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Endometrial cells from women with endometriosis have increased adhesion and proliferative capacity in response to extracellular matrix components: towards a mechanistic model for endometriosis progression

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BACKGROUND: Endometriosis, classified as the presence of endometrial cells in ectopic sites, is a debilitating disease causing pain and infertility in ∼10% of women of reproductive age. It is associated with the aberrant expression of extracellular matrix (ECM) components and their receptors, integrins. METHODS: We analysed the expression of integrins in stromal cells derived from peritoneal, ovarian and deeply infiltrating endometriotic lesions and from endometrium from women with and without endometriosis *in vitro*, using quantitative immunocytochemistry. The adhesive and proliferative capacity of each of the cell types in response to ECM components was assessed by *in vitro* assays of cell attachment and DNA synthesis. RESULTS: We demonstrate that eutopic and ectopic endometrial stromal cells from women with endometriosis exhibit an aberrant integrin profile *in vitro* compared with stromal cells derived from healthy controls. In addition, the former display increased adhesion and proliferative capacity in response to specific ECM components. CONCLUSIONS: We propose that the increased adhesive and proliferative potential of cells from endometriotic lesions may be a key feature in the pathogenesis of endometriosis. Furthermore, the elevated responsiveness of eutopic cells from women with endometriosis may contribute to the predisposition of some women to the disease.

Keywords: extracellular matrix; proliferation; endometrium; endometriosis

Introduction

Endometriosis represents a major healthcare problem which causes pain and infertility to ∼7–10% of women, and for which there is no effective medical treatment. It occurs mostly as, in order of prevalence, peritoneal surface lesions, ovarian lesions and deeply infiltrating lesions of the rectovaginal septum or gut. Endometriotic lesions are commonly defined by the presence of both endometrial-like stromal and epithelial cells, but cases of only stromal endometriosis have also been reported (Clement *et al.*, 1990). The pathogenesis of endometriosis is not clearly understood. The most commonly held theory is that exfoliated menstrual endometrial cells attach to the peritoneal surface, and their subsequent proliferation and invasion into the underlying tissue results in endometriotic lesions (Witz *et al.*, 1999,2001b; Nisolle *et al.*, 2000).

The extracellular matrix (ECM) has a fundamental role in diverse cell processes. Endometrial cell adhesion and proliferation that occurs in the peritoneal environment in the pathogenesis of endometriosis is likely to be modulated by the interaction of ECM components with integrin receptors. It is well established that the proliferative capacity of many cell types is strongly influenced by the ECM. For example, activation of integrins by ECM components contributes to cyclin activation thereby regulating cell cycle entry (Lee and Juliano, 2004; Hodkinson *et al.*, 2006).

Endometriotic lesions exhibit some of the characteristics of tumours. Indeed the presence of endometriotic cells in lymph nodes of women with deeply infiltrating lesions brings into question whether or not endometriosis is a clinically benign disease (Abrao *et al.*, 2006). Previous studies have shown that ovarian cancer cells attach to the mesothelial monolayer, causing disruption of the mesothelium and exposing the underlying ECM, providing a substrate for cell attachment, invasion and subsequent proliferation (Sawada *et al.*, 1994; Gardner *et al.*, 1995). Similarly, endometrial cells adhere rapidly to mesothelium (Witz *et al.*, 2001b) and appear to invade the

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mesothelium via degradation of the underlying peritoneal ECM (Spuijbroek *et al.*, 1992; Witz *et al.*, 2003). Peritoneal mesothelial cells express ECM components at their surface, which facilitate the adhesion of ovarian cancer cells and endometrial cells to the peritoneum, and this is in part mediated through integrins (Lessan *et al.*, 1999; Strobel and Cannistra, 1999; Dechaud *et al.*, 2001). Both the mesothelial and endometrial ECMs contain collagen type IV, laminin, vitronectin and fibronectin (Witz *et al.*, 2001a). The ECM of the stroma of endometriotic lesions (Aplin *et al.*, 1988; Beliard *et al.*, 1997; Harrington *et al.*, 1999) is similar but not identical to that of the endometrial stroma. Tenascin-C, which is mostly present in the endometrium at the proliferative stage of the menstrual cycle is the only ECM component reported so far to be aberrantly expressed in endometriosis in that it is elevated in peritoneal surface and ovarian lesions (Harrington *et al.*, 1999).

Previous studies have demonstrated that the plasminogen activator system and matrix metalloproteinases (MMPs) are elevated in the endometrium from women with endometriosis. This could result in the enhanced proteolytic potential of endometrial fragments undergoing retrograde menstruation, increasing invasiveness and facilitating development of endometriotic foci (Sillem *et al.*, 1997; Gilabert-Estelles *et al.*, 2003). In addition, menstrual endometrium is reported to synthesize relatively high levels of MMP-3 (Koks *et al.*, 2000b), and the corresponding cleavage products, procollagen type III proteolytic fragments, are elevated in the peritoneal fluid (PF) of women with endometriosis compared with the levels observed in PF from women without the disease (Spuijbroek *et al.*, 1992).

Thus ECM and ECM turnover may be important modulators of ectopic endometrial cellular processes that occur in the peritoneal environment in the pathogenesis of endometriosis. The experiments reported here were designed to investigate the potential of ECM components to modulate the adhesive and proliferative capacity *in vitro* of stromal cells derived from endometriotic lesions and from endometrium from women with and without endometriosis. Stromal cells were exposed to immobilized or soluble ECM components to mimic conditions in the peritoneal environment in which ECM might be presented to ectopic endometrial cells, either as a result of mesothelial injury (immobilized) or as components of PF (soluble). We demonstrate that specific ECM components modulate the adhesive and proliferative capacity of eutopic and ectopic endometrial cells from women with endometriosis.

Material and Methods

Tissue samples

All tissue samples were obtained with informed consent in accordance with the requirements of the Oxfordshire Research Ethics Committee. Samples of endometriotic peritoneal surface lesions ($n = 8$), ovarian lesions ($n = 10$) and deeply infiltrating lesions ($n = 8$) were obtained from women aged 21–48 years undergoing laparoscopy for pain or other benign indications. Endometrium at different stages of the menstrual cycle was obtained from fertile women (aged 20–49 years) with ($n = 5$; 1 proliferative and 4 secretory stage) or without ($n = 5$; all secretory stage) endometriosis, with the latter comprising the control group. The samples were obtained by pipelle biopsy during diagnostic laparoscopy or sterilization or by endometrial curettage of the bisected uteri obtained at hysterectomy for benign indications. The absence of visible endometriosis in the control group was confirmed by the surgeon performing the operation. None of the women had received hormonal medication in the preceding three months.

Isolation and culture of eutopic and ectopic ESCs

Endometriotic tissue was dissected away from the adjacent host tissue, and diagnosis was confirmed by histological examination. Endometriotic and endometrial tissues were processed for (ectopic and eutopic) endometrial stromal cell (ESC) culture by collagenase digestion and purification through a percoll gradient as described previously (Chobotova *et al.*, 2002). The purified ESCs were plated into 75 cm^2 tissue culture flasks (10^6 cells per flask) and maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Paisley, UK) supplemented with 10% heat-inactivated fetal bovine serum (FCS, Autogen Bioclear Ltd, Calne, UK) and 50 IU/ml–50 μg/ml penicillin–streptomycin (Invitrogen) (DMEM complete). Cultures were maintained at 37°C in a humidified environment with 5% CO_2 in air unless stated otherwise. The ESCs were used between passages 2 and 6.

Cytospins and characterization of cell lines

Cytospins (Shandon Southern Products Ltd., Runcorn, UK) of eutopic and ectopic ESCs were prepared at derivation and at passages 2, 4, 6 and 10 to assess expression of integrin levels with time in culture. The purity of the isolated ESCs was assessed by staining with a panel of markers including THY-1, vimentin, cytokeratin 18, CD45, CD68, CD10 and progesterone and estrogen receptors. The percentages of THY-1-and vimentin-positive cells were ~98 and 99%, respectively, for endometriotic stromal cells and 84 and 92%, respectively, for ESCs, with <2% contamination by epithelial and bone-marrow derived cells as detected by cytokeratin 18, CD45 and CD68, respectively, and as quantified in cytospins; this was consistent with our previous report (Klemmt *et al.*, 2006). The cell lines derived from all three types of lesion and endometrium were positive for CD10, and estrogen and progesterone receptors, and these markers were expressed consistently and at the same level with passage and irrespective of the stage of the cycle at which they were derived, as we have shown previously (Klemmt *et al.*, 2006).

A panel of integrin antibodies (as indicated in Table 1) was used to detect expression of integrins at different passages using the Alkaline Phosphatase-Anti-Alkaline Phosphatase staining method (Dako, Ely, UK) following the manufacturer's instruction. Cells (400 in each cytospin) were scored for positive or negative staining and the results were expressed as percentage positive.

Cell attachment assays

Microtitre plates were coated in triplicate with 10 μg/ml fibronectin (human plasma, Sigma, Poole, UK), collagen type I (rat tail, Roche, Lewes, UK), collagen type IV (human placenta, Sigma), laminin (human placenta, Sigma), vitronectin (human plasma, Becton Dickinson, Oxford, UK) or tenascin-C (human tumour cell line, Chemicon, Hampshire, UK) in phosphate-buffered saline (PBS) and incubated overnight at 4°C. Wells were washed twice with 100 μl sterile PBS, blocked with 100 μl 0.05% milk-powder in PBS for a minimum of 2 h at room temperature, and washed with PBS.

Subconfluent, human eutopic and ectopic ESCs were dissociated with 1 × trypsin-EDTA, followed by inhibition with DMEM containing 1 mg/ml soybean trypsin inhibitor (both from Sigma). Cells were washed in PBS and seeded in ECM-coated 96-well plates at a

Table I. Details of antibodies.

Marker	Clone	Working dilution, $\mu g/ml$	Source	Reference
Integrin α1	TS2/7	10	Serotec	Grosskinsky *et al*. (1996), Koks *et al.* (2000a)
Integrin α4	9F10	10	PharMingen Int.	
Integrin α5	P1D6	10	Chemicon	Grosskinsky *et al*. (1996)
Integrin α6	4F10	10	Serotec	Hwang *et al*. (2002)
Integrin β1	3S3	10	Chemicon	Gao *et al*. (1995)
Integrin αvβ3	LM609	10	Chemicon	Koks *et al.* (2000a)
Vinculin	hVIN-1	1:400	Sigma	Chen *et al*. (2003)
Mouse IgG	2T8-2F5	10	Coulter	

density of 1×10^4 cells per well in 100 μl serum-free DMEM. Cells were incubated for 2 h at 37°C in a humidified environment with 5% CO_2 in air, washed and fixed in 100 μl 4% glutaraldehyde/4% formaldehyde in PBS for 20 min at room temperature. Cell attachment was quantified by measurement of incorporation of crystal violet as described previously (Mardon and Grant, 1994). The results were expressed as the percentage attachment relative to attachment of ESCs from women without endometriosis.

Immunocytochemistry

Immunocytochemistry was performed according to a protocol described previously (Chobotova *et al.*, 2002). Briefly, sterile 13 mm diameter glass coverslips size 0 (Chance Proper, Birmingham, UK), in 4-well plates, were coated overnight with 10 μg/ml ECM components in PBS and non-specific antibody-binding sites were blocked with 1% bovine serum albumin for 1 h at room temperature. Eutopic and ectopic ESCs were seeded at a density of 6×10^3 cells per well in 500 μl serum-free DMEM (Invitrogen) and incubated for 2 h at 37°C. Coverslips were incubated in 1:400 dilution anti-vinculin antibodies (Sigma) or mouse IgG, followed by incubation with 15 μg/ml of donkey anti-mouse fluorescein isothiocyanate-conjugated IgG (Jackson ImmunoResearch Laboratories Inc., PA, USA) and 5 U/ml of fluorescein-phalloidin (Invitrogen) to visualize polymerized actin. Staining was assessed using a Leitz DMRBE microscope (Leica Microsystems, Werzlar, Germany) and Openlab imaging software (Improvision, Coventry, UK).

DNA synthesis assays

DNA synthesis assays were performed with a modification of a method described previously (Atkinson *et al.*, 1996). Subconfluent human eutopic and ectopic ESCs were serum-starved overnight and dissociated as described for cell adhesion assays. Assays were performed in medium containing phenol-red free DMEM (Invitrogen) supplemented with 2 mM L-glutamine, 50 IU/ml penicillin–streptomycin solution (Sigma) and 0.2% FCS. Cells were washed in PBS and seeded into either (i) ECM-coated 96-well plates at a density of 1×10^4 cells per well in 100 μl medium; (ii) uncoated 96-well plates in 100 μl medium followed by the addition of 10 μl 100 μg/ml ECM components (selected and sourced as for cell attachment assays, above) in triplicate or (iii) uncoated 96-well plates for 24 h in DMEM complete, followed by serum-free DMEM for 24 h, followed by medium containing 10 μg/ml ECM components. In each experiment DNA synthesis was determined after 24 h by the use of 5-bromo-2′-deoxyuridine incorporation with ELISA (Roche) following the manufacturer's instructions. The optical density measurements were normalized to that in the control (no ECM present) and expressed as percentage relative to the proliferation of ESCs from women without endometriosis, and of ESCs cultured in the absence of ECMs.

Statistical analyses

The data were assessed by one-way analysis of variance followed by either Tukey's Multiple Comparison post-test or Dunnett's Multiple post-test where applicable. Differences with an α level of <0.05 were considered significant.

Results

Integrins are aberrantly expressed in cultured endometriotic stromal cells

The expression levels of integrins in the endometrium and in endometriotic tissues are different *in vivo*. We investigated the *in vitro* expression and sustained retention of integrin subunits in ESCs over time in culture using quantitative immunocytochemistry of ESC cytospins at different passages (Fig. 1). The proportion of cells positive for integrin subunit α4 was significantly lower for stromal cells derived from peritoneal surface and ovarian lesions compared with control ESCs. The percentage of integrin α6-positive cells was significantly lower in cytospins of cells derived from all three types of endometriotic lesion compared with control ESCs reflecting the comparable levels of integrin α6 previously recorded in these tissues *in vivo* (Rai *et al.*, 1996). The profile of integrin expression for all types of ESCs was retained with passaging. Our results demonstrate that endometriotic stromal cells display an aberrant integrin profile compared with control ESCs, and this is largely retained during culture *in vitro* up to passage 10.

Endometriotic and ESCs from women with endometriosis display increased attachment on ECM components compared with cells from controls

ESCs are anchorage-dependent and adhesion of both shed menstrual cells in the pelvic cavity and the cells in endometriotic lesions is likely to be important in the development and progression of the lesion. We compared the adhesive capacity of stromal cells isolated from established endometriotic lesions with ESCs from women with (two from proliferative and three from secretory stages of the cycle) and without (one from proliferative and five from secretory stages of the cycle) endometriosis on immobilized ECM components (Fig. 2A).

ESCs from deeply infiltrating lesions exhibited an adhesive capacity similar to control ESCs on all ECM components, whereas ESCs from ovarian lesions exhibited a 2.5–3-fold increase in attachment to all the ECM components compared with control ESCs. Stromal cells derived from peritoneal surface lesions exhibited a 1.5–2-fold increase in attachment

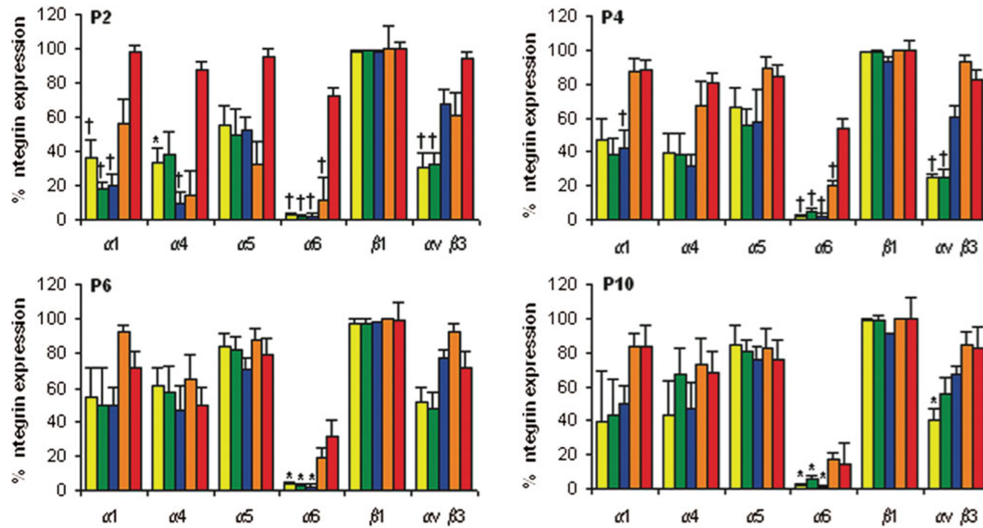

Figure 1: Integrin profile of cultured stromal cells
Quantitative immunocytochemistry of integrin expression in eutopic and ectopic ESCs at passages 2, 4, 6 and 10. Data are expressed as percentage positive cells and bars represent mean ± SEM. Integrin expression was similar in the three different endometriotic lesions throughout passaging with loss of α6 in endometriotic versus control ESCs (*$P < 0.05$, †$P < 0.001$). ESCs were derived from peritoneal surface lesions (yellow, $n = 8$); deeply infiltrating lesions (green, $n = 5$); ovarian lesions (blue, $n = 10$); endometrium from women with endometriosis (orange, $n = 5$) and endometrium from control group (red, $n = 5$). P, passage number of cells

to fibronectin and collagens type I and IV compared with control ESCs. Cells from women with endometriosis exhibited a 1.5-fold increase of attachment to fibronectin, collagen type I, collagen type IV and vitronectin. The most striking observation in these experiments was a 3.5-fold increase in adhesive capacity of ESCs from women with endometriosis on tensascin-C, compared with control ESCs.

We performed immunocytochemistry on eutopic and ectopic ESCs adherent to ECM components to investigate whether the difference in adhesive capacity was reflected in focal contact assembly (Fig. 2B). ESCs derived from all three types of endometriotic lesions exhibited similar results thus ovarian lesion ESCs are shown as a representative. The cell shape, focal contact assembly and actin cytoskeleton were similar in eutopic and ectopic ESCs cultured on fibronectin, laminin and vitronectin (Fig. 2B, a–i). Focal contacts had assembled between 60 and 90 min after adhesion to ECM components in all ESCs. Vinculin was localized in focal contacts clustered in filopodia that were connected by actin stress fibres. However, ESCs derived from ovarian lesions and from the endometrium of women with endometriosis displayed cell spreading and formation of focal contacts on tenascin-C,

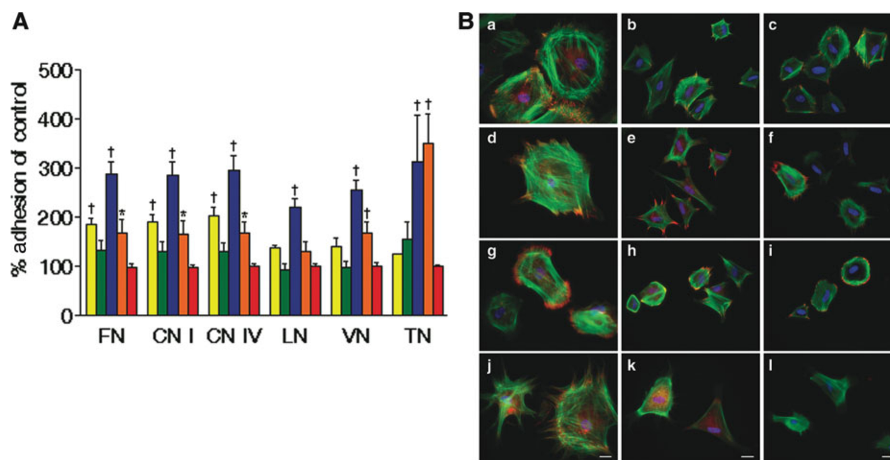

Figure 2: Quantitative and qualitative analyses of adhesion of eutopic and ectopic ESCs to ECM components
(**A**) Percentage attachment compared with control ESCs (100% attachment). Bars represent mean ± SEM, and *$P < 0.05$ and †$P < 0.01$. ESCs were derived from peritoneal surface lesions (yellow, $n = 6$); deeply infiltrating lesions (green, $n = 8$); ovarian lesions (blue, $n = 8$); endometrium from women with endometriosis (orange $n = 5$) and endometrium from control group (red, $n = 5$). FN, fibronectin; CN I and IV, collagens I and IV; LN, laminin; VN, vitronectin; TN, tenascin-C. (**B**) Ovarian lesion ESCs (a, d, g, j) and eutopic ESCs derived from women with endometriosis (b, e, h, k) and without endometriosis (c, f, i, l) adherent to fibronectin (a–c), laminin (d–f), vitronectin (g–i) or tenascin-C (j–l) stained for vinculin (red) and actin (green)

while control ESCs on tenascin-C displayed more diffuse pericellular distribution of vinculin (Fig. 2B j–l).

The proliferative capacity of ESCs derived from women with endometriosis is elevated in response to immobilized ECM components

We predict that the progression of endometriotic lesions involves proliferation of ESCs subsequent to their attachment to the peritoneal tissue. We investigated the effect of the ECM on proliferation of ESCs. First, we analysed DNA synthesis in eutopic and ectopic ESCs in response to immobilized ECM components. The results were expressed as the percentage of control ESCs for each ECM component (Fig. 3A), or the percentage of cells in the absence of ECM for each cell type (Fig. 3D). The DNA synthesis in ESCs derived from peritoneal surface lesions was significantly reduced in response to immobilized fibronectin, collagen type I and collagen type IV compared with control ESCs. ESCs isolated from ovarian lesions exhibited a 2–4-fold increase in DNA synthesis on fibronectin, laminin, vitronectin and tenascin-C compared with control ESCs. DNA synthesis in ESCs from women with endometriosis displayed a 1.5–3-fold increase in DNA synthesis when plated on collagen type IV, laminin, vitronectin or tenascin-C compared with control ESCs.

The effect of ECM on DNA synthesis was analysed by comparison of ESCs cultured in the presence and absence of ECM (Fig. 3D). All six ECM components induced an increase in DNA synthesis in ESCs from women with endometriosis. Immobilized fibronectin, laminin and vitronectin resulted in >4-fold increase in DNA synthesis in ESCs from ovarian lesions and women with endometriosis compared with ESCs in uncoated wells (Fig. 3D). Interestingly, ESCs from deeply infiltrating lesions did not show modulation of DNA synthesis in response to any of the ECM components, and ESCs from peritoneal lesions only responded to laminin.

DNA synthesis in ESCs from ovarian and deeply infiltrating lesions and from women with endometriosis is elevated in response to soluble ECM components

Soluble ECM components that are present in PF were added to freshly plated ESCs (Fig. 3B and D). Compared with control ESCs, the addition of soluble fibronectin, collagen type I and collagen type IV to ESCs from peritoneal lesions resulted in lower levels of DNA synthesis, whereas deeply infiltrating ESCs were not modulated by these ECMs. There was a 2-fold increase in DNA synthesis in ESCs derived from women with endometriosis in response to both vitronectin and laminin, and a 2-fold increase in ESCs from ovarian lesions in response to soluble vitronectin only.

In comparison to ESCs cultured in the absence of soluble ECMs (Fig. 3D), all cell types responded to laminin with an increase in DNA synthesis of between 1.5- (peritoneal lesion ESCs) to >4- fold (ESCs derived from ovarian lesions). Similar to laminin, a 2 >4-fold increase in DNA synthesis in response to vitronectin is observed in all cell types with the exception of peritoneal lesion ESCs. Furthermore eutopic ESCs exhibited a 2-fold increase in DNA synthesis in response to fibronectin and ESCs from women with endometriosis additionally responded with a 3-fold increase of DNA synthesis to collagen type IV.

Adherent ESCs from women with endometriosis exhibit elevated DNA synthesis in response to soluble collagen types I and IV and laminin

We explored the possibility that soluble ECM components present in the peritoneal cavity may provide a proliferative stimulus to stromal cells already adherent in the peritoneum (Fig. 3C). Compared with control ESCs, cells from women with endometriosis exhibited a 2-fold increase in DNA synthesis in response to soluble collagen types I and IV, and laminin, and to a lesser extent to tenascin-C (Fig. 3C).

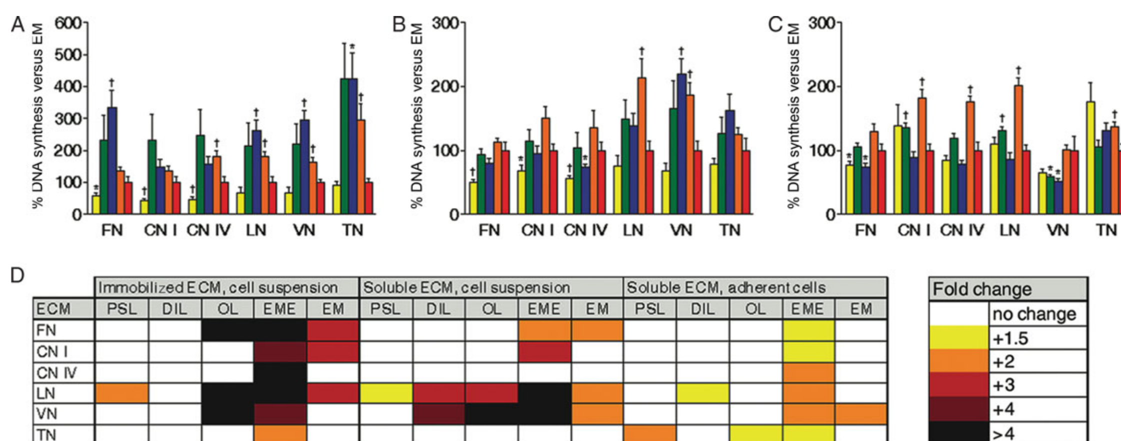

Figure 3: Proliferative capacity of freshly plated and adherent eutopic and ectopic ESCs in response to ECM components
DNA synthesis in freshly plated eutopic and ectopic ESCs that were exposed to (**A**) immobilized and (**B**) soluble ECM components, or (**C**) adherent stromal cells that were exposed to soluble ECM components. Data are normalized to the proliferation rate of ESCs observed in uncoated control wells, and expressed as percentage of control ESCs in each experimental setting. Bars represent mean ± SEM, with $*P < 0.05$ and $†P < 0.01$. The fold changes in proliferative capacity of ESCs normalized to uncoated control wells are displayed in (**D**). ESCs were derived from peritoneal surface lesion (PSL) (yellow, $n = 5$); deeply infiltrating lesion (DIL) (green, $n = 5$); ovarian lesion (OL) (blue, $n = 5$); endometrium from women with endometriosis (EME) (orange, $n = 5$) and endometrium from control group (EM) (red, $n = 3$). FN, fibronectin; CN I and IV, collagens I and IV; LN, laminin; VN, vitronectin; TN, tenascin-C

Deeply infiltrating ESCs exhibited a <1.5-fold increase in DNA synthesis in response to collagen type I and laminin, whereas the addition of soluble vitronectin to these ESCs and ESCs derived from ovarian lesions resulted in a significant reduction of DNA synthesis relative to control ESCs.

The addition of soluble ECM components to most types of ESCs did not affect levels of DNA synthesis compared with the ESCs cultured in the absence of exogenous soluble ECM components. The exception to this was ESCs from women with endometriosis, which exhibited a 1.5–2-fold increase in response to all six ECM components. Control ESCs also exhibited a 2-fold increase in response to vitronectin, and addition of tenascin-C to adherent peritoneal surface and ovarian lesion ESCs resulted in a 2- and 1.5-fold increase, respectively, compared with the same cells in the absence of ECM.

Discussion

Endometriosis represents a major therapeutic challenge, largely because the aetiology of the disease remains unknown. In this study, we investigate the function of the ECM in the adhesion and proliferation events that occur during the pathogenesis of the disease. We demonstrate that, in comparison to ESCs derived from fertile, healthy controls (i) ESCs derived from three different types of endometriotic lesions exhibit an aberrant integrin profile, that is sustained in *in vitro* culture; (ii) stromal cells derived from peritoneal surface and ovarian lesions and from the endometrium of women with endometriosis exhibit an increased adhesive phenotype on ECM components and (iii) stromal cells derived from ovarian and deeply infiltrating lesions and from the endometrium of women with endometriosis have an increased proliferative phenotype in response to specific ECM components.

Research into the underlying molecular and cellular mechanisms of endometriosis is impeded because there are few robust experimental models available. The occurrence of endometriosis is highly, although not exclusively, dependent on menstruation since the disease is restricted to humans and subhuman primates and therefore primate models are ideal, although problematical. Rodent models can be very informative but inevitably have a limited application to the pathophysiology in humans. The manipulation of the cell culture system that we describe here is one of the few relevant experimental *in vitro* models available for the study of endometriosis (Klemmt *et al.*, 2006).

There is evidence to suggest that both the stromal and epithelial cell subtypes from endometrium adhere to amnion and peritoneum (van der Linden *et al.*, 1998; Lucidi *et al.*, 2005). Others have shown that the ESCs invade the peritoneal mesothelium (Witz *et al.*, 2002), thus providing a putative mechanism for establishing endometriotic lesions. Other investigators have reported that the adhesion of clumps of menstrual endometrial cells is dependent upon the presence of structurally intact integrins (van der Linden *et al.*, 1998; Koks *et al.*, 2000a). It is thus important that we were able to demonstrate that cultured stromal cells retain integrin expression *in vitro*, validating the use of cultured ESCs as a model for investigating the function of these molecules in endometriosis. The integrin profile of endometriotic stromal cells from peritoneal surface and ovarian lesions we observe in this study is comparable to that reported previously for stroma in the lesions *in vivo* (Lessey *et al.*, 1994; van der Linden *et al.*, 1994; Lessey and Castelbaum, 1995; Rai *et al.*, 1996). In addition, we have delineated a similar integrin profile for stromal cells from deeply infiltrating lesions of the recto-vaginal septum.

Previous findings suggest that both immobilized and soluble ECM components in the peritoneal environment may influence the capacity of endometrial cells to form endometriotic lesions. Our rational for testing stromal cell lines derived from endometriotic lesions, as well as endometrium, for their adhesive capacity, and for the effect of soluble ECM components on DNA synthesis was 4-fold. First, the adhesive capacity of the cells within a lesion on different ECMs will directly influence processes that are critical in the pathogenesis and growth of a lesion: these include cell proliferation, differentiation, cell migration and invasion and apoptosis. Second, some cells within the lesions are exposed to soluble ECMs that are present in the PF, thus the effect of such factors will similarly influence cell behaviour. Third, it is possible that cells escape the lesions and seed new lesions at other sites within the peritoneal cavity, being exposed along the way to these factors. Fourthly, soluble ECM will be generated in the local microenvironment within the lesions during the process of invasion. Finally, the development of new-targeted treatments for endometriosis will require further knowledge of the adhesive and proliferative properties of cells in established lesions.

Peritoneal (Witz *et al.*, 2001a) and ovarian (Smith *et al.*, 2002; Rodgers *et al.*, 2003) ECM contains collagen type I and type IV, fibronectin and laminin. These substrates may be exposed as a result of mesothelial injury, or after rupture of the follicle in the ovary, thus potentially providing sites for primary attachment of menstrual endometrial cells. The follicular fluid also contains soluble proteolytic fragments of ECM proteins such as laminin, collagen type IV, versican and hyaluronan, which are released into the peritoneal cavity during ovulation (Rodgers *et al.*, 2003). These molecules may thus influence the adhesive or proliferative behaviour of ESCs that arrive in the peritoneal cavity as a result of retrograde menstruation. The attachment and proliferation assays we describe here were designed to investigate these hypotheses (as shown in the scheme depicted in Fig. 4).

Our data indicate that, in comparison to control ESCs, stromal cells derived from peritoneal and ovarian lesions and eutopic ESCs from women with endometriosis are significantly more adhesive. Notably, ESCs from ovarian lesions have increased adhesive capacity to all ECM components tested, and eutopic ESCs from women with endometriosis are much more adhesive on tenascin-C. It is interesting to note that the levels of tenascin-C are higher in the stroma of ovarian lesions compared with endometrium (Harrington *et al.*, 1999). The formation of ovarian lesions requires dramatic tissue remodelling accompanied by local inflammatory reactions in which tenascin-C may have an important function (Chiquet-Ehrismann and Chiquet, 2003). Interestingly, stromal cells derived from deeply infiltrating lesions and normal endometrium exhibit similar adhesive capacities on

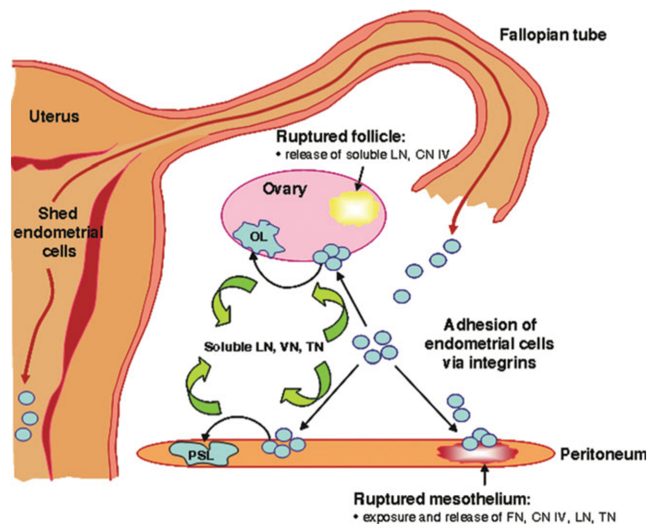

Figure 4: Model for the interaction of ECM components with endometrial cells in the pathogenesis of endometriosis
Cells, or clumps of cells from endometrium are retrogradely transported through the fallopian tubes into the pelvic cavity. Endometrial cells in suspension in the PF adhere to exposed ECM following mesothelial injury. Soluble, or proteolytic fragments of ECM in the PF modulate the proliferative capacity of endometrial cells either in the PF, adherent on the peritoneal lining, or already established in endometriotic lesions

all the ECM components tested. The fact that the process of invasion, involving transient and weaker interactions with ECM, is a prominent feature of deeply infiltrating lesions may be relevant to this observation.

Integrin-mediated cell adhesion to ECM ligands promotes both cell survival and also cell cycle progression. Fibronectin, collagen type I and type IV have been shown to increase the proliferation rate of various cell types (Atkinson *et al.*, 1996; Chamoux *et al.*, 2002; Kijima *et al.*, 2003). Previous reports have demonstrated that endometriotic lesions exhibit an enhanced capacity to proliferate *in vivo* compared with endometrium from women without endometriosis (Li *et al.*, 1993; Nisolle *et al.*, 1997).

Our data suggest that ESCs isolated from ovarian lesions and deeply infiltrating endometriosis exhibit relatively high proliferative capacity both on immobilized ECM and in the presence of soluble ECM components compared with those isolated from peritoneal surface lesions and eutopic, normal endometrium. Specifically, the ESCs from the peritoneal surface lesions display a reduction in DNA synthesis in response to fibronectin, collagen type I and type IV compared to control ESCs. This is in accordance with the observation that the stromal compartment of red and black peritoneal lesions have fewer proliferating cells compared with ovarian lesions *in situ* (Nisolle *et al.*, 1997; Scotti *et al.*, 2000).

Conversely, the proliferative capacity of cells from women with endometriosis was consistently higher than control ESCs in response to both immobilized and soluble ECM components laminin, collagen type IV, vitronectin and tenascin-C in all three experimental settings, indicating that the microenvironment is critical for proliferation of these cells. This observation is in agreement with the previous reports that cell proliferation is increased in the endometrium of women with endometriosis (Wingfield *et al.*, 1995; Nisolle *et al.*, 1997; Meresman *et al.*, 2002).

Laminin has been shown to advance the malignant phenotype of most cancer cells and stimulate tumour growth by promoting cell proliferation and protease activity (Patarroyo *et al.*, 2002). Similarly, vitronectin and tenascin-C stimulate the proliferation and motility of mammary, endometrial and ovarian cancer cells (Chiquet-Ehrismann *et al.*, 1986; Yamanaka *et al.*, 1996; Huang *et al.*, 2001; Hapke *et al.*, 2003). Thus our results lend support to the notion that the development of endometriotic lesions may have features in common with those of invasive cancer (Koninckx *et al.*, 1999).

The concentration of cellular ECM components in the circulation varies according to physiological status, but ranges from 0.4 to 2 μg/ml in healthy individuals and can reach up to 7 μg/ml in patients (Ylatupa *et al.*, 1995; Katayama *et al.*, 2003; Castellanos *et al.*, 2004). The release of intact and progressively fragmented laminin, collagen type IV, fibronectin and tenascin through proteolytic degradation into the circulation also occurs in inflammatory diseases and tumour invasion (Ylatupa *et al.*, 1995; Riedle and Kerjaschki, 1997; Davel *et al.*, 1999; Katayama *et al.*, 2003; Castellanos *et al.*, 2004). Fibronectin and vitronectin are circulating in the plasma at much higher levels (~300 μg/ml). A concentration of 10 μg/ml of soluble ECM components was chosen in the experiments we report here, however, we acknowledge that the concentrations of different ECM components is likely to vary physiologically.

Guidice and Kao (2004) proposed a scheme for some of the events that occur in the peritoneal environment to mediate the establishment of an endometriotic lesion. The data we present in this study suggest that specific ECM components may have an important function in the onset and progression of endometriotic lesions and thus in the pathogenesis of endometriosis. We propose that retrogradely flushed menstrual endometrial cells interact with ECM components present in the peritoneal cavity in both soluble and insoluble forms (Fig. 4; Guidice and Kao, 2004). The establishment of an endometriotic lesion is then likely to be mediated by the exposure of ECM substrates in the peritoneal cavity, and extensive protease-mediated remodelling of the ECM as the lesion progresses. This model now provides us with a basis for the further dissection of the molecular mechanisms mediating endometriosis.

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