Glycodelin levels in uterine flushings and in plasma of patients with leiomyomas and polyps: implications for implantation

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BACKGROUND: Glycodelin, a glycoprotein, is present in both blood plasma and uterine flushings. It has been implicated in the process of implantation and angiogenesis. During the secretory phase, progesterone secretion is related to glycodelin production. METHODS AND RESULTS: We obtained uterine flushings, prospectively, from 47 infertile patients during the proliferative phase. Patients were recruited from our university practice. Transvaginal ultrasound and sonohysterography permitted the stratification of patients into control, leiomyoma or polyp groups. Total plasma and uterine flushing glycodelin was measured with enzyme-linked immunosorbent assay. Blood was also analysed for progesterone. Uterine flushing glycodelin levels were significantly increased in patients with polyps when compared with controls. An increase in uterine flushing glycodelin levels was noted in patients with leiomyomas compared with controls, though not statistically significant. Plasma glycodelin levels were significantly increased in patients with leiomyomas and polyps when separately compared with controls. There was a significant relationship between plasma glycodelin production and progesterone levels in patients with polyps. CONCLUSIONS: Leiomyomas and polyps are growing tumours and thus produce significant plasma glycodelin levels. Uterine glycodelin flushings are elevated in patients with both polyps and leiomyomas. Elevated glycodelin levels in the follicular and peri-ovulatory period may impair fertilization and implantation.

Key words: endometrium/glycodelin/menstrual cycle/polyps/progesterone

Introduction

Glycodelin is a 28 kDa glycoprotein, containing 180 amino acids (Julkunen et al., 1988), encoded via a single gene located to chromosomal region 9q34 (Van Cong et al., 1991). Glycodelin-A is one of three isoforms, an endometrial-derived protein consisting of unique sialylated and fucosylated LacdiNac (GalNAc β1–4GlcNAc) oligosaccharide sequences (Dell et al., 1995).

Glycodelin is located in the glandular and surface epithelium of the endometrium (Julkunen et al., 1986a). It is the principal secretory phase product of endometrial epithelial cells. Glycodelin is lowest in the follicular phase of the cycle. On post-ovulatory day 4–5, significant glycodelin secretion is associated with an increase in ovarian progesterone secretion. Glycodelin levels peak by post-ovulatory day 12 (Julkunen et al., 1986a). During pregnancy, glycodelin levels in both decidualized endometrium and amniotic fluid peak at 10–18 weeks (Julkunen et al., 1985). Glycodelin is secreted into the uterine cavity and has been recovered in uterine flushings, rising 6 days after the LH peak (Li et al., 1993). Glycodelin has been implicated, among other proteins, as a facilitator of implantation (Clark et al., 1996). This rise in glycodelin, during ovulatory cycles, is probably due to progesterone (Julkunen et al., 1986b).

Physiologically, glycodelin has been implicated in many biological processes. Glycodelin inhibits, in a dose-dependent manner, the binding of human sperm to the zona pellucida (Oehninger et al., 1995). The absence of glycodelin around ovulation may be important for successful fertilization (Clark et al., 1996). In addition to glycodelin’s ‘contraceptive’ properties, it has been hypothesized that glycodelin is immunosuppressive, suppressing natural killer (NK) cell activity (Okamoto et al., 1991). At the time of implantation, glycodelin levels are elevated (Julkunen et al., 1985), and may protect the embryo, at the endometrial level, from NK cell destruction. Uterine flushings for glycodelin reveal increased glycodelin, in the uterine cavity, at the time of implantation (Li et al., 1993). If conception ensues, glycodelin levels remain elevated.

Glycodelin has recently been shown to be angiogenic (Song et al., 2001). We have shown that both benign and malignant gynaecological tumours produce elevated levels of plasma glycodelin and messenger RNA within their tissues (Horowitz et al., 2001).
et al., 2001). Moreover, a synthetic peptide mimic of glycodelin increases both the migration of human umbilical vein and arterial endothelial cells as well as capillary tube-like formation, both important components of angiogenesis (Song et al., 2001). Rapidly growing tumours and tissues may be producing glycodelin and thus promoting cell growth and the angiogenesis necessary to support this growth.

Our preliminary studies (unpublished data) reveal intense glycodelin staining in endothelial blood vessels within the myometrium surrounding leiomyomata, while myometrium that is some distance from leiomyoma stains poorly for glycodelin. We also noted an increase in blood vessels in the myometrium surrounding leiomyomases when compared with controls without leiomyomas. In the present study we investigated the production of glycodelin from patients with normal uterine cavities utilizing uterine flushing fluid obtained by sonohysterography. In addition, plasma levels of glycodelin were obtained from these patients. Subjects or patients with normal cavities were compared with abnormal cavities containing polyps and leiomyomases. We hypothesise that rapidly growing polyps and leiomyomases will produce significant levels of glycodelin in uterine flushings and plasma, when compared with patients without these lesions. The location and nature of the lesion will also influence glycodelin levels. Furthermore, we hypothesise that progesterone levels are not correlated with glycodelin levels.

Materials and methods

Patients

Forty-seven patients met inclusion criteria into this study. Patients who were undergoing infertility evaluation were recruited from the Reproductive Endocrinology and Infertility department at Emory University/Crawford Long Hospital from June 2000 to December 2001. Uterine flushings were obtained, via sonohysterography, during the proliferative phase (cycle day 5–14). The first day of menstruation was designated as day 1. Patient age ranged between 20 and 47 years (median = 35.4). Study patients had not taken steroid hormones 2 months prior to sonohysterogram, and had regular menstrual cycles 24–35 days in length. Prior to performing a sonohysterogram, a complete history and physical examination was performed. Patients were excluded from sonohysterography if they had an active uterine, vaginal, or pelvic infection. The Emory University Human Investigations Committee approved collection of uterine flushings and blood samples and the experimental protocol. Patients signed an informed consent for sonohysterography and uterine flushings as well as blood collection.

Uterine flushings

A complete transvaginal ultrasound was performed, including an evaluation of the uterus, adnexa, and cul de sac. A Pedersen speculum was then placed, and the cervix was cleansed with 0.9% bacteriostatic sodium chloride. This removed any excess mucus from the external os and cervical canal. A 5-F Hystero-Salpingography Catheter (Medical Device Technologies, Gainesville, FL, USA) was placed into the uterine cavity. The catheter balloon was inflated with 2.0 ml of air. Gentle traction was placed on the catheter to seal off the uterine cavity from the cervical canal. Under transvaginal ultrasound, 2.0 ml of 0.9% sodium chloride was injected (using a 10 ml luer lock syringe), over 10 s through the catheter side arm.

This fluid was recovered via the same syringe using gentle suction. Ultrasound was utilized to ensure that the 2.0 ml of fluid represented a true uterine flushing and not a contaminant from the Fallopian tubes or cervical canal (both of which produce glycodelin). This procedure was repeated five times, with a new syringe and a fresh 2.0 ml of sodium chloride. A total of 10 ml was utilized to retrieve these uterine flushings. Samples were stored at −80°C for subsequent glycodelin enzyme-linked immunosorbent assay (ELISA).

After the uterine flushings were recovered, ~20 ml of sodium chloride were placed in the uterine cavity with the catheter in order to evaluate for possible lesions (i.e. leiomyomases, polyps and adhesions).

Plasma samples

After the uterine flushings and ultrasound were complete, 10 ml of blood were obtained in a lithium heparin tube. The plasma was immediately stored at −80°C for glycodelin ELISA assay and progesterone evaluation.

Uterine flushing samples

The volume of fluid retrieved from the uterus for the 44 samples ranged from 2.5 to 9.5 ml. The retrieved volume was also variable in other studies (Hamilton et al., 1998). The total protein content was estimated in the lyophilized uterine flushing. The amount of total protein measured was adjusted per 1 ml of the volume retrieved in all the samples, and the final value was expressed as ng of glycodelin per ml of uterine flushing.

Enzyme-linked immunosorbent assay

100 µl of plasma and uterine flushing from each patient was added in separate triplicate wells of the ELISA plate, and incubated overnight at 37°C. The next day, plates were washed three times with phosphate-buffered saline (PBS), and blocking was done at room temperature for 1 h using 1% milk. Wells were washed three times with PBS and incubated for 2 h at 37°C with 100 µl of affinity column purified chicken anticylglycodelin antibody at a dilution of 1:50. Wells were washed three times with PBS and blocked with 1% milk for 1 h. The wells were again washed three times and secondary antibody (rabbit antichicken IgG conjugated with alkaline phosphatase) was added at a dilution of 1:35 000 in PBS and incubated at 37°C for 2 h. The wells were washed again and the substrate para-nitrophenyl phosphate was added. The optical density was measured at 405 nm after 30 min. The ELISA assay has an interassay variation of 7.92%, while the intra-assay variation was 6.82% (Poddar et al., 1998).

Statistical analysis

Normal distribution plots were applied to all three groups (normal cavity, leiomyomases and polyps) with regard to uterine flushing glycodelin, plasma glycodelin, and progesterone levels. Normality was not observed within the groups. Thus, non-parametric statistical analysis was used to compare means between groups. The hypothesis was that lesion groups would have increased protein production, thus a one-tailed alternative was used in all tests of significance comparing means. Spearman correlations between continuous variables were evaluated using SAS statistical program. P < 0.05 was considered statistically significant.

Results

A total of 44 subjects were included for evaluation. Three subjects were not included in the final analyses since their uterine cavities had adhesions. Twenty controls had no polyps or leiomyomases in their myometrium or uterine cavity, while
and intra-assay coefficients of variation were 1.7 and 1.9% respectively for progesterone.

There was no significant difference in the glycodelin or progesterone levels by age, ethnicity, or menstrual cycle day. Data were therefore aggregated for subsequent analyses.

**Variation in retrieved flushing volume (Table I)**

The retrieved volume ranged between 2.5 and 9.5 ml. The mean volume of retrieved fluid for the entire study was 7.03 ± 2.1 ml.

**Glycodelin levels in leiomyomas**

Comparison was made between patients with leiomyoma that distorted the cavity (submucosal protruding and submucosal pedunculated) and leiomyomas that did not distort the cavity (submucosal and intramural). Patients with ‘submucosal leiomyomas’ had leiomyomas adjacent to the endometrium that did not distort the uterine cavity. We found no significant differences in plasma or flushing glycodelin levels between these two groups.

Since the sample size of each type of leiomyoma was small (see Table II) and there were no significant differences between them with regard to flushing or plasma glycodelin, we pooled all the leiomyoma together and compared them with the control. ELISA revealed a statistically significant increase in plasma glycodelin levels, but not uterine glycodelin flushing levels, when compared with controls (Figures 1 and 2). Though uterine flushing levels were not significantly elevated statistically, the leiomyoma group showed a much larger increase than the controls (534 ± 354.8 versus 359.1 ± 200.4 ng/ml).

**Progesterone levels and leiomyomas**

There was no significant difference between lesion location and progesterone levels. This comparison was made between patients with leiomyoma that distorted the cavity (submucosal protruding and submucosal pedunculated) and leiomyomas that did not distort the cavity (submucosal and intramural). No differences in progesterone levels were seen in pooled patients with leiomyoma compared with controls.

**Glycodelin levels in polyps**

Uterine cavities with polyps produced significantly more plasma and uterine flushing glycodelin when compared with the controls (Figures 1 and 2). Since polyps distort the uterine cavity, there was a significantly larger increase in flushing glycodelin levels when compared with controls (Figures 1 and 2).

Table I. Patients’ characteristics

<table>
<thead>
<tr>
<th>Patients’ characteristics</th>
<th>Total no. of patients</th>
<th>Age (years)</th>
<th>Menstrual cycle daya</th>
<th>Uterine flushing retrieved (ml)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (normal cavity and myometrium)</td>
<td>20</td>
<td>34.9 ± 4.2</td>
<td>9.6 ± 2.0</td>
<td>6.5 ± 2.3</td>
</tr>
<tr>
<td>Intramural myoma (normal cavity)</td>
<td>5</td>
<td>37.2 ± 2.3</td>
<td>9.2 ± 2.4</td>
<td>7.4 ± 2.9</td>
</tr>
<tr>
<td>Submucosal myoma (adjacent to endometrium, normal cavity)</td>
<td>2</td>
<td>37.5 ± 0.71</td>
<td>10.5 ± 0.71</td>
<td>9.8 ± 0.35</td>
</tr>
<tr>
<td>Submucosal protruding leiomyoma</td>
<td>3</td>
<td>34.7 ± 4.9</td>
<td>8.7 ± 1.2</td>
<td>8.0 ± 0.87</td>
</tr>
<tr>
<td>Submucosal pedunculated leiomyoma</td>
<td>2</td>
<td>37.5 ± 3.5</td>
<td>10.5 ± 0.71</td>
<td>8.5 ± 0.70</td>
</tr>
<tr>
<td>Polyp</td>
<td>12</td>
<td>34.9 ± 7.5</td>
<td>10.4 ± 2.2</td>
<td>6.9 ± 1.5</td>
</tr>
<tr>
<td>Mean totals</td>
<td>44</td>
<td>35.4 ± 5.0</td>
<td>9.8 ± 2.0</td>
<td>7.03 ± 2.1</td>
</tr>
</tbody>
</table>

Values are means ± SD.

aMenstrual cycle variability did not affect flushing or plasma glycodelin levels.

bPatients with fibroids had significantly more uterine flushing retrieved when compared with controls (P = 0.01).

Table II. Classification of control and lesion subjects

<table>
<thead>
<tr>
<th>Lesion</th>
<th>Flushing glycodelin (ng/ml)</th>
<th>Plasma glycodelin (ng/ml)</th>
<th>Plasma progesterone (ng/ml)</th>
<th>Lesion volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal cavity and uterus (n = 20)</td>
<td>359.1 ± 200.4</td>
<td>36.5 ± 13.9</td>
<td>1.12 ± 2.6</td>
<td>Lesion-free</td>
</tr>
<tr>
<td>Intramural (n = 5) (normal cavity)</td>
<td>486.4 ± 336.9</td>
<td>48.4 ± 23.6</td>
<td>0.75 ± 0.2</td>
<td>447.47 ± 541.33</td>
</tr>
<tr>
<td>Submucosal (n = 2) (adjacent to endometrium, normal cavity)</td>
<td>255 ± 92.6</td>
<td>47.5 ± 30.41</td>
<td>1.03 ± 0.38</td>
<td>1160.4 ± 1481.3</td>
</tr>
<tr>
<td>Submucosal protruding (n = 3)</td>
<td>703.66 ± 552.48</td>
<td>68.67 ± 32.35</td>
<td>0.60 ± 0.1</td>
<td>120 ± 45.27</td>
</tr>
<tr>
<td>Submucosal pedunculated (n = 2)</td>
<td>680 ± 128.69</td>
<td>47.5 ± 20.51</td>
<td>0.29 ± 0.27</td>
<td>36.17 ± 34.1</td>
</tr>
<tr>
<td>Total for leiomyomas (n = 12)</td>
<td>534 ± 354.8</td>
<td>53.2 ± 23.9</td>
<td>0.73 ± 0.25</td>
<td>415 ± 676.64</td>
</tr>
<tr>
<td>Polyps (n = 12)</td>
<td>519.1 ± 293.4</td>
<td>70.3 ± 25.3</td>
<td>0.62 ± 0.7</td>
<td>1.09 ± 0.91</td>
</tr>
</tbody>
</table>

Values are means ± SD.

24 had leiomyomas or polyps. The characteristics of these patient groups are summarized in Table I. Table II presents patients with polyps and leiomyomas based on their location with regard to uterine flushing glycodelin, plasma glycodelin and progesterone. Samples from cavities with adhesions were collected (n = 3), but were not included in the final analysis. The ELISA assay used for detection of glycodelin levels in uterine flushings and serum plasma, has previously been validated in our laboratory. Serum progesterone concentrations were determined by a commercially available assay (Immulite; Diagnostic Products Corp., Los Angeles, CA, USA). The inter- and intra-assay coefficients of variation were 1.7 and 1.9% respectively for progesterone.
There does not appear to be a relationship between uterine glycodelin and progesterone with regard to the leiomyoma group (Figure 1). Patients with polyps produced significantly more plasma glycodelin than leiomyoma patients (Figure 1).

Relationship between uterine flushing glycodelin, plasma glycodelin and progesterone with regard to polyps

There is a significant relationship between plasma glycodelin production and progesterone levels ($r = -0.65$, $P = 0.023$). There is no relationship between plasma glycodelin and uterine flushing glycodelin ($r = 0.35$, $P = 0.27$, not significant). No relationship exists between uterine flushing glycodelin and plasma progesterone ($r = 0.08$, $P = 0.81$, not significant).

Relationship between uterine flushing glycodelin, plasma glycodelin and progesterone with regard to the leiomyoma group

There does not appear to be a relationship between uterine flushing glycodelin and progesterone ($r = -0.27$, $P = 0.39$, not significant) or between plasma glycodelin production and progesterone levels ($r = -0.20$, $P = 0.54$, not significant), or plasma glycodelin and uterine flushing glycodelin ($r = 0.09$, $P = 0.78$, not significant).
polyp volume and uterine glycodelin flushing \((r = 0.23, P = 0.48, \text{not significant})\).

**Discussion**
Uterine leiomyomata are the most common benign pelvic neoplasms of women and are found in ~25% of women in their childbearing years. Their causal relationship to infertility and pregnancy loss is controversial. Studies have been conducted to clarify the relationship between leiomyomata and reproductive outcome within IVF populations. There appears to be a relationship between implantation and pregnancy rates and leiomyoma location. In our experience, and that of others (Fahri et al., 1995), when the uterine cavity is distorted with a leiomyoma, implantation and pregnancy rates are decreased. However, there is controversy in the literature with regard to intramural leiomyomas that do not distort the uterine cavity. In recent retrospective (Eldar-Geva et al., 1998) and prospective (Hart et al., 2001) studies, implantation and pregnancy rates were significantly lower when intramural patients were compared with controls without leiomyomas. In contrast, Surrey et al. reported a decrease in implantation rates, but not live birth rates, in women aged 40 years with intramural leiomyomas (Surrey et al., 2001).

The data concerning polyps and implantation rates or pregnancy loss is scant. In our practice, polyps are often present prior to IVF. Lass et al. analysed IVF data from patients with polyps of \(<2 \text{ cm}\). Pregnancy rates were not decreased, but there was a trend toward increased pregnancy loss (Lass et al., 1999).

The present study measured glycodelin in plasma and uterine flushings in patients with leiomyomas. These samples were collected in the proliferative phase of the menstrual cycle, at which time, both plasma and uterine flushing glycodelin levels are extremely low. Both uterine flushing and plasma glycodelin levels rise in the secretory phase with flushing levels significantly elevated above serum levels. In order to compare patients within the polyp and leiomyoma groups with regard to glycodelin, we first noted that cycle day was not correlated with glycodelin or progesterone production.

After obtaining roughly double the amount of control samples when compared with patients in the leiomyoma and polyp groups, we decided to analyse our data. The sample size of the individual leiomyoma lesion types was small. Thus, patients with leiomyomas, as a whole, were compared with controls (without leiomyomas or cavity abnormalities). This control group represents a sample of our infertile population and they are not true normal controls. There was a significant increase in glycodelin plasma levels in the leiomyoma and polyp groups. Our previous work indicates that glycodelin is a potent angiogenic factor (Song et al., 2001), and is significantly expressed in the serum of patients with leiomyomas (Horowitz et al., 2001). In addition, blood vessel endothelial cells in the myometrium surrounding leiomyomas stain intensely for glycodelin when compared with control myometrium (without leiomyomas) (unpublished data). These data further support these assumptions.

We hypothesize that glycodelin contributes to leiomyomata and polyp growth by facilitating neovascularization, and thus aiding in the nutrient transport needed for growth. The cell of origin for glycodelin in leiomyoma is unclear. It is either ‘trapped’ into blood vessel endothelial cells like human umbilical cord endothelial cells (Zhou et al., 2000), or is synthesized locally within blood vessel endothelial cells surrounding the leiomyoma.

When location of leiomyomata was analysed, intramural and submucous (non-distorting, \(n = 7\)) versus submucousal protruding and pedunculated (distorting the cavity, \(n = 5\)), no significant difference was noted in uterine flushing glycodelin levels. Patients with leiomyoma (\(n = 12\)) produced uterine flushing glycodelin levels that were increased (though not significant) \((534 \pm 354.8 \text{ ng/ml})\) when compared with controls \((359.1 \pm 200.4 \text{ ng/ml})\).

Polyps are growths of endometrial tissue covered by epithelium. They have glands, stroma and blood vessels (Kurman and Mazur, 1994), all of which point to their ability to produce glycodelin within the uterine cavity. Subjects with polyps produced significantly increased amounts of uterine glycodelin flushings. We presume that glycodelin is able to ‘leak’ out of the blood vessels surrounding polyps and leiomyomas and become soluble within the 0.9% sodium chloride uterine flushing. This is the first study to note increased glycodelin flushings in cavities with known leiomyomas and polyps. We hypothesize that an intramural leiomyoma, which is intensely enveloped within myometrium would produce more plasma and uterine flushing glycodelin when compared with a pedunculated or protruding myoma. With a larger data set, this may be borne out.

Glycodelin has been shown to inhibit sperm–oocyte binding and NK cell activity. In ovulatory human endometrium, glycodelin levels are very low 6 days before, and 5 days after, ovulation (peri-ovulatory period). Low glycodelin levels may facilitate or permit fertilization to take place. During implantation (6 days after ovulation) glycodelin secretion into the uterine cavity increases significantly. This increase may be pivotal in suppressing NK cell activity and rendering the endometrium receptive to implantation. There have been many proposed mechanisms for decreased implantation in patients with both distorting and non-distorting leiomyomas and polyps. Even though glycodelin was measured in the middle and late proliferative phase of the menstrual cycle, the present data allow speculation that fertilization and endometrial receptivity may be altered by increased glycodelin production in the uterine cavity of patients with leiomyomas and polyps at the time (peri-ovulatory) when uterine glycodelin levels should be absent or low. In this study, peri-ovulatory patients with polyps and leiomyomas (distorting and non-distorting), produced elevated uterine glycodelin levels, compared with controls.

In the proliferative phase of an ovulatory cycle, both serum progesterone and glycodelin levels are very low and start rising after ovulation. The rise in progesterone precedes the significant rise in glycodelin after ovulation. All of our samples were taken in the proliferative phase. In the polyp group, there was a significant negative relationship between plasma glycodelin production and progesterone production.
addition, plasma glycodelin levels were significantly elevated in patients with polyps compared with controls. This would suggest that progesterone is not the major regulatory factor in the follicular phase as it is for the secretory phase. In addition we see a relationship, though not significant, between plasma glycodelin production and uterine glycodelin flushings in patients with leiomyomas. Li et al. also noted a relationship between serum and uterine flushing glycodelin levels in patients with endometrial adenocarcinomas (Li et al., 1998). Similar to polyps and leiomyomas, adenocarcinoma cells may produce glycodelin or growth factors that initiate the production of glycodelin. This uterine glycodelin is then absorbed into the plasma. Recently, we reported that lysophosphatidic acid induces glycodelin gene expression in tumour cells (Ramachandran et al., 2002). Results from our previous studies and others have also reported that phorbol myristate acetate stimulates studies and others have also reported that phorbol myristate acetate stimulates glycodelin gene expression in K562 cells. These studies would suggest that glycodelin synthesis during the early proliferative phase of the menstrual cycle might be under non-hormonal regulation.

The data presented here are from a pilot study evaluating both uterine flushing and plasma glycodelin levels, in relation to progesterone levels, in patients with leiomyomata and polyps. The major limitation of our study is the small sample size in the lesion groups. Even with a ‘small sample size’, we were able to show a statistically significant increase in both plasma (P < 0.0001) and uterine flushing glycodelin (P = 0.038) levels in polyp patients when compared with controls. Patients with leiomyoma had significant plasma glycodelin levels (P = 0.0224) when compared with controls. Uterine glycodelin flushings were increased, but not statistically significantly, in the leiomyomata group when compared with controls (0.45 power). Using a power analysis, we would need to quadruple the leiomyoma sample size in order to detect a difference with 0.80 power. Thus, this pilot study provides evidence that glycodelin and its regulation in leiomyoma and polyps is worthy of further study.

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References


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