and invasion into the peritoneum remain unanswered. Studies elsewhere using laparoscopy have demonstrated that retrograde menstruation is a nearly universal phenomenon in women with patent Fallopian tubes (Halme et al., 1984; Liu and Hitchcock, 1986). However, factors that promote attachment, invasion and growth of endometrium in the peritoneal cavity are unknown.

A putative role for activin A in the pathogenesis of the endometriotic lesion has recently been espoused. The endometrium and the endometriotic tissue produce activin A (Florio et al., 1998; Leung et al., 1998; Jones et al., 2000, Florio et al., 2003). Activin A can be found in high concentrations in the peritoneal fluid (PF) and in the endometriotic cyst (Reis et al., 2001). Activins and inhibins are members of the transforming growth factor-β (TGF-β) superfamiliy that result from the assembly of subunits α (18 kDa) and β (14 kDa). Inhibins A and B are formed by a combination of a common α with a β subunit. Activins A, B and AB are homodimers of β subunits (βA+βA, βB+βB and βA+βB, respectively) (Ling et al., 1986; Vale et al., 1986) and act through a cell surface receptor (ActRII) (Mathews and Vale, 1991,1993; Mathews et al., 1992). Following the binding of activin, ActRII recruits ActRII, promoting its activation (Tsuchida et al., 1993). Activated ActRII phosphorylates members of the Smad family (Smad 2 or Smad 3), which interacts with Smad 4 and this complex translocates to nucleus, where it promotes gene expression (Wrana and Attisano, 2000).

Follistatin and inhibins are activin antagonists and classically exert their biological effects indirectly, by counteracting activin induced events. Follistatin is a single chain glycoprotein (Ueno et al., 1987) found in multiple tissues. Follistatin is produced in a coordinated way with activin, and is the major regulator of activin bioactivity. It binds to activin with high affinity and blocks its interaction with ActRII (de Winter et al., 1996). Inhibin antagonizes activin’s actions through competitive binding to receptor type II (Lebrun and Vale, 1997), that is sequestered in an inactive complex with inhibin and the co-receptor betaglycan (Gray et al., 2002). Endometriotic...
tissue expresses follistatin in higher amounts than eutopic endometrial tissue (Torres et al., 2007), while inhibin A is found in high concentrations in endometriotic cysts and in the PF of women with endometriosis (Reis et al., 2001).

Activin A has been shown to stimulate the expression of matrix metalloproteinases (MMPs) by cultured endometrial stromal cells (ESCs) and endometrial epithelial cells (EECs) (Jones et al., 2006). Increased MMP expression has been associated with cell invasion and migration. In addition, in some cancer cells, activin A stimulation leads to alterations in the expression of cadherins which affects the invasive phenotype (Yoshinaga et al., 2004). The present study investigates the effect of activin A on the rate of endometrial-PMC attachment and transmesothelial invasion of endometrial cells using an in vitro model of the peritoneum.

Materials and Methods

Approval for this study was granted by the Institutional Review Board of the University of Texas Health Science Center at San Antonio. The authors report no conflict of interest in the performance of this study.

Endometrial cell culture

Proliferative phase endometrium was obtained by aspiration biopsy using a Pipelle (Prodimed, Unimar Inc., Neuilly-En-Thelle, France) or immediately following hysterectomy performed for benign conditions in women without endometriosis. Hysterec- tomy was performed for patients with pelvic prolapse or myomatous uterus and Pipelle biopsy specimens were obtained from patients undergoing elective interval sterilization or infertility evaluation. Patients had not undergone hormonal treatment for three months prior to collection of endometrium.

Monolayer cultures of ESCs and EECs were established as previously described (Kirk and Irwin, 1980; Dechaud et al., 2001; Witz et al., 2002). Briefly, the endometrium was mechanically dispersed with a scalpel then enzymatically digested with 0.1% collagenase type 1 and 0.05% DNase. EECs were separated from the stromal cells by gravity sedimentation. The stromal cell-rich supernatant was placed in a culture flask and cells were allowed to adhere for 20 min then washed with medium. Adherent stromal cells were cultured as monolayers in flasks with Dulbecco’s modified Eagle Medium (DMEM)/F12 (1:1) (Sigma, St Louis, MO, USA) containing antibiotics and antimycotics, 5 µg/ml insulin (Sigma) and 10% fetal calf serum (Hyclone, Logan, UT, USA).

The epithelial-rich cell pellet was dispersed and plated in flasks for 20 min. The non-adherent epithelial-rich supernatant was recovered and plated in a new flask. EECs were then grown as monolayers in an enriched medium containing (volumes per liter of solution): MCDB 131 (Sigma—Aldrich, St Louis, MO, USA, 330 ml); Medium 199 (Sigma, 335 ml), Minimal Essential Medium—alpha modification (JRH Biosciences, Lenexa, KS, USA, 222 ml), antibiotics and antymycotics (10 ml), 10 µg/ml insulin (1 ml), D-glucose 0.3 µg/ml (667 µl) and fetal calf serum (100 ml) (Merviel et al., 1995; Witz et al., 2003).

After the second passage, epithelial and stromal cells were placed on eight-well chamber slides. Purity of culture was morphologically determined by hematoxylin–eosin staining, and immunocytochemically by incubation with monoclonal antibodies for human cytokeratin, vimentin, CD45 and Von Willbrand factor. Cultured ESCs were fusiform, expressed vimentin, and did not express cytokeratin, von Willebrand factor, or CD45. EECs were polygonal, expressed cytokeratin, and did not express vimentin, von Willebrand factor, or CD45. Using these techniques, we have obtained greater than 97% purity of ESCs and EECs. After the third passage, however, EECs started losing their typical shape and became more fusiform, so they were not used in the experiments from this stage onwards.

Evaluation of activin receptors in cultured ESCs and EECs

The expression of activin receptors by ESCs and EECs was determined using immunocytochemistry. ESCs and EECs were grown on chamberslides or lifted, dispersed and placed on a slide with Cytospin (Thermo Scientific, FL, USA). Chamberslides were washed in phosphate-buffered saline (PBS) twice and then fixed in cold acetone (−20°C) and kept in a −70°C freezer until processing. For the cytospin slides, cells were spun in Cytospin Collection Fluid (Thermo Scientific), air-dried and kept at 4°C until staining procedures.

The peroxidase–avidin–biotin technique was used for staining (Vectastain Elite Universal Kit—Vector Laboratories, Burlingame, CA, USA). The antibodies for ActRIB and ActRIIA (kindly donated by Dr W. Vale, Salk Institute, USA) were used under standard conditions (overnight incubation, at 4°C, after endogenous peroxidase and biotin blocking, and incubation in normal goat serum), followed by biotinylated goat anti-rabbit secondary antibody and by peroxidase–avidin–biotin complex (Vector Laboratories). The antiserum against ActRIB is specific for this receptor subtype (Tsuda et al., 1995), whereas the antiserum against ActRIIA has a weak cross-reaction with the other variant of type II activin receptor (ActRIB) but none with type I receptor or other TGF-β superfamilies members (Mathews and Vale, 1993).

After the peroxidase development with 3,3-diaminobenzidine (Sigma), the slides were mounted with Entellan New (Merck, São Paulo, Brazil). Non-immune mouse immunoglobulin substituted for the primary antibody served as a negative control. Staining was graded as absent, mild, moderate, or strong.

Activin, inhibin and follistatin treatment

After the first or second passages, EECs and ESCs were grown to subconfluence and culture medium was changed to one containing DMEM/F12 with 10% heat inactivated, charcoal stripped fetal calf serum (stripped medium) with or without activin A, inhibin A or follistatin for 24 h.

Initial experiments were performed, as described in the subsequent section, to determine the effect of activin A (R&D Systems, Minneapolis, MN, USA) on the rate of transmesothelial invasion by EECs and ESCs using concentrations of 6.25–50 ng/ml (EECs, n = 5; ESCs, n = 6). Subsequent experiments were performed to assess the consequence of treatment with activin A in the presence of its antagonists, inhibin and follistatin. In these experiments, EECs (n = 9) or EECs (n = 10) were cultured with activin A (25 ng/ml) with or without inhibin A, 50 ng/ml (Diagnostic Systems Laboratories, Webster, TX, USA) or follistatin, 250 ng/ml (R&D Systems).

EECs and EECs were collected using an enzyme free cell dissociation solution (Sigma) and washed with their respective medium. The effect of activin A, in the presence or absence of inhibin A or follistatin, on the rate of endometrial-PMC attachment, transmesothelial migration by endometrial cells, proliferation and expression of cadherins was evaluated as described in the subsequent paragraphs.

Mesothelial cell culture

Two models were used to quantify the proportion of endometrial attachment to PMCs as well as the rate of subsequent transmesothelial invasion. Our prior investigations have demonstrated similar rates of endometrial cell binding to commercially available LP9 PMCs (NIH Aging Cell Repository, Corelli Institute for Medical Research, Camden, NJ, USA) and PMCs derived from parietal peritoneum and ovarian surface epithelium (Lucidi et al., 2005). This suggests that LP9 PMCs are an appropriate experimental surrogate for patient derived PMCs. The LP9 PMCs were grown in MCDB-131/Medium 199 (1:1) (Sigma) supplemented with epidermal growth factor (20 ng/ml), t-glutamine (2 mM), hydrocortisone (400 ng/ml), 1% antibiotics and antymycotics, HEPES buffer and 15% fetal calf serum.

Peritoneal model

A previously described assay was used to evaluate the rate of EEC and ESC attachment to LP9 PMCs (Lucidi et al., 2005). In brief, ESCs or EECs were labeled with calcein-AM (Molecular Probes, Eugene, OR, USA) (5 µM) for 20 min at 37°C. EESCs or EECs were plated at 20 000 cells per well over 96 well plates with confluent LP9 PMCs. Plates were then cultured at 37°C for 1 h in 5% CO2 in air. The plates were inverted, submerged in a bath of PBS containing calcium and magnesium (Invitrogen, Carlsbad, CA, USA), and incubated at 37°C in 5% CO2 in air for 15 min on an orbital mixer (Barnstead/Thermolyne, Dubuque, IA, USA) set at 20 rpm allowing non-adherent endometrial cells to precipitate under gravity. Fluorescence readings were taken for each well before and after washing. Each assay was run in a minimum of six replicates. Each data point was calculated as the average of the six replicates. The percentage of attached endometrial cells was calculated for each
well ([Fluorescence value after washing/Fluorescence value before washing] × 100).

The rate of transmural implantation of endometrial cells was determined by growing LP9 PMCs on growth factor reduced Matrigel™ coated 24-well invasion chambers containing membranes with 8 µm pores (BD Bioscience, San Jose, CA, USA). LP9 ESCs or EM42 cells were grown to near confluence, labeled with CellTracker Green® (Molecular Probes), and placed over the LP9 covered membranes (25 000 cells/well). Preliminary experiments demonstrated that 25 000 endometrial cells per well produced a uniform distribution of endometrial cells without crowding or stacking of cells. The invasion chambers were incubated at 37°C in 5% CO₂ in air and cultures were interrupted at 24 h. Cells not invaded, on the upper surface of the membranes, were mechanically removed with a cotton tip applicator, and the membranes were fixed in cold formaldehyde. Each invasion assay was run in triplicate.

The membranes were then treated with Hoechst 33342 (Invitrogen, Grand Island, NY, USA), a fluorescent nuclear stain, to identify cell nuclei. The number of invaded cells on the bottom of the coated membranes was determined using a fluorescence microscope with a 20× objective. Images were obtained from eight standardized, non-overlapping fields representing ~40% of the total surface area. The number of invaded endometrial cells was counted, as we have previously described (Nair et al., 2007) by identifying a Hoescht labeled nucleus surrounded by CellTracker® Green labeled cytoplasm.

**Proliferation assay**

Increased number of cells on the undersurface of the invasion chamber could be due to an increased rate of cell division by invaded cells rather than an absolute increased number of invaded cells. To exclude this possibility, a cell proliferation assay was performed. EECs or ESCs were plated in a 96-well plate at the concentration of 20 000 cells/well. Cells were incubated with or without activin-A alone or in combination with inhibin-A or follistatin for 24 h. Proliferation rates were compared using the MTT assay (ATCC, Manassas, VA, USA), in which MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] is oxidized to purple formazan by active mitochondrial reductases. Formazan production is directly related to the number of viable cells. Cells were lysed with a detergent, and absorbance was measured at 570 nm using a microtiter plate reader (Molecular Devices, Downingtown, PA, USA) as recommended by the manufacturer.

**Real-time polymerase chain reaction**

RNA was extracted from cell samples using RNAqueous—Micro kit (Ambion-Applied Biosystems, Austin, TX,) and reverse transcription was performed using Clontech Sprint PowerScript Single Shots; Random Hexamer Primers (Clontech, Takara, CA, USA). Reaction was carried out in a thermocycler at 42°C for 1 h and then at 99°C for 5 min. RT-PCR was carried out in an ABI-Prism 7700 Sequence Detection System using the fluorescent dye SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). All samples were run in duplicate on 96-well optical PCR plates (Applied Biosystems) in a final reaction volume of 25 µl. The PCR parameters were 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, 40 cycles at 95°C for 15 s and 60°C for 1 min.

The primers used for PCR amplification of E- and N-cadherin are listed in Table I. The gene encoding the ribosomal protein S26 was used as an internal control (Bonne-Duquennoy et al., 2006). Primers were designed to span intron–exon borders and thus anneal only to cDNA. No amplification of fragments occurred in negative control samples prepared without reverse transcriptase. The specificity of PCR products was confirmed by single peak dissociation curves and by gel electrophoresis showing that the amplicons had the expected molecular weight.

A standard cDNA sample prepared from mid-secretory endometrium was diluted serially to construct relative standard curves that were used to quantify the PCR results. The threshold cycle (Ct) for amplification of each sample was used to calculate its input amount of target cDNA through the linear equation generated by the standard curve. To adjust for the internal control, the results are expressed as the ratio between the calculated input amount of cadherin cDNA and that of S26 cDNA, expressed in arbitrary units (Catalano et al., 2007).

**Statistical analysis**

Invasion, attachment and proliferation assays were run in triplicate or quadruplicate, whereas RT-PCR was run in duplicate. Data were tested for normality and for homogeneity of variances and did not depart significantly from normal distribution. Thus, the results are presented as means ± standard error of the mean (SEM) and differences between treatments were assessed by paired t-test with Bonferroni correction. P < 0.05 was considered statistically significant.

**Results**

**Demonstration of activin receptors in endometrial cell cultures**

EECs and ESCs from all primary cultures stained positive for activin receptors ActRIIA and ActRIIB. The intensity of staining for both receptors was moderate and was similar in stromal cells and epithelial cells.

**Effects of activin A on the invasion of endometrial cells into modeled peritoneum**

Activin A increased invasion of both EECs and ESCs through the modeled peritoneum in a dose-dependent fashion, with the highest invasion rates achieved with the 25 ng/ml concentration. When treated with 25 ng/ml there was an approximate 2-fold increased invasion of EECs and ESCs in presence and absence of activin A alone or in combination with inhibin A or follistatin (Fig. 2). To evaluate the effect of the antagonists to activin A, inhibin A and follistatin, subsequent experiments were performed with activin A (25 ng/ml). The increased rate of invasion induced by activin A treatment was abrogated by both inhibin A and follistatin (Fig. 2).

**Effects of activin A on the attachment of endometrial cells to modeled peritoneum**

Activin A alone and combinations of activin A plus inhibin A or follistatin had no effect on the attachment rates of EECs or ESCs to the LP9 PMC monolayer (Fig. 3).

**Effects of activin A on the in vitro proliferation of endometrial cells**

A proliferation assay for 24 h of EECs and ESCs in presence and absence of activin A alone or in combination with inhibin A or follistatin, demonstrated no significant effect on the rate of proliferation (Fig. 4).
Effects of activin A on N- and E-cadherin mRNA expression in cultured endometrial cells

EECs expressed both E- and N-cadherin mRNA, while ESCs expressed N-cadherin mRNA in control conditions. Epithelial cells treated with activin A demonstrated a 77% decrease in E-cadherin relative gene expression \((P, 0.05, \text{Fig. 5A})\). Concomitant treatment with inhibin A or follistatin did not reverse the effect of Activin A on EEC E-cadherin expression (Fig. 5A).

Treatments with activin A, activin A plus inhibin, A and activin A plus follistatin did not effect N-cadherin mRNA expression in EECs or ESCs (Fig. 5B and C).

Discussion

The present study demonstrates that activin A stimulates transmesothelial invasion of both EECs and ESCs suggesting a possible role in the histogenesis of the endometriotic lesion. In our model, increased numbers of invaded cells could be the result of an increased rate of endometrial cell-PMC attachment, an increased rate of invasion, or both an increased rate of attachment and invasion. That activin A had no effect on the rate of attachment suggests that the increased number of cells on the bottom of the invasion chamber membranes resulted from stimulation of an invasive phenotype. In addition, the increased number of cells seen in our model could have resulted from an increased rate of invasion, stimulation of cell proliferation (i.e. proliferation of cells on the underside of the invasion chamber), or both. Activin A had no demonstrable effect on the rate of cell proliferation over a 24 h time period.

Activin A has been implicated in cell invasion in physiologic processes such as embryo implantation as well as in endometrial cancer. In both cytotrophoblasts and endometrial cells, activin A stimulation induces production of the gelatinases MMP-2 and MMP-9 (Caniggia et al., 1997; Jones et al., 2006). These MMPs degrade type IV collagen (i.e. basement membrane collagen), being implicated in the pathogenesis of endometriosis (Abdallah et al., 2006; Collette et al., 2006).

Activin A has been implicated in the pathogenesis of endometriosis. Both activin A and inhibin A are measurable in PF at concentrations much higher than their respective serum concentrations. However, PF concentrations of activin A and inhibin A are similar in women with and without endometriosis. The mRNAs for \(\alpha\), \(\beta\)A and \(\beta\)B inhibin subunits, and activin type II receptors (ActRI and ActRII) have been found in peritoneum of healthy women and in endometriotic cells (Florio et al., 1998). Furthermore, activin A concentration in endometriotic ovarian cysts is higher than peritoneal concentrations and five times the blood value (Reis et al., 2001). In a recent study, Rombauts et al. (2006) demonstrated that eutopic endometrial cells from women with endometriosis produce more activin A than those from patients without endometriosis.

In the present study, the increased rate of EEC and ESC transmesothelial invasion induced by activin A was diminished by inhibin A.
and follistatin, although the decrease only reached statistical significance in the group of epithelial cells treated with follistatin. While the effect of inter-individual variation cannot be ruled out, the impaired response to follistatin in ESCs compared to EECs might be due to differences in the molecular phenotype exhibited by the cells. Studies have shown that fibronectin, an adhesion molecule that the endometrium expresses only in the stroma (Béliard, 1997), is able to bind follistatin (Maguer-Satta, 2006). Thus, it may be speculated that part of the follistatin added to the ESCs was neutralized by binding to fibronectin.

Inhibin A is present in the endometrium and in the PF (Florio et al., 1998; Petraglia et al., 1998). Follistatin is also produced by the endometrium (Jones et al., 2002). Increased tissue and serum levels of activin A have been reported in women with endometrial cancer (Petraglia et al., 1998; Otani et al., 2001), while the expression of inhibin α subunit is decreased (Mylonas et al., 2004). Thus, it is conceivable that there is a homeostatic balance involving activin A, inhibins and follistatin that affects the invasiveness of EECs and ESCs. Alterations in this homeostasis could contribute to the histogenesis of the endometriotic lesion.

Our results show that cultured EECs express both E- and N-cadherin and ESCs express N-cadherin mRNA. N-cadherin has been identified in endometriotic cysts as well as normal endometrium, mainly in epithelial cells and stromal cells in the proliferative phase (Poncelet et al., 2002; Tsuchiya et al., 2006). Although it is possible that N-cadherin contributes to the establishment of stable contacts between extrauterine endometrial cells and the mesothelial lining (Poncelet et al., 2002), we did not observe any difference in the expression of this cadherin among the groups.

Studies have linked activin to cadherin activity (Ramos et al., 1996; Yoshinaga et al., 2004). Here, we report a decreased E-cadherin mRNA expression in cultured EECs treated with activin A. This mechanism might contribute to the increased invasion rate observed in activin A-stimulated epithelial cells. In fact, the lack of E-cadherin expression by endometrial cells has been associated with increased invasive behavior in vitro (Gaetje et al., 1997; Starzinski-Powitz et al., 1998, 1999; Zeitvogel et al., 2001; Poncelet et al., 2002). Endometriotic implants contain an invasive cell type with the ability to invade collagen gels, which is seldom found in cultures from eutopic endometrium, and the majority of invasive cells from endometriotic lesions are E-cadherin negative (Gaetje et al., 1997). However, other mechanisms are likely to be involved in the pro-invasion effect of activin A, particularly in ESCs, in which the expression of N-cadherin mRNA was not altered by activin A, while E-cadherin has not been detected thus far (Tsuchiya et al., 2006).

In conclusion, our results show that activin A increases invasion of EECs and ESCs into modeled human peritoneum suggesting that this cytokine is able to facilitate the process by which endometrial cells invade the peritoneum to form endometriotic implants. The invasive behavior induced by activin A may be at least partially due to the down-regulation of E-cadherin expression in EECs. Further studies should evaluate whether these effects of activin A differ between

Figure 4: Proliferation rates of endometrial epithelial cells (A) and endometrial stromal cells (B) following treatment with activin A 25 ng/ml alone (Act) or in combination with inhibin A (Act + Inh) or follistatin (Act + Fst). Data are means ± SEM of the proliferation assay absorbance readings.

Figure 5: E-cadherin (A) and N-cadherin (B,C) mRNA expression in endometrial epithelial cells (A, B) and endometrial stromal cells (C) stimulated with activin A 25 ng/ml alone (Act) or in combination with inhibin A (Act + Inh) or follistatin (Act + Fst). Data are means ± SEM of the gene expression adjusted to the internal control S26. *P < 0.05 versus control.
normal cells and eutopic endometrial cells from women with endometriosis, thus providing a comprehensive view of the role of activin-A in the genesis of the endometriotic lesion.

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