Human Reproduction vol.13 no.4 pp.1030–1035, 1998

Endometrial vascular and glandular expression of integrin $\alpha_v\beta_3$ in women with and without endometriosis

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The integrin $\alpha_v\beta_3$ functions in both cell–cell and cell–extracellular matrix adhesion, and has reported roles in platelet aggregation, immune function, tissue repair, tumour invasion, angiogenesis and uterine receptivity. The aim of this study was to use immunohistochemistry to describe the vascular and glandular expression of integrin $\alpha_v\beta_3$ in formalin fixed, paraffin embedded endometrium obtained from women with ($n = 29$) and without ($n = 24$) endometriosis. The results showed a significant increase in the percentage of vessels expressing $\alpha_v\beta_3$ in the endometrium of women with endometriosis compared with controls ($P = 0.0001$). This difference was more pronounced in the secretory phase ($P = 0.001$) than the proliferative phase ($P = 0.016$). There was no correlation between vascular $\alpha_v\beta_3$ expression and the endothelial cell proliferation index ($P > 0.05$). Vascular sprouts were not observed in any of the 53 endometrial tissues obtained from women with or without endometriosis throughout the menstrual cycle. Results from semi-quantitative scoring of gland immunostaining showed that neither controls ($P = 0.3329$) nor the endometriosis group ($P = 0.2260$) had any significant changes in terms of $\alpha_v\beta_3$ expression between the different stages of the menstrual cycle. There was also no difference in glandular $\alpha_v\beta_3$ expression between women with and without endometriosis ($P = 0.4302$). These results provide evidence for increased endometrial angiogenesis in women with endometriosis compared with controls, and suggest that glandular expression of $\alpha_v\beta_3$ is not related to uterine receptivity *per se*.

Key words: angiogenesis/endometriosis/endometrium/integrin $\alpha_v\beta_3$/uterine receptivity

Introduction

The integrins are a family of cell adhesion molecules that function in both cell–cell and cell–substratum adhesion. They are heterodimeric glycoproteins consisting of non-covalently associated α and β subunits (Hynes, 1987; Hemler, 1990). Integrins have been shown to bind to a specific amino acid sequence Arg–Gly–Asp (RGD), and to RGD related sequences found in ligands (Ruoslahti and Pierschbacher, 1987; Albelda and Buck, 1990).

Integrin $\alpha_v\beta_3$ is found on many cell types, including endothelial cells (EC), and is known to bind at least seven different ligands; vitronectin, fibrinogen, von Willebrand factor, fibronectin, thrombospondin, laminin and thrombin (Dejana *et al.*, 1993; Luscinskas and Lawler, 1994). Recent studies have shown that the ligation of integrin $\alpha_v\beta_3$ was required for the survival and maturation of newly formed blood vessels, with expression increasing four-fold during angiogenesis on chick chorionallantoic membrane (Brooks *et al.*, 1994a). The growth of new blood vessels induced by basic fibroblast growth factor (bFGF), tumour necrosis factor-α (TNF-α) and human melanoma fragments was blocked by monoclonal antibody (mAb) to $\alpha_v\beta_3$, but the mAb had no effect on pre-existing vessels (Brooks *et al.*, 1994b; Friedlander *et al.*, 1995). Other studies also support a role for integrin $\alpha_v\beta_3$ as an angiogenic marker. Sepp *et al.* (1994) showed that the expression of $\alpha_v\beta_3$ integrin on human microvascular EC could be increased by bFGF. Enenstein *et al.* (1994) documented that the αv subunit concentration, which was normally low in the microvasculature, was increased on angiogenic vascular sprouts. A recent study has provided evidence that integrin $\alpha_v\beta_3$ colocalizes with matrix metalloproteinase (MMP)-2, a family of zinc requiring matrix-degrading enzymes, on angiogenic blood vessels *in vivo* (Brooks *et al.*, 1996).

The process of angiogenesis involves a sequence of steps that includes EC activation, basement membrane breakdown, EC migration and proliferation to form sprouts, and sprout fusion and tube formation (Furcht, 1986). However, it has been shown that vascular sprouting can occur without proliferation of EC or *vice versa* (Ausprunk *et al.*, 1974; Sholley *et al.*, 1984; Nagy *et al.*, 1995; Vernon *et al.*, 1995). Previous work performed in our laboratory demonstrated two significant peaks of the EC migratory signal in endometrial explants, one during the early proliferative phase and the other during the mid–late proliferative phase of the normal human menstrual cycle (Rogers *et al.*, 1992). Subsequent studies (Goodger and Rogers, 1994) found that endometrial EC proliferation did not follow a consistent pattern across the menstrual cycle, and that endometrial EC migratory signal production did not correlate with EC proliferation. Furthermore, in rat and human endometrial EC proliferation studies (Goodger and Rogers, 1993, 1994; Macpherson and Rogers, 1995), vascular sprouts were not observed but several EC in the same vessel profile were seen to be proliferating concurrently. A recent study (Wingfield *et al.*, 1995) showed a significant increase in the endometrial EC proliferation index in women with endometriosis (12.9 ± 2.6%) compared with controls (4.0 ± 3.2%).

Many members of the integrin family are expressed in the endometrium during the menstrual cycle. It has been reported

Table I. Total number (*n*) of endometriosis and control endometrial tissues at each menstrual stage for vascular (V) and glandular (G) studies

		M	EP	EMP	MP	LP	ES	MS	LS	Total
Endometriosis										
[V]	(*n*)	3	4	5	6	2	2	2	5	29
[G]		2	4	5	5	2	2	2	5	27
Control										
[V & G]	(*n*)	2	3	1	4	1	7	4	2	24

M, menstrual; EP, early proliferative; EMP, early-mid proliferative; MP, mid proliferative; LP, late proliferative; ES, early secretory; MS, mid secretory; LS, late secretory.

that integrin α1, α4 and β3 subunits show spatial and temporal variation in glandular and luminal epithelial expression by human endometrium during the menstrual cycle (Lessey *et al.*, 1992; Tabibzadeh, 1992). These three integrins have been proposed as markers of uterine receptivity (defined as the time when the uterus is receptive to the implanting embryo) because of the temporal pattern of their expression around the time of implantation (Lessey *et al.*, 1994a, 1995). The integrin β3 subunit, which associates with αv and gpIIIa (Fitzgerald *et al.*, 1987), was shown to appear on the glandular epithelial cells abruptly after day 20 and its expression extended throughout the secretory phase. It has also been reported that integrin β3 expression was delayed in endometrium that is histologically dated as out of phase, while aberrant or diminished expression was observed in patients with minimal or mild pelvic endometriosis despite the presence of in-phase histological features (Lessey *et al.*, 1992, 1994b). Albers *et al.* (1995) have also found β3 to exhibit cycle dependency. In contrast, other workers have reported that β3 expression occurred only during the proliferative phase in endometrium of women with and without endometriosis (Bridges *et al.*, 1994). These contradictory findings on the cycle specific expression of integrins have cast a doubt on their credibility as markers of the 'implantation window', the brief period of time when the uterus is receptive to the implanting blastocyst.

The aims of the present study were to quantify vascular and glandular expression of integrin $\alpha_v\beta_3$ throughout the menstrual cycle, and to compare and contrast the expression of $\alpha_v\beta_3$ between endometria from women with and without endometriosis.

Material and methods

Patient groups

Archival, formalin fixed, paraffin embedded endometrial tissue blocks from a previous study (Wingfield *et al.*, 1995) were used to obtain sections for both control and endometriosis subjects. Controls were defined as women with a normal pelvis at laparoscopy performed for tubal sterilization or for infertility investigation. Endometriosis was also laparoscopically proven and the subjects staged according to the American Fertility Society (AFS) score (1985). All women had normal menstrual cycles, had not received any hormonal therapy, had used an intrauterine device, had not been pregnant, or had not lactated during the previous 2 months. There was a total of 10 women with minimal endometriosis (AFS stage I), nine with mild (stage II), six with moderate (stage III) and two with severe (stage IV). Two women with confirmed endometriosis had unknown AFS staging and were not included in the glandular study, but were used in the vascular study, since an AFS stage was not required for this part of the work. Both control ($n = 24$) and endometriosis ($n = 29$) endometria were histologically grouped into eight stages of the menstrual cycle according to the criteria of Noyes *et al.* (1950) as shown in Table I.

Immunohistochemistry

Formalin fixed paraffin embedded tissues were sectioned at 5 μm, adhered to 2% aminopropyltriethoxysilane (Sigma, Castle Hill, NSW Australia) coated slides and allowed to air dry. After dewaxing, tissue sections were washed with phosphate buffered saline (PBS, pH 7.4) and immersed in 0.1 M trisodium citrate buffer (BDH, Merck, Victoria, Australia) at room temperature for 2 h. Tissues were rehydrated in PBS before quenching with 3% hydrogen peroxide in 50% methanol. After a PBS wash, 10% normal rabbit serum (Australian Laboratory Services, Victoria, Australia) was applied at 4°C overnight. The next day, anti-integrin $\alpha_v\beta_3$ (clone LM609; Chemicon, San Francisco, CA, USA) was applied at 0.8 μg/ml at 4°C overnight. After a PBS rinse, the sections were immersed in rabbit-anti-mouse secondary antibody (Zymed, CA, USA) for 20 min, followed by another PBS rinse. Biotinylated horseradish peroxidase–streptavidin (HRP-Strep; Zymed) was added for 20 min, the sections were rinsed with PBS and AEC chromogen (Zymed) was applied. The sections were then washed with distilled water and mounted in an aqueous mounting medium (ASAP; Zymed). The positive control was endometrial tissue that had known reactivity to anti-integrin $\alpha_v\beta_3$ and the negative control was immunostained with the matching type and concentration mouse IgG. A small number of malignant and benign ovarian tumour sections was also stained with antibody to $\alpha_v\beta_3$ and used as control for the identification of vascular sprouts. A small number of endometrial tissues from which both frozen and fixed samples was available were used to confirm that immunostaining for $\alpha_v\beta_3$ was similar in both types of section.

Total vessel numbers were calculated from serial sections stained with antibody to endothelial cell marker CD34 (Clone QBEND10; Serotec, Oxford, UK). Following dewaxing and rehydration in PBS, tissues were quenched, rinsed with PBS and incubated with primary antibody to CD34 (0.2 μg/ml) at 37°C for 40 min. After a PBS wash, secondary Ab, HRP-Strep and AEC chromogen were applied sequentially for 10 min each separated by a PBS wash. Tissue sections were counterstained with haematoxylin (Harris', Australian Biostain, Victoria, Australia) and mounted with ASAP.

Scoring and statistics

Scoring of glandular staining and quantification of vessel staining was performed using a Zeiss stereo microscope (BH-2, Olympus Optical Co., Tokyo) linked via a colour CCD video camera (MW-Fi5E, Panasonic; Matsushita Electrical Industrial Co. Ltd, Osaka, Japan) to a video monitor. Counts or scores were made from the monitor screen by the operator. To ensure unbiased scoring, the operator was blinded as to the subject group or menstrual cycle stage of each tissue section. All vessel profiles expressing integrin $\alpha_v\beta_3$ were counted in 10 fields per section at ×400 magnification of each endometrial tissue. Each field had an area of 0.019 mm^2. Endothelial expression of $\alpha_v\beta_3$ was further confirmed at ×1000 magnification under oil immersion. Total vessel number was quantified from a CD34 antibody stained serial section. Results were expressed as the percentage of vessels expressing $\alpha_v\beta_3$ (no. vessel – $\alpha_v\beta_3$/total vessel –CD34). The final result for each tissue was the mean of all 10 fields. Integrin $\alpha_v\beta_3$ results for each subject were correlated with endothelial cell proliferation index (EC PI) which was taken from a previously published study (Wingfield *et al.*, 1995). Endothelial cell PI was the

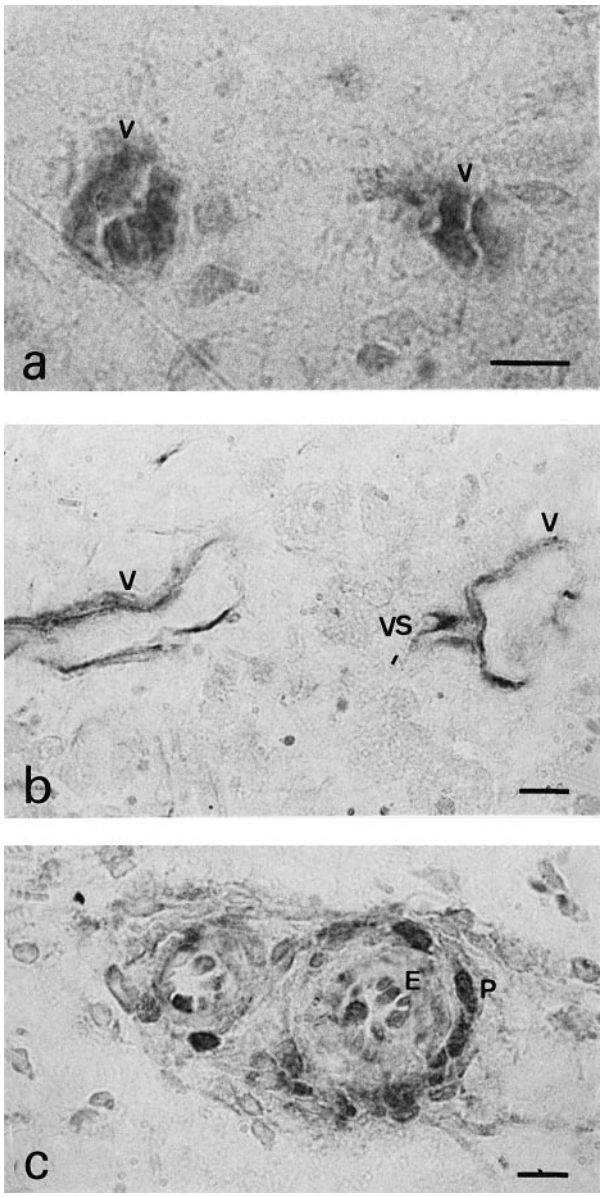

Figure 1. Blood vessels as localized by anti-integrin $\alpha_v\beta_3$ (LM609). (**a**) Blood vessels (V) seen in early–mid-secretory phase endometrium from a subject with endometriosis. (**b**) Blood vessel in a malignant ovarian tumour (V) has what appears to be a vascular protrusion or sprout (VS) from the pre-existing vessel as seen. (**c**) Some endothelial cells (E) of a mid-secretory endometriosis endometrium express $\alpha_v\beta_3$ while others do not. Some perivascular cells (P) are also stained for $\alpha_v\beta_3$. Scale bar = 10 μm. Oil immersion.

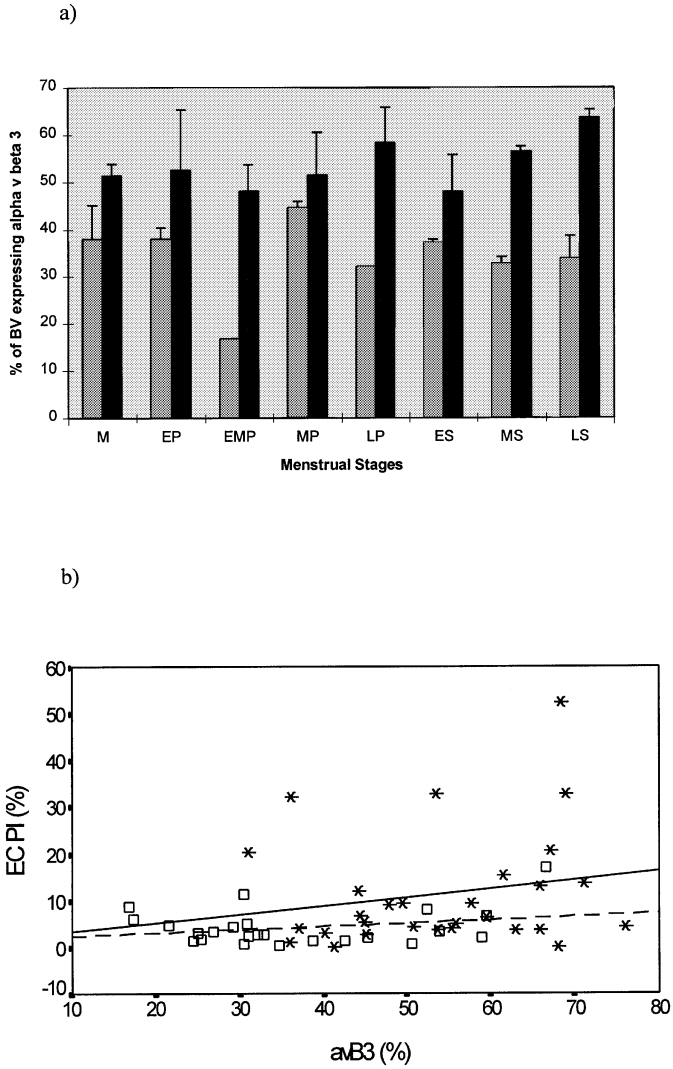

Figure 2. (**a**) Vascular expression of integrin $\alpha_v\beta_3$ in control endometrium (light bars) and endometrium from women with endometriosis (dark bars). All data are mean ± SEM. (**b**) Correlation of vascular $\alpha_v\beta_3$ expression with endothelial cell proliferation index (EC PI) in the endometrium of women with (—, R^2 = 0.035) and without (---, R^2 = 0.0645) endometriosis. □, Control; *, endometriosis.

percentage of EC expressing proliferation cell nuclear antigen (PCNA; clone PC10, Novocastra Laboratories, Newcastle upon Tyne, UK). The EC PI was calculated by dividing the number of proliferating EC as localized by PCNA by the total number of EC nuclei as localized by CD34 and haematoxylin.

Semi-quantitative scoring of endometrial glands was performed in five fields at ×100 magnification for each tissue. Each field had an area of 0.304 mm^2. For some of the smaller endometrial tissues, it was only possible to score three or four fields. For each field, glandular staining was expressed as 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (intense staining). The final score for each tissue was the mean of all five fields. The result for each of the eight stages of the menstrual cycle was the mean of all the tissues from that stage.

Numerical data were analysed using Excel (version 5.0; Microsoft Corporation, Redmond, WA, USA) and SPSS (Version 6.1.2, Microsoft Corporation). The Levene homogeneity test was used to measure variance and the K-S Lilliefors/Shapiro–Wilks test was used to test normality to determine whether parametric or non-parametric tests were to be used in further analyses. The *t*-test was used to compare between groups when data were normal and variance was homogeneous; otherwise, the Mann–Whitney-*U*–Wilcoxon rank sum *W*-test was used. Correlation was performed using regression analysis. The Kruskal–Wallis one-way analysis of variance was used to examine changes between stages of the menstrual cycle.

Results

The endothelial expression of integrin $\alpha_v\beta_3$ was predominantly cytoplasmic (Figure 1a). All the EC within the same vessel

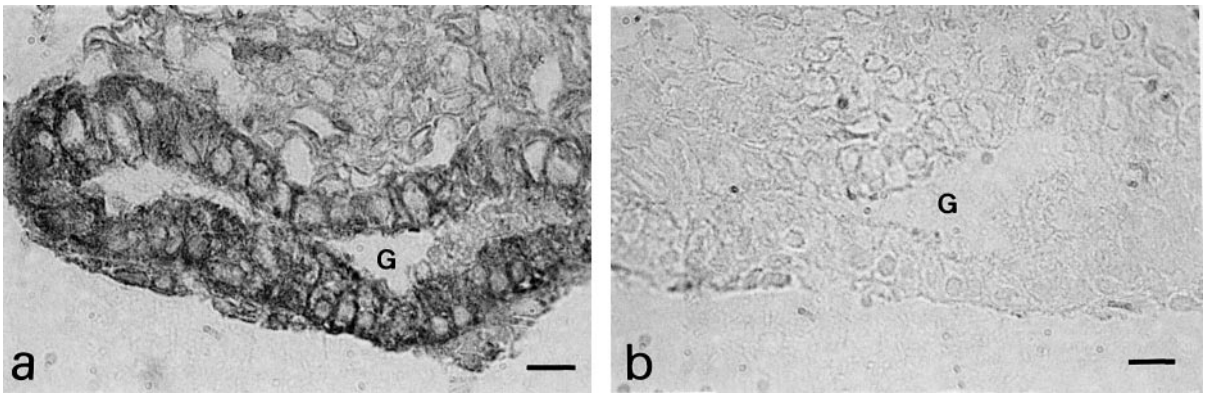

Figure 3. Positive control and negative control of a human endometrial gland. (**a**) The gland (G) shows immunoreactivity to anti-integrin $\alpha_v\beta_3$, LM609. (**b**) The same gland shows no immunoreactivity when LM609 is substituted with matching concentration of mouse IgG1. Scale bar = 10 μm. Oil immersion.

profile often did not stain positively for $\alpha_v\beta_3$ in both control and endometriosis groups. In some vessels, as few as one to two EC were stained, whereas in others, as many as five to six EC expressed this integrin. Structures which may have been vascular sprouts (Figure 1b) were seen in ovarian tumour sections, but were not observed in any endometrial tissues obtained throughout the menstrual cycle.

At least two different types of perivascular cell also stained positively for $\alpha_v\beta_3$ (Figure 1c). The first type of perivascular cell was round and was sometimes found inside vessels. The second type was more elongated and always surrounded the vessels.

Vascular expression of $\alpha_v\beta_3$ was seen in control and endometriosis groups throughout the eight stages of the menstrual cycle (Figure 2a). However, the percentage of endometrial vessels expressing $\alpha_v\beta_3$ in endometriosis subjects was significantly increased above controls (53 versus 36%; $P = 0.0001$). Statistical analysis showed that the difference was more marked in the secretory phase ($P < 0.001$) than the proliferative phase ($P = 0.016$). $\alpha_v\beta_3$ expression was not different in the proliferative compared with the secretory phase in either the control ($P = 0.653$), or endometriosis groups ($P = 0.146$). Neither the control nor endometriosis groups showed a significant correlation between vessel expression of $\alpha_v\beta_3$ and EC proliferation index (Figure 2b). However, there was a trend for the control samples to be distributed toward the lower ends of each axis, whereas the endometriosis samples were distributed around the higher ends of each axis.

Glandular localization of integrin $\alpha_v\beta_3$ was primarily cytoplasmic (Figure 3a), while the negative control was completely unstained (Figure 3b). In the endometriosis group, there was no significant difference ($P = 0.376$) in endometrial glandular $\alpha_v\beta_3$ expression between women with minimal or mild endometriosis (stages I and II) and women with moderate to severe endometriosis (stages III and IV). Thus, all four endometriosis groups were combined for further comparison with controls. Endometrial glands of both control and endometriosis groups showed $\alpha_v\beta_3$ expression during the proliferative and secretory phases of the menstrual cycle (Figure 4). There were no significant differences across the menstrual cycle in the glandular expression of $\alpha_v\beta_3$ in either the control ($P = 0.3329$) or the endometriosis groups ($P = 0.2260$). When all

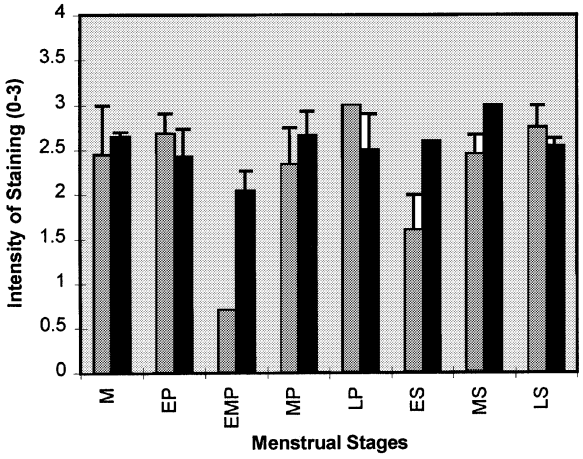

Figure 4. Endometrial glandular expression of integrin $\alpha_v\beta_3$ in women with (dark bars) and without (light bars) endometriosis. All data are mean ± SEM. Intensity of staining (0–3) and menstrual stages (M, EP, EMP, MP, LP, ES, MS, LS) are detailed in Materials and methods.

proliferative phases combined were compared with all secretory phases combined, there were no differences in either controls ($P = 0.363$) or women with endometriosis ($P = 0.5157$). No significant difference was observed between women with and without endometriosis in terms of glandular $\alpha_v\beta_3$ expression ($P = 0.4302$).

Most endometrial tissues showed either no or minimal stromal staining and this was interpreted as background resulting from the overnight primary antibody incubation. A few endometrial sections showed intense stromal $\alpha_v\beta_3$ staining; however, no definite pattern of expression or obvious biological conclusion could be drawn in regards to the menstrual cycle. Similarly, in some sections, there was evidence of glandular and endothelial nuclear staining for $\alpha_v\beta_3$ integrin. Once again, no obvious pattern or biological interpretation could be placed on these observations.

Discussion

The major novel finding from this study is that the endometrium from women with endometriosis has significantly higher vascular expression of integrin $\alpha_v\beta_3$ than control

endometrium. This finding adds to the increasing evidence that endometrium from women with endometriosis has a number of fundamental differences to that from normal controls. Reported differences include higher levels of EC proliferation (Wingfield *et al.*, 1995), increased heterogeneity in the surface epithelium and reduced glandular and stromal mitoses and basal vacuolated cells (Fedele *et al.*, 1990), increased secretion of complement-3 (Isaacson *et al.*, 1990), increased resistance to the cytotoxic effect of heterologous lymphocytes (Oosterlynck *et al.*, 1991), reduced glycogen synthase and glycogen content (Ishihara *et al.*, 1991) and increased production of CA-125 (McBean *et al.*, 1993). In an earlier study (Wingfield *et al.*, 1995), we have postulated that elevated EC proliferation in the endometrium of women with endometriosis may lead to an enhanced ability of clumps of endometrial cells that reach the peritoneum through retrograde menstruation to induce an angiogenic response and thus successfully implant and survive. The present findings that the vascular expression of integrin $\alpha_v\beta_3$, a marker shown to be associated with angiogenesis in other tissues (Brooks *et al.*, 1994a), is elevated in the endometrium of women with endometriosis further supports the concept that angiogenic factors may play a significant role in the aetiology of endometriosis.

In our previous studies of EC proliferation and vascular growth, we have postulated that angiogenesis in human endometrium may not occur via the classical steps of basement membrane breakdown, EC migration and proliferation, sprout formation and subsequent tube formation (Macpherson and Rogers, 1995). This statement was based on the observation that proliferating EC nearly always appeared inside existing endometrial vessels, rather than being associated with structures that could be identified as vascular sprouts. By contrast, proliferating EC could readily be seen in association with sprout-like structures during vascularization of the corpus luteum. From these observations, we postulated that endometrial angiogenesis most likely occurs through a process of vessel elongation and expansion. The results of the present study provide further support for this hypothesis. Throughout all endometrial sections we only observed integrin $\alpha_v\beta_3$ on EC that appeared to be within existing blood vessels. While it is not possible to state conclusively from our results that vascular sprouts do not occur during angiogenesis in the human endometrium, it seems highly improbable that integrin $\alpha_v\beta_3$, a marker that specifically localizes to vascular sprouts in other tissues (Enenstein *et al.*, 1994), will stain blood vessels but not sprouts in the endometrium. Further support for the idea that there are no vascular sprouts in endometrium was provided by the ready identification of sprout-like structures using integrin $\alpha_v\beta_3$ in ovarian tumour tissue, in contrast to their complete absence using the same immunostaining protocol in the endometrial tissue.

Within a blood vessel that is growing by elongation and expansion, there is presumably a need for EC to migrate as vascular remodelling occurs. Integrin $\alpha_v\beta_3$ has been shown to have a role in vascular basement membrane breakdown and its expression is transient (Brooks *et al.*, 1996). In the present study, it was observed that only some vessels expressed $\alpha_v\beta_3$, while others did not, and that not all EC within a vessel were positive. It is possible that vessels that expressed integrin $\alpha_v\beta_3$ were growing lengthwise or widthwise, while those that did not express $\alpha_v\beta_3$ remained dormant. More specifically, it is possible that within a growing vessel, some EC are involved in the remodelling process and some are quiescent.

Previous studies (Goodger and Rogers, 1994) reported that endometrial EC migratory signal production did not correlate with EC proliferation. The results of the present study showed that EC $\alpha_v\beta_3$ expression did not significantly correlate with the EC proliferation index in either the control or endometriosis groups. Assuming integrin $\alpha_v\beta_3$ to be a marker for migrating EC, this finding suggests that EC proliferation and EC migration do not occur concurrently during angiogenesis in endometrium, unlike some other tissues. If correct, this observation implies that EC proliferation and migration are under separate control mechanisms in the endometrium, rather than being part of the same process.

Integrin $\alpha_v\beta_3$ was also observed in perivascular cells of the human endometrium. Integrin $\alpha_v\beta_3$ has been reported to colocalize with smooth muscle cells (SMC; Brown *et al.*, 1994; Hoshiga *et al.*, 1995). The elongated cells surrounding the arterioles as shown in Figure 1c could be SMC, but further immunohistochemistry is required to confirm this. The round perivascular cells also remain unidentified, but they are most likely to be leukocytes as these cells have been shown to express $\alpha_v\beta_3$ integrin to enable them to attach to CD31/platelet endothelial cell adhesion molecule (PECAM) of the endothelium during extravasation (Piali *et al.*, 1995).

In contrast to previous studies that demonstrated β_3 integrin appearing on the luminal and glandular epithelium only during the proliferative phase (Bridges *et al.*, 1994) or only during the secretory phase (Lessey *et al.*, 1992; Albers *et al.*, 1995), the present study did not observe temporal or spatial alterations in glandular $\alpha_v\beta_3$ expression. One possible explanation for this difference could be the different antibodies used. In previous studies α_v and β_3 subunits were localized by separate individual antibodies. This present study, however, utilized the mAb LM609 (Cheresh and Spiro, 1987) which specifically targets the unbound $\alpha_v\beta_3$ integrin heterodimer, and this may be the cause of the differences between this study and previous ones. The epitope that mAb LM609 recognizes does not appear until the two subunits come together to form a heterodimer. As it has been documented that integrins exist in both active and inactive states and that alteration between these states requires complex conformational change in order for ligand binding and signal transduction (Humphries, 1994), the earlier studies of individual integrin subunits may not provide the complete picture. In a similar vein, however, the fact that LM609 only localizes $\alpha_v\beta_3$ when the integrin is unbound or remains in free form, means that results of this study may not provide the whole picture as to the distribution of this molecule.

This study has also demonstrated that there was no difference in the glandular expression of $\alpha_v\beta_3$ integrin between normal control and endometriosis endometria. The endometrium of women with minimal or mild endometriosis (AFS stage I and II) did not show diminished or aberrant glandular expression of $\alpha_v\beta_3$. Again, in contrast to previous studies (Lessey *et al.*, 1994b) which found β3 expression to be diminished or non-

existent in women with minimal or mild endometriosis, this study observed similar glandular $\alpha_v\beta_3$ expression in all eight menstrual stages throughout the cycle in women with endometriosis.

The present study is the first to report the use of anti-integrin $\alpha_v\beta_3$ (mAb LM609) on archival, formalin fixed paraffin embedded tissues. It has been suggested that LM609 is only useful on frozen sections, however the results of the present study indicate that fixed tissues can also now be studied.

Acknowledgements

Thanks are due to Maxine Orre for immunostaining of malignant and benign ovarian tumour sections. This study was supported by Grant No. 960041 from The Australian National Health and Medical Research Council.

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Received on April 18, 1997; accepted on January 19, 1998