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# Inhibition of CD44 N- and O-linked Glycosylation Decreases Endometrial Cell Lines Attachment to Peritoneal Mesothelial Cells

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## Abstract

Attachment of endometrial epithelial cells (EECs) and endometrial stromal cells (ESCs) to peritoneal mesothelial cells (PMCs) with and without inhibition of N- and O- linked glycosylation, viability of EECs and ESCs, and expression of CD44 surface density was evaluated. Inhibition of CD44 N- and O-linked glycosylation by using tunicamycin and/or B-GalNAc, significantly inhibits endometrial cell attachment to peritoneal mesothelial cells suggesting a role in establishment of early endometriotic lesions.

### Keywords

Endometriosis; CD44; glycosylation

The pathogenesis of endometriosis remains poorly understood. Sampson's Theory proposes that retrograde menstruation is the source of endometrial tissue that implants in the pelvis. Peritoneal cells secrete hyaluronan (HA), a glycosoaminoglycan. CD44, the primary receptor for HA, is a transmembrane glycoprotein which aids in cellmigration and adhesion. (3,4) Alternate splicing and post-translational glycosylation of CD44 is one regulatory step in CD44 binding to HA.(5,6) CD44 glycosylation either increases or decreases affinity to HA depending on specific glycosylation and cell type.(7,8)

The process of attachment of endometrial cells to peritoneal mesothelial cells (PMCs) is assisted by extracellular interactions. This is a possible novel target for pharmacologic therapy aimed to inhibit ectopic endometrial cells from adhering to PMC's.

Dechaud reported decreased adhesion of endometrial cells to PMCs after hyaluronidase treatment suggesting a role for CD44/HA in the attachment of endometrial cells to PMCs.(9)

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No prior study has assessed glycosylation in attachment of endometrial cells to PMCs. The purpose of this study was to evaluate the role of N- and O-linked glycosylation in attachment of endometrial cell lines to PMCs.

This study was approved by the Institutional Review Board at the University of Texas Health Science Center San Antonio.

A human immortalized cell line of endometrial epithelial cells (EECs), EM42, were grown in MCDM 131:Medium 199:alpha-MEM, 40:40:20, containing 10% fetal bovine serum (FBS) at 37°C. (10–12)

Immortalized human endometrial stromal cells (ESCs), CRL-4003 were grown in DMEM/F12 10% FBS, 1% BD Insulin, Transferrin, Selenous (ITS) +Premix Universal Culture Supplement (Catalog#354352, BD, Franklin Lakes, NJ), at 37°C. (13,14)

PMCs (LP9 line) were obtained from National Institutes of Health Aging Cell Repository Coriell Institute for Medical Research, Camden, NJ and grown in MCDB-131/Medium 199 (1:1) (Sigma-Aldrich, St. Louis, MO), epidermal growth factor (20 ng/mL), L-glutamine (2 μM), hydrocortisone (400 ng/mL), 1% antibiotic/antimycotic, and 15% FBS at 37°C. (10,15–17)

The attachment assay was performed as previously described.(10,15,18–19) Briefly, cells were harvested using non-enzymatic cell dissociation solution (Sigma-Aldrich, Catalog #5914). Viable cells were labeled with 5 uM Calcein-AM (Invitrogen) for 20 minutes at 37°C, and then were plated at 20,000 cells/well in 100 μl of charcoal stripped heat-inactivated 10% fetal bovine serum media (SHIS), over confluent PMC's in 96-well plates and cultured at 37° C for 1 hour. Fluorescence was determined using a Thermo-Forma Fluoroskan fluorometer with Ascent Software (Thermo-Fisher Scientific, Milwaukee, WI). The plates were submerged and inverted in a bath of phosphate-buffered saline containing calcium and magnesium (Invitrogen), and incubated at 37 °C for 15 minutes on an orbital mixer (Barnstead/Thermolyne, Dubuque, IA) at 20 rpm, allowing non-adherent endometrial cells to precipitate. SHIS (100 μl) was added to each well and fluorescence was assessed.

Cells were grown to subconfluence and treated with benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (B-GalNAc), an O-linked glycosylation inhibitor (78 μg/ml, 156 μg/ml, or 233 μg/ml), or tunicamycin (Tunica), an N-linked glycosylation inhibitor (0.5 μg/ml, 1 μg/ml, or 5 μg/ml) for 24 hours on 96-well plates with SHIS media. The fluorocein-conjugated lectin (20 μg/ml), Artocarpus integrifolia (Jacalin) which binds selectively to O-linked glycosylated sites was added to wells treated with B-GalNAc for 30 minutes at 37 °C. Similarly, Ricinus communis agglutinin-1 (RCA; 20 μg/ml) which binds N-linked sites was added to wells treated with Tunica. Plates were washed with PBS containing calcium and magnesium (Invitrogen, Carlsbad, CA), andfluorescence was assessed..

Cell viability was assessed using CellTiter-Glo® Luminescent Cell Assay (Promega, Madison WI). This assay determines the number of viable cells based on quantification of the ATP. (20) The cells were grown to sub-confluence on 96well plates with SHIS media. The concentrations of B-GalNAc (78, 156, and 233 μg/ml) and Tunica (0.5, 1.0, 5.0 μg/ml) were similar to previously reported studies.(21,22) Cells were lysed with the addition of 100 μl of the CellTiter-Glo® assay mixture for 30 minutes at room temperature, and luminescence was assayed.

CD44 surface density was assessed by flow cytometry. Cellswere treated with B-GalNAc or Tunica for 24 hours. Flasks were harvested, washed, and treated with CD44 monoclonal antibody, 5F12 clone (Lab Vision, Thermo Fisher Scientific), 0.6 μg/$10^6$ cells, followed by

CY-5 fluorescent conjugated secondary antibody staining (Jackson ImmunoResearch, West Grove, PA).

Each assay was run with 12 replicates. Analysis of variance (ANOVA) and Tukey's post-hoc analysis were preformed where appropriate.

The attachment of both lines to PMCs was decreased after Tunica treatment ($p<0.01$). There was a 4%, 25%, and 32% decrease in attachment of EECs compared to controls after treatment with the 0.5, 1, and 5 μg/ml doses of Tunica, respectively. There was a 54%, 68%, and 64% decrease in attachment of ESCs to PMC's after treatment with the 0.5, 1, and 5 μg/ml doses, respectively.

The attachment of EEC's ($p<0.01$) and ESC cells ($p<0.01$) to PMCs was decreased after B-GalNAc treatment. There was a 21%, 31%, and 25% decrease in attachment of EECs after treatment with the 78, 156, and 233 μg/ml doses of B-GalNAc,. There was a 16%, 21%, and 34% decrease in attachment of ESCs to PMCs after treatment with the 78, 156, and 233 μg/ml doses, respectively. When both Tunica and B-GalNAc were used to treat cell lines at the middle doses, there was a significant decrease in attachment compared to the control, but it was not more than either drug individually.

Both lines showed a decrease in glycosylation after Tunica treatment ($p<0.001$). There was a 10%, 31%, and 54% decrease in glycosylation of EECs after treatment with increasing doses. There was a 39%, 42%, and 48% decrease in glycosylation after treatment of ESC cells with increasing doses.

Both lines showed a decrease in glycosylation after B-GalNAc treatment ($p<0.001$). There was a 12%, 37%, and 34% decrease in glycosylation after treatment of EECs with increasing doses. There was no significant difference with the 78 μg/ml dose of B-GalNAc; however, there was an 18% and 12% decrease in glycosylation after treatment of ESC cells with the 156 and 233 μg/ml doses.

There was a decrease in proliferation following Tunica treatment in both EECs and ESCs. There was a 4%, 12%, 21% decrease in proliferation of EECs with increasing doses. ESCs showed a decrease of a 26%, 22%, and 23% in proliferation with increasing doses. B-GalNAc did not decrease proliferation of EECs or ESCs.

There were no differences in expression of CD44 surface density on either cell line with Tunica or B-GalNAc.

This study demonstrates that inhibition of N- and O-linked glycosylation inhibits EEC and ESC attachment to PMCs. We confirmed deglycosylation with lectins that specifically bind to N- and O-linked glycosylation sites. B-GalNAc and Tunica did not affect cell surface expression of CD44. B-GalNAc did not affect cell proliferation; however, Tunica did decrease proliferation. While Tunica decreases the cell viability, this does not affect the attachment or glycosylation results and analysis since only viable cells are used in these assays.

Glycosylation of the CD44 molecule causes conformational changes which may affect its ability to bind to HA. Different splice variants will have a differing quantity and type of glycosylation sites.(23,24) The exact mechanism whereby glycosylation of CD44 affects binding to HA is unclear. Several studies have found that N-linked glycosylation is an important regulator of binding in several cell lines. N-linked glycosylation inhibition can either increase or decrease binding depending on the cell line. Lesley found that CD44 expression did not correlate with binding to HA in ovarian cancer cell lines, but treatment

with Tunica decreased the adhesion to hylauronan.(25,26). They concluded that glycosylation of CD44 was more important than the amount of CD44 expression in determining adhesive potential, but these results could not be replicated in other cell types.(26,27) Katoh found that treatment of ovarian cells with a degylcosylating enzyme decreased CD44 binding to HA.(28) Bartolazzi reported that inmelanoma cells, mutation the CD44 N-linked glycosylation sites inhibited CD44 binding to HA.(6) O-linked glycosylation is also important in the interaction between CD44 and HA.(29–32) Dasgupta reported that blocking O-linked glycosylation in colon carcinoma cells, increased CD44 binding to HA.(29)

While there is still much to discover about these unique extracellular interactions, there is growing evidence that the interaction between CD44 and HA is involved in the attachment of endometrial cells to PMCs.(9,33) We recently demonstrated that there is increased expression of CD44 variant isoforms 6,7,8, and 9 in menstrual endometrium from women with endometriosis compared to women without endometriosis.(34) The additional exons increase glycosylation sites which may lead to increased ability to attach to PMCs.

In summary, deglycosylation of N- and O-linked sites decreases attachment of EECs and ESCs to PMCs. These findings suggest a role for CD44 N- and O-linked glycosylation in the development of early endometriotic lesions and has potential to lead to novel pharmacologic therapy to treat or prevent endometriosis.

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