Altered glycosylation in peri-implantation phase endometrium in women with stages III and IV endometriosis

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BACKGROUND: Endometriosis is a common cause of infertility and pelvic pain. Lectin histochemistry has shown that glycan expression is a sensitive marker of differentiation in the normal endometrium. Endometrial biopsies were taken during the implantation window from women with subfertility and advanced (stage III and IV) endometriosis to evaluate specific glycans bound by lectins from *Dolichos biflorus* agglutinin (DBA) and *Vicia villosa* agglutinin (VVA), which detect related but distinct glycan sequences regulated by progesterone action.

METHODS: Endometrial tissue from 12 women with subfertility and advanced endometriosis and 11 healthy controls were taken on days 19–24 of the menstrual cycle and processed into either epoxy resin or paraffin wax. Lectin histochemistry was analysed using light microscopy to quantify the amount of glandular reaction product.

RESULTS: There was a significant ($P = 0.011$) reduction in DBA binding to endometrium from patients with endometriosis compared with controls, which was not seen with VVA ($P = 0.135$). Three stage IV biopsies and one stage III biopsy completely failed to bind DBA and, of these, three showed moderate glandular binding of VVA. DBA and VVA binding differed significantly ($P = 0.0039$) in the endometriosis specimens whereas in controls no significant difference was detected ($P = 0.812$).

CONCLUSION: Secretory phase glycosylation in women with advanced endometriosis differs from that in healthy women with a reduction in fucosylated $N$-acetylgalactosamine sequences bound by DBA. Shorter VVA-binding glycans are not significantly affected. In addition to indicating abnormalities of epithelial differentiation, these findings may be directly relevant to implantation failure, as blastocyst attachment requires a critical interaction with the epithelial glyocalyx.

Key words: endometriosis / glycosylation / human / implantation window / infertility

Introduction

Endometriosis occurs in 6–10% of women of reproductive age and rises to 30% in women experiencing infertility (Giudice and Kao, 2004; Bulun, 2009). Subfertility in women with endometriosis remains unexplained; hypotheses range from distorted pelvic architecture (Amer, 2008) to defective endometrial maturation (Sharpe-Timms, 2001) with progesterone resistance (Burney et al., 2007) and expression of inappropriate biochemical markers during the window of implantation, the period of ~48 h around days 20–22 of the menstrual cycle when implantation occurs (Sharkey and Smith, 2003).

Specific integrins, selectin ligands and other adhesion molecules are up-regulated in normal endometrium during the implantation window (Genbacev et al., 2003; Aplin, 2006, Singh and Aplin, 2009), and a proposed model of blastocyst adhesion suggests the involvement of selectins and trophinin in initiating implantation (Fukuda and Sukihara, 2008). Sugars may play a role in ligand-receptor interaction between the blastocyst and the endometrium (Aplin, 1991; Jones and Aplin, 2009). In addition to selectin ligands (Margarit et al., 2009), there are other apical epithelial surface glycans that show increased abundance in the mid-secretory phase in normal women (Aplin et al., 1998; Jones et al., 1998, 2009). One of these is a group of structures recognized by *Dolichos biflorus* agglutinin (DBA). DBA binds to the blood group A moiety $\text{GalNAc}_1\text{Fuc}_1\text{Gal}_1\text{GlcNAc}_1$; Etzler and Kabat, 1970] as well as to a related sialo-oligosaccharide containing the four NeuAc$\text{GalNAc}_1\text{Gal}_1\text{GlcNAc}_1$ antennae and known as Sda$^+$ (Klisch et al., 2008). During the proliferative...
phase, DBA binding is not detectable, but after appearing in the mid-secretory phase it increases in intensity in the glandular epithelium and secretions (Jones et al., 1998, 2009).

The lectin from *Vicia villosa* agglutinin (VVA) shows a similar increase in reactivity in secretory phase endometrium (Kupryjanczyk, 1989; West and Cope, 1989; Jones et al., 1998). VVA is a tetramer containing A and B subunits; the dominant isolectin in our preparations (Vector Laboratories, Peterborough, UK) appears to be B4. In haemagglutination inhibition assays, the Sd^a^ active Tamm–Horsfall glycoprotein, containing the serologically active structure, GalNAc^b^1,4(NeuAc^a^2,3)Gal^b^1,4GlcNAc^b^3Gal, inhibited B4 lectin agglutination of both Cad and Tn-exposed erythrocytes (Tollefsen and Kornfeld, 1987). However, VVA binds more weakly to the longer oligosaccharides recognized by DBA (Tollefsen and Kornfeld, 1987), whereas recognizing terminal GalNAc in shorter structures, including (GalNAc^a^1,3Gal^b^1) and peptides containing two adjacent O-linked GalNAc residues (Tn antigen; Tollefsen and Kornfeld, 1983a, b; Puri et al., 1992).

In the current study, eutopic endometrial biopsies from healthy controls and women with advanced endometriosis were evaluated, comparing the binding patterns of DBA and VVA lectins, in order to test the hypothesis that specific secretory phase epithelial Golgi-associated terminal glycosyltransferase activities may be altered in eutopic endometrium from infertile women suffering from endometriosis.

### Materials and Methods

Endometrial biopsies between 2 and 30 mm long and ~2 mm in cross-sectional diameter were taken from 12 subfertile women with regular menstrual cycles of between 28 and 32 days (study group, mean age 34, range 22–41 years). All women had visually and biopsy-proven endometriosis and had undergone endometrial curettage and laparoscopic excision of endometriosis (nine for stage III and three for stage IV endometriosis) between days 19 and 24 of the cycle based on histological dating and the first day of their last menstrual period (LMP). The control group consisted of 11 women (mean age 35, range 29–40 years) of proven fertility, with regular menstrual cycles and without pelvic endometriosis, as confirmed by laparoscopy, which was performed for other indications. Endometrial biopsies from all 11 women were taken between days 20–24 of the cycle. Five of these specimens were specifically collected for this study, with dating confirmed by microscopic examination and LMP, whilst the other six specimens were appropriately dated, Bouin’s fixed, wax-embedded specimens from the archives of the Histopathology Department at Central Manchester University Hospitals NHS Foundation Trust. Spearman’s Rank Correlation showed there to be no significant difference between the age ranges of the two groups of women (P = 0.45). All of the women with endometriosis complained of one or more of the following symptoms: chronic pelvic pain, dysmenorrhoea and/or deep dyspareunia. Neither the study subjects nor the controls had received hormonal therapy in the 3 months before laparoscopy. Endometriosis at the time of biopsy was staged according to the revised American Fertility Society (rAFS) system and confirmed by stereological examination.

The specimens were from two sites: Department of Obstetrics and Gynaecology, University of Padua, Italy (courtesy of Dr P Litta, University of Padua) and Department of Reproductive Medicine, Central Manchester University Hospitals NHS Foundation Trust, Manchester, UK and were fixed and processed into epoxy resin as previously described (Jones et al., 2009). The study was approved by the Local Research Ethics Committee (Ref No 06/Q1407/173) as well as by the Universities of Padua, Italy and Manchester, UK. All women gave written, informed consent to participate in the study.

### Lectin histochemistry

Plastic embedded material was sectioned at 0.5 μm and stained with 1% toluidine blue in 1% borax on a hotplate in order to identify suitable areas for staining. Sections were then cut at 0.75 μm, mounted on APES coated slides (Maddox and Jenkins, 1987) and dried at 50°C for 2 days before staining with DBA and VVA as previously described (Jones et al., 2009). The wax blocks were cut at 4 μm and similarly mounted on APES-coated slides before staining with the same lectins using a similar protocol adapted for wax sections (Jones et al., 1998). In both cases, negative controls were carried out by substituting 0.05 M TBS pH 7.6 containing 1 mM CaCl_2 for lectin. The inclusion of material from healthy women constituted a positive control for the staining technique.

### Image analysis

Sections were coded with regard to their disease or control status and analysed using light microscopy by two independent observers (D.L.M. and C.J.P.J.) blind to the identity of the tissue. The quantity of stain associated with the endometrial glands over the entire section was ranked quantitatively from − (negative) to ++++ (extensive) and the location of the staining (secretions, apical surface, intracytoplasmic) noted. In the case of the plastic blocks, these consisted of between one and six transversely cut pieces of tissue to give sections of stained tissue 3 x 3 to 3 x 7 mm in size, the diamond knife used to cut the sections being 3 mm wide. The number of gland profiles available for examination ranged from 30 to 180. The tissue strips in wax sections ranged from ~10 x 10 to 25 x 15 mm in size and contained between 160 and over 3000 gland profiles. The Mann–Whitney *U*-test was used for comparing the same stain across control and disease samples and Wilcoxon Signed Rank Test for comparing the two different stains within either the control or study samples.

### Results

#### Control group

The binding to endometrial glands of DBA and VVA was assessed during the mid-secretory phase (Figs 1A, B and 2). Table I shows the overall level of glycan binding, along with the location: in the cytoplasm, on the glandular surface or secreted into gland lumens. The majority of DBA and VVA binding occurred on the epithelial surface or in glandular secretions, with a modest amount in the cytoplasm. The quantity of DBA and VVA staining was approximately equal. All of the control women showed DBA and VVA binding, with only one sample showing stronger DBA staining than VVA. There was no significant difference between the DBA and VVA binding in the control group (Wilcoxon Signed Ranks test; *P* = 0.812, 95% CI = −0.17 to 0.33).

#### Endometriosis samples

The binding of DBA and VVA was assessed in the mid-secretory phase eutopic endometrium of women with stage III and IV endometriosis (Figs 1C–F, 2 and Table II). Binding of both lectins was more intense in glandular secretions and on the surface of gland cells than in cytoplasm. Overall there was greater binding of VVA than DBA in these biopsies: all three stage IV biopsies completely failed to bind DBA, whereas two of these specimens weakly bound VVA, the
other stage IV sample failing to bind both lectins. One stage III sample failed to bind DBA, but had strong expression of VVA. All other samples from the group of endometriosis patients had weak binding of DBA lectin, and moderate to strong VVA binding. Statistical analysis showed a significant difference in staining with the two lectins within the endometriosis group (Wilcoxon Sign Ranks test; \( P = 0.0039, 95\% \text{ CI} = -0.67 \text{ to } -0.165 \)).

Control versus endometriosis specimens

Comparison of DBA and VVA binding between control and endometriosis groups showed a significant reduction in DBA staining (Mann–Whitney \( U \)-test; \( P = 0.011, 95\% \text{ CI} = 0.33 \text{ to } 1.67 \)) and a smaller, non-significant reduction in VVA staining (\( P = 0.135, 95\% \text{ CI} = -0.17 \text{ to } 1 \)). In all cases, no staining was observed in the negative controls.

Discussion

Although there is some controversy in the literature regarding the incidence of DBA and VVA-binding glycans (Lee and Damjanov, 1985; Soderström, 1987; Yen et al., 1986, Kupryjancyzk, 1989), previous studies agree that DBA binding is increased during the mid-secretory phase in healthy fertile women (Duncan et al., 1988; Aoki et al., 1989; West and Cope, 1989; Jones et al., 1998). The present study

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**Figure 1** (A, B) Control eutopic endometrium (Day 22): equivalent areas stained with DBA and VVA showing strong staining of the apical epithelial surfaces (short arrows) and secretions, with some cytoplasmic staining also. (C, D) Grade III endometriosis (Day 22): equivalent areas of an endometrial biopsy stained with DBA and VVA showing greatly diminished levels of binding compared with controls, with DBA reduced more than VVA. (E, F) Grade IV endometriosis (Day 20) biopsy stained with DBA and VVA. There is virtually no staining with DBA but vestiges of stain can be seen with VVA. Scale bars: 100 \( \mu \text{m} \).
demonstrates a decrease in DBA-binding mid-secretory phase glycans in women with endometriosis. DBA and VVA-binding glycans are similarly distributed in the control group. DBA binding is significantly reduced in the endometriosis group compared with controls, whereas that of VVA is diminished, but not to a statistically significant extent. At one extreme, women with stage IV endometriosis completely lack DBA binding, but have moderate-to-strong VVA binding, suggesting that as disease severity increases, the imbalance becomes more pronounced. In keeping with earlier pilot data (Jones et al., 2009), there is evidence that transcription of a significant number of glycosyl transferases increases in the secretory phase (Singh and Aplin, 2009). Evidence for progesterone-dependent glycan expression has been reported in both baboon and human where treatment with the anti-progestin RU486 (mifepristone) resulted in the loss of secretory phase DBA staining (Jones et al., 1998). In the mouse uterus, fucosyl transferase appears to be steroid modulated (White and Kimber, 1994; Sidhu and Kimber, 1999).

Loss of DBA binding may be associated with the progesterone resistance experienced by women with endometriosis (Attia et al., 2000; Burney et al., 2007; Bulun, 2009; Jones et al., 2009). Studies in our laboratory (unpublished data), as elsewhere, have been unable to demonstrate significant differences in the distribution of progesterone receptors (PR) between healthy and endometriotic eutopic endometrium (Attia et al., 2000; Jones et al., 1995). However, a marginally significant decreased ratio of progesterone receptor B isoforms has been reported in endometrial tissue from women with endometriosis (Igarashi et al., 2005) and this might not have been detected by localization protocols. Differences in the methylation status of the progesterone receptor B-isoform (PR-B) promoter have been seen between eutopic endometrium and controls (Wu et al., 2006b) but these also did not reach statistical significance. Likewise, PR-B expression was reduced in the eutopic specimens investigated, but not to a significant extent. Despite the presence of PR, there may be defects in recruitment of downstream signalling molecules. Recent evidence shows that several genes targeted by progesterone are dysregulated in women with endometriosis during the window of implantation (Kao et al., 2003, Bulun et al., 2006) and at other times in the menstrual cycle (Wu et al., 2006a; Giudice and Kao, 2004). Our results suggest possible defects in P-dependent up-regulation of enzymes responsible for terminal fucosylation, 2,3-sialylation and, to a lesser extent, α-N-acetylgalactosaminylation in the endometrium of women with endometriosis, which may lead to a reduction in DBA and VVA binding. Restricted activity of 1,2-fucosyltransferase may be particularly important, as a fucose substituent must be present before αGalNAc can be added to the

![Figure 2](image-url)
oligosaccharide chain (Oriol et al., 1992). Residual VVA binding activity can probably be accounted for by terminal α-N-acetylgalactosamine in shorter structures. Sialidase treatment has little effect on DBA staining in human endometrium, but greatly increased VVA binding, indicating the existence of cryptic sites masked by the presence of terminal sialic acid (Jones et al., 1998).

Glycosylation at the maternofetal interface is important in embryomaternal recognition and the establishment of pregnancy (Aplin, 1991, 1999; Jones and Aplin, 2004, 2009). Fucosylated glycoconjugates have been suggested to play a role in uterine receptivity in different species (Hey et al., 1998). Residual VVA binding activity at the glycocalyx at implantation (Aplin, 1999) such as MUC-1 (Hey et al., 1994; Aplin et al., 1998; Meseguer et al., 2001), expression of which is altered in endometriosis (Burney et al., 2001). Thus, although the altered glycosylation reported in this study may be broadly indicative of a disease-associated epithelial phenotype, we suggest that glycosylation changes may pay a direct role in the subfertility experienced by women with advanced pelvic endometriosis.

### Authors’ Roles

D.L.M.: Analysed and interpreted data, co-wrote manuscript. C.J.P.J.: Co-designed study, obtained ethical approval, carried out sectioning and staining of biopsies, co-wrote manuscript. J.D.A.: Contributed to critical discussion and interpretation of data, co-wrote manuscript. L.G.N.: Co-designed study, obtained patient consent, carried out biopsies, contributed to discussion.

### Acknowledgements

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### Table II Distribution and quantity of DBA and VVA staining in women with stage III and IV endometriosis during the peri-implantation phase.

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Staining range from – (absent) to +++++ (extensive).

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### References


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