Integrin cell adhesion molecules in endometrium of fertile and infertile women throughout menstrual cycle

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Received 23 April 1998; revised 4 January 1999

Integrins (ITGs) are ubiquitous cell adhesion molecules that undergo dynamic alterations during the normal menstrual cycle in human endometrium. The distribution of four different subunits, viz. \( \alpha 4, \alpha 6, \beta 3 \) and \( \beta 4 \) in human endometrial tissue at different stages of the menstrual cycle was studied using immunohistochemical, enzyme immunoassay and SDS-PAGE/Western blot techniques. The specificity of each mAb to their respective ligands viz., laminin (Ln), fibronectin (Fn) and vitronectin (Vn) was done by cell adhesion assays. Both \( \alpha 6 \) and \( \beta 4 \) subunits (Ln receptors) expressed primarily on the glandular epithelium, while glandular, stromal and luminal cells expressed predominantly \( \alpha 4 \) (Fn receptor) and \( \beta 3 \) (Vn receptor). The appearance of \( \alpha 4 \) and \( \beta 3 \) ITG subunits was found to be cell and cycle specific. The levels of both \( \alpha 6 \) and \( \beta 4 \) increased throughout the menstrual cycle, while \( \beta 3 \) subunit appeared abruptly on cycle day 19/20. The immunostaining for \( \alpha 4 \) and \( \beta 3 \) was absent in 90% of infertile women. The timing of expression of \( \alpha 4 \) and \( \beta 3 \), the two cycle-dependent ITGs framed the putative window of implantation and suggests a role in the diagnosis of infertility. In conclusion, the absence of \( \alpha 4 \) and \( \beta 3 \) ITG expression in the endometrium of infertility subjects during mid-luteal phase may be associated with defects in uterine function. The defective uterine receptivity may be an unrecognized cause of infertility in these group of women.

Mechanisms of interactions of cells with the components of extracellular matrix (ECM) proteins has become clearer with the isolation and characterisation of cell surface receptors for ECM components. These receptors are termed as integrins (ITGs). ITGs are heterodimers composed of different subunits and a common \( \beta \) subunit and are involved in cell-cell and cell-ECM interactions. To date, 15 \( \alpha \) and 8 \( \beta \) subunits have been identified in a wide variety of cells. The recent discovery that certain endometrial ITGs exhibit cycle and cell specific pattern of expression generated intense interest in this family of cell adhesion molecules.

Human endometrium undergoes a remarkable series of developmental changes during menstrual cycle in preparation for embryonic implantation. The complex structure of endometrium undoubtedly required an array of distinct molecules, which contribute to the cell distribution, adhesion, trafficking and signalling with ECM constituents of the endometrial mesh work.

In an elegant study by Lessey et al., the variety of ITG subunit expressed on the surface of endometrium underwent spatial and temporal changes during menstrual cycle and certain specific alterations have shown in the peri-implantation window, which may be associated with nidation. Now it is believed that lack and/or disruption of ITG expression in the endometrium may be associated with certain types of infertility.

It is known that 15-20% of couples undergoing evaluation for infertility eventually have no cause identified for their failure to conceive. Lack of uterine receptivity is likely a common defect and the final convergent pathway of many different diagnoses such as LPD, habitual abortion, leiomyomata uteri, endometriosis and unexplained infertility. The factors that contribute to infertility due to defects in uterine receptivity rarely have been investigated because of the lack of suitable markers for the fertile endometrium. A better understanding of defects in uterine receptivity may lead to a more direct therapeutic approach to it patients and remove some of the uncertainty that surrounds the diagnosis. The objective of this investigation is to study the localization and quantification of ITG molecules in the endometrial cells of infertile women and compare the results with normal proven fertile subjects using a prospective, controlled study design.

Materials and Methods

Patient selection — The study was carried out with ten normal fertile (Group I) and fifteen infertile women (three anovulatory four unexplained infertility
and eight luteal phase deficiency (LPD; Group II). Both groups of women (age between 25-37 yr) were recruited from the infertility clinic, Institute for Research in Reproduction, Mumbai, as a part of their clinical investigation for the diagnosis of infertility. Women were defined as normal fertile, if they had one or more successful pregnancies. All patients had undergone previous infertility evaluation including assessment of ovulatory status, tubal patency, normal endocrine parameters and husband semen quality (data not shown). All women were cycling normally and not had taken any hormones or used intrauterine device at least >3 months prior to collection of biopsy.

**Endometrial samples** — The collection of endometrial biopsies (EBs) were approved by the Institutional Review Committee. EBs and aspirates were obtained from the women at four defined phases—(1) early follicular (day 9/10), (2) ovulatory (day 14/15), (3) mid luteal (day 19/20) and (4) premenstrual (day 24/25). Twenty four samples were collected from five fertile and seven sub-fertile women during phase 2 and 4 (two EBs/individual/cycle). Similarly twenty six samples were collected from another group of five fertile and eight sub-fertile women during phase 2 and 4 (two EBs/individual/cycle). The samples were divided into two portions one of them was fixed in bouins fluid and prepared for light microscopy and the morphology of the specimens was examined and dated histologically according to the criteria set by Noyes et al. The second portion of tissue was immediately snap frozen in liquid nitrogen and stored at -70°C. These tissues were subsequently thawed in cold phosphate buffered saline (PBS) embedded in Tissue-Tek OCT compound prior to use. Blood was sampled at the time of operation for the estimation of serum estrogen and progesterone levels. The values were in the range of normal concentrations for the corresponding phase of the cycle. Endometrial specimens were evaluated in the context of timing of ovulation and/or the onset of next menstrual period. Samples were judged as 'out of phase' if histologic dating was delayed by >3 days relative to the predictable day of the menstrual cycle.

**Endometrial cell separation and NP-40 extraction** — Endometrial tissues obtained by aspiration were collected in RPMI-1640 medium. Isolation of glandular epithelial and stromal cells was done as described earlier with minor modifications — the tissue was treated with 0.1% collagenase (type IA, Sigma) and 0.1% hyaluronidase (type IS, Sigma) in Ca²⁺ free PBS with stirring at 37°C for 1hr. The cell suspension was filtered through nylon mesh (pore size 105μm) to remove undigested tissue debris. The cells were collected from the filtrate by centrifugation at 800g for 10 min. and washed in the same medium. The cell suspension filtered through a 38-μm pore size sieve which retained glandular elements and excluded the individual stromal cells. The isolated cells were collected by centrifugation and membrane extracts were prepared by adding small volumes (100-200 μl) of 10 mM tris-acetate (pH 8.0), 0.5% NP-40, 0.5 mM Ca²⁺ with 2 mM PMSF to the final pellet and incubated on ice for 15 min. The lysate was centrifuged for 5 min at 16,000g in a microcentrifuge. The resulting supernatant was called NP-40 extract and used for SDS-PAGE and immuno-blot analysis.

**SDS-PAGE and Western-blot (immunoblot)** — The protein concentration of each phase of NP-40 extract was determined. Samples with equal amount of protein were analysed by SDS-PAGE using 10% polyacrylamide gels using non-reducing conditions. The gel was transferred to nitrocellulose membrane according to the sandwich technique. The non-specific reaction was blocked with 2% non-fat dry milk powder for 1 hr at RT. After washing at room temperature each membrane was sequentially incubated with primary antibody, secondary antibody, ABC complex and then developed in a mixture of DAB-H₂O₂ (ref. 15).

**Antibodies** — Monoclonal antibodies (mAbs) of clone HP2/1 directed against α4 subunit was purchased from Serotec, (England). GoH4, a specific mAb directed against α6 subunit was generously provided by Dr. Arnold Sonnenberg (Central Laboratory of the Netherland Red Cross Transfusion Service, Amsterdam). B4 antibody of clone AA8 was gifted by Dr. Vito quaranta (Scrivps Clinic, Lajolla, CA, USA). Antibody BB9 directed against β3 subunit was purchased from ICN (England). Hexapeptides Gly-Arg-Gly-Asp-Ser-pro (GRGDSP) and Gly-Arg- Gly-Glu-Ser-pro (GRGESP) were purchased from Gibco, BRL - Life Technologies (Hongkong).

**Immunohistochemistry (IHC)** — Immunoperoxidase staining was performed on cryostat sections using avidin biotin peroxidase complex (ABC) method of Hsu et al. with slight modifications. Serial sections (4-5μm thick) were placed onto poly-L lysine coated slides, fixed in acetone at 20°C for 10 min and stained.
using vectastain Elite ABC kit (Vector Laboratories, Burlington, CA). After rehydration in PBS, endogenous protein was blocked in 1% BSA-PBS for 10 min. Antibodies to α4 ITG (HP2/1, 1:1000), α6 (GoH1, 1:500), β4 (AAα6, 1:1000) and β3 (BB10, 1:1000) were used. The antibodies were placed on sections for 1 hr at room temperature (RT). Sections were washed in PBS and biotinylated goat antiantibody (rabbit antirat for α6) antibody (1:1000) was applied and allowed for binding at RT for 30 min. After a PBS rinse, endogeneous peroxidase activity was blocked using a 0.3% H₂O₂ in absolute methanol at RT for 20 min. Sections were rehydrated in PBS and ABC was applied and allowed for 30 min at RT. The peroxidase reaction was visualized with 3′-3′ diaminobenzidine –H₂O₂ (Sigma Chemical Co., St. Louis, MO). Sections after 3 min were subsequently washed in PBS, and lightly counterstained with hematoxylin. The slides were dehydrated through alcohols, cleared in xylene and mounted in Entellan for light microscopy. Negative controls included sections stained without the primary antibody, using PBS instead.

The resulting immunostaining for each subunit was evaluated microscopically at higher magnification (400x) by a single blind observer. Staining pattern was judged as absent (−), weak (+), moderate (+) and strong (++), the staining intensity for each ITG was determined using a semiquantitative HSCORE.

HSCORE was calculated using, HSCORE=Pi (i+1), where i is the intensity of staining with a value of 1, 2 or 3 (weak, moderate or strong respectively) and Pi is the percentage of stained epithelial cells for each intensity, varying from 0-100%.

**Quantification of ITG molecules by cell-ELISA** — Expression of cell surface determinants was measured by ELISA to supplement the findings of IHC. Endometrial cell suspension (1 x 10⁶/well) was coated to 96 well microtiter plate. After overnight incubation at 4°C, the cells were fixed with 0.25% glutaraldehyde. Non-specific binding sites were blocked by incubating with 1% BSA for 30 min at RT. Subsequent incubations were carried out sequentially for 30 min at RT followed by washing in PBS-T20 between each step. Incubation with mAb diluted in PBS with BSA was followed by secondary antibodies (as above). Finally the plate was incubated with substrate, orthophenylene diame (OPD)-H₂O₂ in 0.05 M citrate phosphate buffer containing H₂O₂ (3mM). The colour reaction was stopped after 15 min incubation in dark by adding H₂SO₄ (2M). Absorbance of colour product was measured at 490 nm in an ELISA reader.

**Cell adhesion and mAb inhibition assay** — To assess cell attachment to ECM proteins, endometrial cells (5x10⁵) obtained from normal fertile women during mid luteal phase were incubated with ¹²⁵I (Amersham, England) for 4-6 hr at 37°C. The cells were washed sequentially with PBS followed by 1 mM EDTA in PBS and finally serum free RPMI-1640 supplemented with 1% BSA (RPMI/BSA). The cells were resuspended in RPMI/BSA and plated in triplicate (2 x 10⁵ cells/well) for 20-30 min at 37°C on 96 well microtiter plate, that had been previously incubated with Lm, Fn and Vn (1 µg/well, Sigma). After unbound cells were aspirated, the plate was washed (3x) with RPMI/BSA. ¹²⁵I present in 0.1% SDS cell lysates was measured using gamma counter.

For inhibition experiments, dilutions of inhibitor mAbs (1 µg/well) were added to ligand coated microtiter plate before adhesion of labelled cells and adhesion assays were conducted exactly as described above.

Specific attachment of cells to ECM ligands was expressed as cells bound/mm² (area of microtiter plate:32mm²/well). Background binding of radiolabelled cells to BSA-coated control well was <5% of total input counts and was subtracted during calculation of specific attachment.

**Results** — To investigate the potential role of ITGs in the assessment of uterine receptivity in infertile patients, we compared IHC and SDS-PAGE/Western blot data with quantitative analysis done by ELISA. The semiquantitative analysis of the expression of ITG subunits in various endometria is shown in Table 1. Relative intensity of staining for α4, α6, β3 and β4 (designated by HSCORE) is shown in Fig. 1. Immunostaining for each of the antibodies in both glandular and stromal components of the endometrium representing mid luteal phase of the menstrual cycle is shown in Figs 2,3. The quantitative analysis of ITG is shown in Figs 4,5a,b,c. The Western blot immunostaining for α6 and β3 is shown in Fig 6. The specificity of ITG subunits for their respective ligands was carried out by cell adhesion assay (Figs 7a,b,c).

Intensity of staining for all the four ITG subunits was found to change in a cell and cycle dependent
manner. Expression pattern of ITGs in endometrium of normal fertile women significantly differed from that of infertile women. Distribution of β4 and α6 subunits that recognise primarily laminin expressed on the surface of the glandular epithelium throughout the menstrual cycle in fertile (10/10) and in all three types of infertile (15/15) subjects. These two subunits appear to be localized at the basolateral surface, adjacent to the basement membrane of the endometrial glands (Figs 2a,b). Maximum level of these subunits were detected in the secretory phase (day 20) with a marginal decrease in either side of this period (Figs 4,5a,b,c).

The expression of α4 subunit of ITG known to bind fibronectin was quite restricted. The subunit was undetectable above background staining in either glandular epithelium or mesenchyme in the proliferative endometrium (data not shown). The staining was detected in basal and apical glandular cytoplasm from day 14 onwards and reaches to peak during mid luteal phase (days 20; Fig. 3a) and staining intensity was reduced significantly by day 24 of the cycle (expression from days 14-24).

Expression of β3 ITG subunit was not detected in the proliferative endometrium. However, abrupt increase of β3 expression was apparent in a pericellular distribution on glandular epithelium on day 19 or 20 of the menstrual cycle (Fig. 3b). Expression was similar to α4 and it was further noticed that co-expression of α4, α6, β4 and β3 subunits occurred on glandular epithelium only during 4 days interval (days 20-24). This pattern of epithelial ITG expression is shown in Fig. 1, which summarizes the data from 10 fertile endometrial samples taken across the menstrual cycle. This interval is thought to correspond precisely to the putative window of implantation in women. The ELISA results showed, out of 4 ITG subunits measured, the levels of α6 was found to be higher than other three ITGs in all the phases of menstrual cycle.

Table 1—Distribution of ITG subunits in fertile endometrium during the menstrual cycle

<table>
<thead>
<tr>
<th>Cycle Phase (day)</th>
<th>Glandular epithelium ITG subunit</th>
<th>Stroma ITG subunit</th>
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<tbody>
<tr>
<td></td>
<td>α4</td>
<td>α6</td>
</tr>
<tr>
<td>day 9/10</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>day 14/15</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>day 19/20</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>day 24/25</td>
<td>+</td>
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</tr>
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(-- ) Corresponds to absent staining; (+) corresponds to weak staining; (++) corresponds to moderate staining and (+++) corresponds to strong staining

Fig. 1—Relative intensity of staining for α6, α4, β3 and β4 throughout menstrual cycle in fertile women. Immunohistochemical staining was assessed by a blinded observer using the semi-quantitative HSCORE (range from 0-4) and correlated to the estimate of histologic dating. Positive staining for all four ITG subunits was seen only during a 4 day interval corresponding to cycle days 20-24, based on histologic dating criteria of Noyes et al. This interval of ITG co-expression corresponds to the putative window of implantation.
subunit was missing in 14 of 15 (94%) samples, whereas β3 was not expressed in 13/15 (87%) samples. ELISA results were well in agreement with IHC and immunoblot data, where we have showed the absence of α4 and β3 subunits in mid luteal phase of infertile subjects (Figs 5a,b,c, 6). All the three categories of infertile subjects (anovulatory, unexplained infertility and LPD showed negative for

Fig. 2 — Immunostaining for α6 (a) and β4 (b) ITG subunits in secretory phase endometrium (day 20). The staining intensity was judged at ++ for glandular epithelium (arrow). The staining was absent in the stromal cells (asterisks). Control slides (c) were incubated with PBS in place of primary antibodies showing negative for GoHJ and AA6 (x 400).

Fig. 3 — Immunostaining for α4 (a) and β3 (b) ITG subunits in secretory phase endometrium. The staining intensity was judged as ++ for the glandular epithelium and cells (arrow) and + for stromal (asterisks). Control slides (c) were incubated with PBS in place of respective primary antibodies showing negative for HP2/1 and BB10 (x 400).
immunostaining (data not shown) and no difference was seen between them. SDS-PAGE/western blot analysis showed early proliferative and premenstrual phase epithelial cells had no immunostaining at 95 kDa region compared with secretory phase which showed strong staining for β3. Whereas for α6, the immunostaining at 110 kDa region was seen in fertile as well as infertile subjects throughout the menstrual cycle (Fig 6).

Ligand specificity of anti-ITG-mAbs — To assess the functional role of ITGs we have carried out biological assay addressing-(a) interaction of anti-ITG mAbs with Ln, Fn and Vn; and (b) whether cell-ECM adhesion mediated by ITG is RGD dependent (Figs 7a,b,c). Anti-α6 and anti-β4 mAbs significantly inhibited the endometrial cell attachment of Lm,
Discussion

The factors responsible for initial interaction between maternal and fetal epithelium leading to the establishment of pregnancy remains poorly understood. Endometrium may be both permissive or hostile, but the molecular basis for alteration in uterine receptivity still remains unclear. Extensive literature is available on various endometrial proteins and their role in the implantation process. Many of these hormonally regulated secretory proteins (cell surface lectins, progestogen-associated endometrial protein (PEP or PP14), insulin-like growth factor binding protein (IGFBP-1), estrogen and progesterone receptors, leukemia inhibitor factor (LIF), mucin, TGF-α and TGF-β, extracellular matrix proteins and various cytokines) expressed by endometrium are known to differ between fertile and infertile women during different phases of the cycle. The complexity of this orchestrated response by endometrium is indeed impressive and newly discovered proteins continue to be added to this growing list.

Role of ITGs in reproduction has been suggested recently. Several reasons make these molecules very attractive due to their constant involvement from gametogenesis to parturition. We examined the expression of ITGs throughout the menstrual cycle and found interesting changes. The present study indicates that during luteal phase, endometrium of fertile and infertile women exhibits similarities and differences in the presence or absence of ITG molecules. Of all the endometrial components examined, the differences in α4 and β3 subunits observed between fertile and infertile women were related to glandular and surface epithelial cells. The present studies and those of Lessey et al. have demonstrated that dynamic alterations in ITG expression accompany the histological changes that characterize the endometrial cycle. It has been postulated that these differences in distribution denote defined roles for these ITGs, α6 and β4 ITG subunits are known to bind ECM and thus their expression largely on endometrial epithelial cells is not unexpected. Moreover these two subunits are known to promote epithelial cell-cell adhesion and contrast, GRGESP peptide, which is lack of RGD sequence did not inhibit the cell binding to ECM. In general, these results indicated that ITGs may interact with RGD site in their respective ligands.
this function is consistent with their pericellular rather than basal distribution.

Similar to another classical study of Lessey et al., it has been confirmed in this study the cycle variation of α4 and β3 subunit expression. α4 subunit was expressed on the surface of the epithelial and stromal cells in all fertile cases in the post ovulatory as well as secretory phases and was not detected in the proliferative and pFN menstrual phases. Lack of α4 ITG in these cells during proliferative phase as observed here, suggests decrease the cell-matrix interactions and allow the glandular structures to grow deep in the stroma as elongated rectilinear glands. The onset of α4 ITG expression after ovulation may strengthen the anchorage to the stroma and contribute to the coiled appearance of the glands observed in the luteal phase. These observations support the findings of Lessey et al., where the authors showed that ITG plays a role in the implantation process. Alternatively, it may only be a marker for other events that take place during this critical interval of the menstrual cycle (day 20-24). In either case, α4 ITG provides a means to investigate whether shifts or defects in endometrial receptivity may be an important of infertility. Its disappearance on cycle day 24 in normal fertile endometrium coincides closely with closure of the implantation window. It's expression was significantly (P<0.001) reduced in the endometrium of infertile women.

Pattern of β3 expression on endometrial epithelial cells was quite unusual. The abrupt appearance of the subunit, on post ovulatory days 5-6 (days 19-20 of the menstrual cycle) a time that exactly coincides with the opening of the window of endometrial receptivity. Since both α4 and β3 subunits are specifically co-expressed only during the time of maximal uterine receptivity (post ovulatory days 6 to 10), we reasoned that each can be used as a clinical marker for the assessment of endometrial function. The use of ITGs as markers of uterine receptivity can be further supported by the findings of low β3 expression in women with endometriosis.

These studies further suggests that the expression pattern of β3 provides a useful internal landmark of luteal phase development, and its lack and/or delayed expression in infertile patients may provide a new diagnostic modality for the evaluation of defects in endometrial receptivity. Despite the 47 years since the landmark article describing the technique of histologic endometrial dating, we have not had a reliable test to evaluate potential defects in uterine receptivity. Moreover, the traditional endometrial dating may miss a substantial number of such defects because the timing of the biopsy may be inappropriate, or traditional dating methods cannot detect certain functional defects in endometrial ITG expression. We believe this method would be more appropriate if done in conjunction with specific biochemical markers of the endometrial maturation. This facilitate the diagnosis of infertility due to repetitive pregnancy loss or failure of implantation and better direct therapy for affected women.

However, the mechanism by which ITG expression regulates in endometrium is not known. Our preliminary studies conducted towards this direction suggests that α4 and β3 subunits expression appears to be progesterin dependent (unpublished data). Based on recent studies, it would not be surprising to find that growth factors and cytokines play a role in the expression of endometrial ITGs. Studies are in progress to understand signal transduction mechanism(s) operates in embryo-endometrial interactions and implantation at molecular level using rat as an in vivo model.

Acknowledgment

The authors are grateful to Dr. H.S. Juneja, Director of the institute for giving us continued encouragement and guidance in carrying out this work. The authors also thank Arno Hellensberg, M.D. (Central Laboratory of the Netherlands Red Cross Transfusion Service, Amsterdam). Vito Quaranta, M.D. (Scripps Research Institute, La Jolla CA) for their generous donation of monoclonal antibodies.

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