Transforming growth factor β1 enhances adhesion of endometrial cells to mesothelium by regulating expression of integrins

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Supplementary Materials and Methods

Cell culture

Immortalized normal human endometrial cells (HES cells), established by Dr. Krikun (Yale University, New Haven, Connecticut), were kindly provided by Dr. Asgi Fazleabas (University of Illinois, Chicago) (1, 2). Immortalized human endometriotic epithelial cells (12Z) (3) were generously provided by Dr Starzinski-Powitz (Johann-Wolfgang-Goethe-Universitaet, Germany). Human mesothelial Met5A cells were purchased from the American Type Culture Collection (ATCC, VA, USA). HES cells were cultured at 37°C in an atmosphere containing 5% CO₂/air in Dulbecco's Modified Eagle Medium (DMEM; Welgene, Daegu, Korea) containing 10% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich) and 1% penicillin/streptomycin (Gibco, Rockville, MD, USA). 12Z cells were cultured in RPMI1640 (Lonza, USA) containing 10% heat-inactivated FBS and 1% penicillin/streptomycin. Met-5A cells were maintained as monolayers at 37°C in an atmosphere containing 5% CO₂/air in Medium 199 (M199, Welgene, Daegu, Korea) containing 10% heat-inactivated FBS and 1% penicillin/streptomycin.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from HES and 12Z cells using a GeneJET RNA Purification Kit (ThermoFisher Scientific, USA). Equal amounts of total RNA (1 μg) from each sample were
then subjected to reverse transcription with oligo-dT primers using M-MLV reverse transcriptase (ThermoFisher Scientific). cDNA was amplified by PCR using AccuPower® PCR PreMix (Bioneer Co., Daejeon, Korea). Primers used in this study were as follows: *integrin αV*, forward 5′-ATGCTCCATGTAGATCACAAGAT-3’ and reverse 5′-TTCCCAAAGTCCTTGCTGCT-3’; *integrin α6*, forward 5′-AGGTACAGTTGGGCCAGC-3’ and reverse 5′-AGGCTCGCATGAGAATGTCC-3’; *integrin β1*, forward 5′-GTCGTGTGTGTAGTGCAAC-3’ and reverse 5′-GCTGGGGTAATTTGTCCCGA-3’; *integrin β3*, forward 5′-CTGCCGTGACGAGATTGAGT-3’ and reverse 5′-GCTGGGGTAATTTGTCCCGA-3’; *integrin β4*, forward 5′-GAGCTCACCAACCTGTACCC-3’ and reverse 5′-GCCCAATAGGTCGTTGCA-3’; *integrin β5*, forward 5′-ACCTGGAACACGGTGAGA-3’ and reverse 5′-AAAAGATGCGGTGTCCCACA-3’; *CD44s*, forward 5′-AGGGATCTCTCCAGCTTCCTTT-3’ and reverse 5′-AAAGGCATGGGCGAGTCTGTGACT-3’; *ICAM-1*, forward 5′-CAGTGACCATCTCCAGCTTCCTTT-3’ and reverse 5′-GCTGCTACCACAGTGATGACAA-3’; *L-selectin*, forward 5′-AAACCCATGGAACCTGGCAAG-3’ and reverse 5′-CGCAGTCCTCCTTGTCTTCTT-3’; *E-cadherin*, forward 5′-TACAATGGCGCCATCGCTTA-3’ and reverse 5′-AGCTGTGAGGATGCCAGTTT-3’; *β-actin*, forward 5′-CAAGAGATGGCCACCGCTGACT-3’ and reverse 5′-TCCTTCTGCACTCTGTGGCA-3’.

**Western blot analysis**

Total protein was extracted from cells using 1% NP-40 lysis buffer (150 mM NaCl, 10 mM HEPES (pH 7.45), 1% NP-40, 5 mM NaPyrophosphate, 5 mM NaF, 2 mM Na3VO4) containing
a protease inhibitor cocktail tablet (Roche, Germany). Equal amounts (20 μg) of protein from each sample were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Fractionized proteins were transferred by electrophoresis onto nitrocellulose filters (Hybond ECL; GE Healthcare). Filters were blocked with 5% nonfat dry milk at room temperature for 1 h and incubated with primary antibodies against p-Smad, Smad or GAPDH at 4°C overnight. Filters were washed three times and incubated with horseradish peroxidase conjugated-secondary antibodies. Bands representing target proteins were detected using ECL Plus and ImageQuant LAS 4000 (GE Healthcare).

**Cell adhesion assay and antibody neutralization**

HES (5 × 10^5 cells) were seeded in a 6-well plate and cultured for 24 h. 12Z cells (3 × 10^5 cells) were seeded onto a 100 mm culture dish plate and cultured for 24 h. Medium was replaced and cells were incubated in serum free-medium with or without TGF-β for 24 h. HES and 12Z cells were first labeled with 5-chloromethylfluorescein diacetate (CMFDA; Invitrogen, Carlsbad, CA, USA) for 15 min at 37°C, then washed in 1 × phosphate-buffered saline (PBS) and gently transferred onto a Met-5A cell monolayer. After gentle shaking at 20 rpm for 20 min at 37°C, cells were washed three times with 1 × PBS to remove unbound cells. Attached HES and 12Z cells were visualized using a fluorescent microscope (200 × magnification), and quantified using ImageJ software (NIH, Bethesda, MD, USA). The number of cells in 4 randomly chosen areas in each well was used for statistical analysis. In experiments using neutralizing antibodies, HES cells were treated with monoclonal integrin αV, β1 and β4 antibodies (Abcam, Cambridge, UK) or a rabbit IgG control antibody (Abcam, Cambridge, UK) before being transferred onto a Met-5A cell monolayer and analyzed as described above.

**Statistical analysis**
Statistical analysis was performed using a Student’s t-test or one-way analysis of variance with Tukey’s post-hoc test using GraphPad Prism Software (GraphPad, CA, USA). Values are expressed as mean ± SD. Minimum significance level was set at a $P$ value of 0.05. At least 3 independent replications were performed for each experiment.
Supplementary Fig. 1. Schematic representation describing adhesion of endometrial cells to mesothelium by TGF-β1-mediated adhesion molecules expression.

This study shows that the secreted TGF β1 is involved in the increase of endometrial cell adhesion to mesothelium by enhancing expression of adhesion molecules including integrin αV, α6, β1, and β4.
References