Endometrial integrin expression is independent of estrogen or progestin treatment in vitro*

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Objective: To examine the regulation of endometrial integrin expression by estrogens and progestins in vitro.

Design: Immunocytochemical study.

Setting: Academic research unit.

Patient(s): Twenty-five regularly cycling women without endometrial pathology, of whom seven had endometriosis.

Intervention(s): Endometrial cells obtained by aspiration curettage were treated with diethylstilbestrol, promegestone, and antiprogestin. Immunocytochemistry was performed with antibodies directed against integrins $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_3$, and $\beta_3$ integrin subunit.

Main Outcome Measure(s): Semiquantitative staining score

Result(s): Endometrial cells express several integrins in vitro in a consistent and cell specific pattern. Neither differences between treated and untreated cells nor an effect of treatment duration or dosage were observed. Cells from patients with and without endometriosis showed similar patterns.

Conclusion(s): The cellular distribution of integrin expression was similar to that described in vivo. In contrast, a steroid regulated expression could not be detected in vitro. Rather, a derepression by a factor not included in our model could be responsible for the cyclic appearance of some integrins. In endometriosis, no fundamental difference of integrin expression was detected. (Fertil Steril 1997;67:877–82. © 1997 by American Society for Reproductive Medicine.)

Key Words: Endometrium, integrins, cell culture, immunocytochemistry, endometriosis, estrogen, progestin, antiprogestin

Integrins are a family of heterodimeric transmembrane proteins that participate in diverse cell-to-cell and cell-to-extracellular matrix interactions. They consist of an $\alpha$ subunit, of which 11 variants have been described and a $\beta$ subunit, of which 6 variants are known to date. The extracellular domain can act as a receptor for extracellular matrix components, adhesion molecules of other cells, or signaling proteins (1). Furthermore, $\alpha_4\beta_1$, $\alpha_6\beta_3$, and $\alpha_5\beta_3$ integrin influence extracellular matrix turnover via regulation of metalloproteinase expression (2).

In human endometrium, epithelial cells specifically express $\alpha_{2}\beta_1$, $\alpha_{3}\beta_1$, and $\alpha_6\beta_3$, and stromal cells constitutively express $\alpha_5\beta_1$ (3, 4). A role of the studied adhesion molecules in the implantation process of the blastocyst has been proposed (5). This hypothesis was strengthened further by observations that during the “window of implantation” (cycle days 20 to 24), $\alpha_1\beta_1$, $\alpha_4\beta_1$, and $\beta_3$ are coexpressed (6), whereas $\alpha_2\beta_1$ is suppressed specifically, and a lack of $\alpha_4\beta_1$ and $\beta_3$ was observed in infertile women (3, 7–9). In endometriosis, a lack of integrin $\beta_3$ expression in uterine endometrium has been reported by Lessey et al. (10), whereas Bridges and coworkers
found $\beta_3$ in only proliferative and not in secretory-phase endometrium from women with endometriosis.

Mifepristone (RU486; Roussel Uclaf, Paris, France) and onapristone (Zk98299; Schering AG, Berlin, Germany) are competitive P receptor antagonists (12, 13). Mifepristone induces morphologic changes resembling luteal phase defect if administered after ovulation (14).

To study the regulation of integrins in a strictly controlled model, we examined if endometrial cells are capable of integrin expression in vitro. Furthermore, we investigated if this expression is influenced by the estrogen, diethylstilbestrol (DES), a progestin (promegestone), an antiprogestin (mifepristone or onapristone), or a combination of these agents.

**MATERIALS AND METHODS**

**Subjects**

Endometrial biopsies were obtained by using a flexible sampling device (Pipelle de Cornier; Prodimed, Neully-en-Thelle, France) from normally cycling women with endometriosis ($n = 7$) or without endometriosis ($n = 18$) undergoing diagnostic laparoscopy between cycle days 8 and 12. All subjects gave informed consent, and the study was approved by the Ethical Committee of the University of Heidelberg Faculty of Medicine.

**Reagents**

All media, as well as antibiotic antimycotic solution, gentamicin, nonessential amino acids, insulin-transferrin-selenite, phenol red, and trypsin ethylendiaminetetraacetic acid, were from Sigma Chemical Company (Deisenhofen, Germany). Glutamax was obtained from Carl Roth GmbH (Karlsruhe, Germany) and fetal bovine serum (FBS) was from CCPro (Neustadt, Germany). Diethylstilbestrol was obtained from Sigma and promegestone from Dupont (Dreieich, Germany).

For immunocytochemistry, the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) was used, with DAB as chromogenic substrate (Sigma). The antibodies are listed in Table 1. The antibody Bw 495/36 was used for reference staining.

**Cell Culture**

All endometrial samples were collected in Dulbecco's modified Eagle's medium (DMEM)/F12, supplemented with antibiotic antimycotic solution and gentamicin at 4°C, and processed within 24 hours. After the tissue was minced with scalpels, a trypsin digestion was performed until a single-cell suspension was obtained. An aliquot was drawn and counted with the trypan blue (Sigma) exclusion method in a Neubauer hemocytometer. Cells were seeded in 96-well plates at a density of $2 \times 10^4$ per well and cultivated at 37°C and 5% CO$_2$ in DMEM/F12, containing phenol red and supplemented with 10% FBS. Upon confluence, cells were switched to phenol-red-free, serum-free medium, and supplemented with insulin-transferrin-selenite, nonessential amino acids, and glutamax.

**Sex Hormone Treatment**

Hormone treatment was initiated simultaneously as follows (in all cultures, a negative control containing ethanol at 0.1% vol/vol was run): 13 cultures from patients without endometriosis and 7 cultures from patients with endometriosis were treated for 2 days in one well each with DES $10^{-9}$ M, R5020 $10^{-9}$ M, RU486 $10^{-7}$ M, and a combination of DES + R5020, R5020 + RU486, and DES + R5020 + RU486 at the same concentration (standard treatment). This treatment was extended to 4 and 6 days in five cultures. Five other cultures from patients without endometriosis had standard treatment for 2 days and, additionally, 100-fold increase and 100-fold diluted dosages of all hormones were administered in separate wells. In a last set of five cultures from patients without endometriosis, a treatment similar to the standard was performed, except that Zk98299 $10^{-7}$ M was used instead of RU486.

**Immunocytochemistry**

After termination of treatment, cells were left in the wells and fixed in formaldehyde for 2 minutes and in ice-cold methanol for 6 minutes and rinsed in phosphate-buffered saline (PBS) three times for 5 minutes. Nonspecific binding sites were blocked with 5% FBS in gelatine-PBS at 20°C. Cells were incubated with primary antibody for 24 hours, rinsed with PBS as above, and incubated for 45 minutes with secondary antibody. After repeat rinsing, cells were incubated with avidin-biotin complex for 30 minutes and stained with diaminobenzidine (DAB) for 5 minutes. The staining reaction was stopped with PBS. For negative controls, nonspecific immunoglobulin G of the same subclass as the primary antibody was used. Endometrial epithelial cells stain strongly and specifically with the monoclonal antibody Bw 495/36 (15). Hence, this antibody was used as reference for the semiquantitative assessment of staining with integrin antibodies by light microscopy at $\times 100$ and $\times 250$ magnification. The intensity was graded as absent (= 0, = negative control), weak (= 1), moderate (= 2), or strong (= 3, = reference staining) and the entire well was evaluated.
Table 1  Primary Antibodies

<table>
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<tr>
<th>Species</th>
<th>Specificity*</th>
<th>Clone</th>
<th>IgG*</th>
<th>Type</th>
<th>Manufacturer</th>
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<td>κ</td>
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<tr>
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<td>IgG1</td>
<td>κ</td>
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<tr>
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<td>κ</td>
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<td></td>
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</tr>
<tr>
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<td>Anti-human CD49d (integrin subunit α1)</td>
<td>Polyclonal</td>
<td></td>
<td></td>
<td>Chemicon</td>
</tr>
</tbody>
</table>

Antibody for reference staining

| Mouse | Anti-Human Filament Cytokeratin | Mak 495/36 | IgG3 | κ    | Behring Institut, Marburg, Germany |

Antibodies for negative controls

<table>
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<th>IgG3</th>
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<tr>
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<td>Anti-human (nonspecific)</td>
<td>Polyclonal</td>
<td>IgG</td>
<td>Chemicon</td>
</tr>
</tbody>
</table>

* IgG, immunoglobulin G.

An independent observer, blinded to the treatment of cells, regularly repeated the scoring in the beginning of the experiments and, after complete agreement was noted, at random intervals during the course of the study.

Statistics

For all identical cell types and treatments, mean values were calculated. For statistical analysis, Wilcoxon’s signed rank test, Friedman’s test, Mann-Whitney’s U test, or Kruskall Wallis test were performed as appropriate, and the level of significance was defined as $P \leq 0.05$.

RESULTS

Staining for α2β1 and α6β1 integrin

Staining for α2β1 integrin was significantly more intensive in epithelial than in stromal cells ($P < 0.05$, Friedman’s test). Overall, a less-intensive staining was obtained for α3β1, and this was almost exclusively expressed in stromal cells (difference between epithelial and stromal cells: $P < 0.001$, Wilcoxon’s test), and, again, no differences were seen between the different treatments. Furthermore, an identical staining pattern was observed in endometrial cells from women with and without endometriosis. Figure 1A shows the immunocytochemical staining for α2β1 integrin. Figure 2, panels A and B, shows the mean staining scores for α2β1 and α3β1 in cells from patients without endometriosis are represented.

Integrins α3β1 and α6β1

Integrins α3β1 and α6β1 were equally strongly expressed in epithelial and in stromal cells (staining similar to reference). Figure 1B shows the staining of α4β1. No effects of any of the hormone treatments were observed and, again, no differences were seen between cells from women with and without endometriosis.

Integrin α2β1 and Subunit β3

The integrin subunit β3 was expressed with moderate intensity in epithelial cells and weakly in stromal cells (difference between cells significant at $P < 0.01$, and difference between stromal cells and negative control significant at $P < 0.005$). None of the treatments showed a significant influence on this staining pattern. Integrin α2β3 showed the lowest mean staining intensity of all integrins tested. Again, these findings were independent of the presence or absence of endometriosis. Figure 2C and D depicts the mean staining intensities for subunit β3 in cells from women with and without endometriosis. Expression of α2β3 was observed in the epithelial compartment in 2 of 7 samples from endometriosis patients compared with 7 of 18 from women without endometriosis (difference not significant).

Hormone Dosage

For all integrins, the dosage of the different hormone treatments also was tested in 100-fold increased concentration and in 100-fold diluted con-
centation compared with standard treatment. Again, no effect could be observed. The standard treatment was extended to 4 and 6 days and this did not change the expression of any of the integrins. No different effects were observed if onapristone was used instead of mifepristone.

**Controls**

All reference stainings uniformly showed the expected intensity in epithelial cells, whereas no nonspecific staining was observed in any negative controls. Replicates of identical wells were identical as far as detectable with our semiquantitative scoring.

**Figure 2** Mean staining intensities for different integrins (y axis). ■, epithelial cells; □, stromal cells. Vertical t-bars indicate SEM. D, DES 10⁻⁸ M; R, promegestone 10⁻⁸ M; Ru, mifepristone 10⁻⁷ M; Zk, onapristone 10⁻⁷ M; Eth, ethanol, 0.01%. (A) integrin α₄β₁ in normal endometrium; (B) integrin α₄β₁ in normal endometrium; (C) integrin subunit β₃ in normal endometrium; (D) integrin subunit β₃ in endometriosis.

**DISCUSSION**

The immunocytochemical findings presented in our study demonstrated that endometrial cells are capable of expressing integrins in an in vitro environment. Furthermore, a cell specificity observed in immunohistochemical studies (3, 4) was preserved in our model. Both findings were a necessary prerequisite for studying integrin regulation in vitro.

Several authors reported a cycle-specific appearance of some integrins in vivo, albeit the findings are somewhat controversial as to which integrin is expressed at what time (3, 7–9). The reported differences may, in part, be because of the choice of different clones of monoclonal antibodies directed against integrin subunits or dimers. According to these findings, a direct steroid dependence of integrin expression seemed a possibility that could be best tested under strictly controlled conditions in vitro. However, none of the different single hormonal treatments nor any of the combinations induced an integrin expression that was different from that after exposure to vehicle alone. Similarly, variations in hormone dosage or treatment duration were without effect. Different explanations for these unexpected findings are conceivable.

First, the hormone sensitivity of our model could be questioned. This would be of particular importance if we had observed a lack of expression in vitro compared to in vivo. In contrast, we found that the expression of β₃, α₁, and α₄β₁, which could be attributed to P action during the secretory (luteal) phase in vivo, were expressed with equal intensity in the absence of a progestin, or even of any hormone, in vitro. Furthermore, numerous effects that are a result of steroid application have been reported in similar in vitro models (16).

Second, the spatial relationships and therefore the polarity, especially of the epithelial cells, is altered
in monolayer cell cultures. Hynes (17) reported that both the cytoskeleton and the extracellular matrix environment influence integrin expression. This may have obscured the action of a putative “integrin suppressing factor” in the absence of a progestin in our model, that is, a factor that would be active during most of the cycle and inactive during the “window of implantation.”

Third, factors that modulate steroid action may have been absent from the in vitro system. In particular, cytokines that are secreted by endometrial cells, as well as by transient leukocyte populations of the endometrium in vivo, are likely candidates in this respect. It seems possible that the above-mentioned putative “integrin suppressing factor” is a result of the action of cytokines. For example, an integrin modulation by transforming growth factor β (TGFβ), which is also present in the endometrium, has been reported (18, 19). Presently, we are testing this assumption.

Last, it seems interesting in this context that Taskin and coworkers (20) were unable to demonstrate an effect of high doses of exogenous steroids on the endometrium in the late luteal phase.

Two antiprogestins with slightly different modes of action on the cellular level, mifepristone and onapristone, did not show an effect on integrin expression, neither in the presence nor in the absence of agonist. Therefore, it seems unlikely that the inhibitory action on implantation or the induction of abortion through these agents (21, 22) is directly related to a disruption of integrin expression. However, integrins that are detectable immunologically may be functionally impaired (23). Again, we are exploring this possibility.

Lessey and coworkers (10) found a high correlation between the absence of β3 expression, in in-phase endometrium and the diagnosis of minimal or mild endometriosis, which might provide an explanation for the reduced fertility of these patients (24). Furthermore, an altered integrin expression in retrogradely menstruated endometrial fragments might increase their adhesiveness and therefore facilitate the development of endometrial implants (25). In vitro, however, endometrial cells from patients with endometriosis express both β3 and α5β3 to an extent similar to women without endometriosis. Of course, this does not rule out the possibility that β3 is down-regulated in vivo by factors other than steroids. Again, Lessey and coworkers (10) name TGFβ as one possible candidate.

In conclusion, we found a consistent cell-specific expression of integrins in vitro, but were unable to demonstrate an effect of sex hormones in this system. Further studies directed at the role of cytokines and the ability of integrins to bind their ligands under different in vitro conditions are necessary.

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