Introduction

Endometriosis is a gynaecological disorder defined by the presence of extraterine endometrial epithelial glands and stroma. Although histologically similar, distinct biochemical differences have been noted between ectopic and eutopic endometrial tissues (for a review, see Sharpe-Timms, 1997).

Evidence is also accumulating which suggests that eutopic endometrium from women with endometriosis has intrinsic qualitative and quantitative differences as compared with endometrium of women without this disorder (Fedele et al., 1990; Isaacson et al., 1990; Ishihara et al., 1991; McBean and Brunsted, 1993; Lessey et al., 1994; Akoun et al., 1995; Wingfield et al., 1995; Noble et al., 1996; Osteen et al., 1996; Tseng et al., 1996; Sugawara et al., 1997; Gebel et al., 1998; Piva et al., 1998). Despite their potential importance, the role of most of these factors in endometriosis is poorly understood.

Our prior research has demonstrated that in vitro, both rat and human endometriotic lesions synthesize and secrete a protein which we called endometriosis protein-I (ENDO-I; Sharpe and Vernon, 1993; Sharpe et al., 1993). We have subsequently shown that partial ENDO-I amino acid sequence and full-length ENDO-I cDNA sequence are virtually identical to those of hepatic haptoglobin (Sharpe-Timms et al., 1998; Piva and Sharpe-Timms, 1999). As recent data suggest that hepatic haptoglobin possesses novel immunosuppressive and angiogenic activities in association with certain disease states (for a review, see Dobryszycza, 1997), expression and localization of extrahapatic haptoglobin (ENDO-I) in the peritoneal cavity may contribute to the pathogenesis of endometriosis by altering immune function or promoting neovascularization of developing endometriotic lesions. Therefore, this study evaluated in vivo expression and localization of ENDO-I in human endometrial and endometriotic tissues.

Materials and methods

Tissue procurement

Matched endometrium and peritoneal endometriotic lesions were collected from 22 women with endometriosis (proliferative stage, n = 7; secretory stage, n = 15) at laparoscopic evaluation for endometriosis. Additional endometrial tissues (controls) were obtained from 20 women without endometriosis (proliferative stage, n = 10; secretory stage, n = 10) at the time of elective laparoscopic sterilization or following hysterectomy for benign reasons. Patients were not taking steroid hormones or steroid-modulating medications at the time of sample collection. Endometrial biopsies were collected while patients were under general anaesthesia using a Pipelle (Unimar, Wilton, CT, USA) endometrial suction curette. Full-thickness endometrial sections were obtained from hysterectomy specimens. Peritoneal endometriotic lesions, including red petechiae and reddish-brown lesions, were elevated with biopsy forceps, and the area was circumscribed by either laser or sharp dissection. Endometrial tissues were classified as proliferative (cycle days 4 to 14) or secretory (cycle days 16 to 28) according to the date of the last menstrual period. Menstrual cycle stage was histologically confirmed (Noyes et al., 1950) by the Department...
of Pathology at our university. The presence of endometriosis was also confirmed histologically. Informed consent, as approved by the University of Missouri Institutional Review Board–Health Sciences Section, was obtained from all patients before collection of tissues.

**In-situ hybridization**

Site-specific ENDO-I mRNA expression was identified in tissue sections by non-isotopic in-situ hybridization using digoxin (Dig)-labelled antisense and sense riboprobes generated from cDNA as previously described in detail and validated (Olson et al., 1997). The riboprobes used in these experiments were originally developed to study the extrahepatic haptoglobin called glycoprotein-42 (GP-42), a gene expressed by rabbit endometrium at the time of embryo implantation (Hoffman et al., 1996; Olson et al., 1997). Briefly, the haptoglobin 5` cDNA probes were prepared by polymerase chain reaction (PCR) amplification. The 5` PCR primer design was based on a highly conserved region of the β-chain N-terminal amino acid sequences, especially between residues 7 and 13, contained in GP-42 (Olson et al., 1997), rabbit hepatic haptoglobin (Chow et al., 1983) and human hepatic haptoglobin (Maeda, 1985). This region was also conserved in the ENDO-I β-chain N-terminal amino acid sequence (Sharpe-Timms et al., 1998). The program Primer Designer (Scientific and Educational Software, Durham, NC, USA) and the human hepatic haptoglobin β-chain nucleotide sequences (Maeda, 1985) were used to find a compatible 3` primer (Olson et al., 1997). The sequence for the 3` primer was also confirmed within the human ENDO-I nucleotide sequence (Piva and Sharpe-Timms, 1999).

In contrast to previous studies (Olson et al., 1997) in which frozen tissue sections were prepared, formalin-fixed, paraffin-embedded tissues were used in this study. Tissues were sectioned at 5 µm and dried on glass slides at 60°C for 50 min. Tissue sections were de-waxed in two changes of fresh xylene for 5 min each, re-hydrated through a series of ethanol (100%, 100%, 95%, 70%) for 1 min each, and washed twice in two changes of diethylpyrocarbonate (DEPC-treated water. In-situ hybridization using the Dig-labelled antisense (positive detection) and sense (negative control) riboprobes (diluted 1:800) and subsequent immunological detection (1:500 dilution of alkaline phosphatase-conjugated anti-Dig antibody) was then performed as described previously (Olson et al., 1997). Endometrial and endometriotic glandular epithelium also served as an internal negative control for this procedure, as we have previously shown that isolated endometrial glands do not synthesize ENDO-I de novo (Sharpe et al., 1993).

**Immunohistochemistry**

Immunohistochemistry was used to identify site-specific ENDO-I protein localization. Semi-adjacent sections (5 µm) of the formalin-fixed, paraffin-embedded tissues used for the in-situ hybridization were prepared for immunohistochemistry. Immunohistochemistry was performed using an avidin–biotin complex peroxidase procedure according to manufacturer’s instructions (Vectastain ABC, Vector, Burlingame, CA, USA) and as used previously in our laboratory to study immunolocalization of granulocyte-macrophage colony-stimulating factor in similar tissues (Sharpe-Timms et al., 1994). A polyclonal anti-haptoglobin antibody (Dako, CA, USA) which we have shown previously to recognize ENDO-I protein (Sharpe-Timms et al., 1998), was used as the primary antibody for the ENDO-I immunohistochemical staining. Tissue sections were also incubated with blue haematoxylin counterstain) localized in the functionalis zone of the endometrial stroma, but not in the basalis region or the endometrial epithelial glands (levels of endometrial ENDO-I mRNA and protein expression are listed in Table I).

Figure 1. Pattern of ENDO-I mRNA expression and protein localization in endometrial tissues. (A) In-situ hybridization using the antisense probe demonstrated ENDO-I mRNA expression (punctate purplish staining) in the functionalis zone of the endometrial stroma, but not in the basalis region or the endometrial epithelial glands (absence of ENDO mRNA in the endometrial glands also served as an internal negative control). (B) In a semi-adjacent section to the tissue shown in (A), no signal was detected by in-situ hybridization using the sense probe (negative control). (C–F) Immunohistochemical staining using an anti-haptoglobin antibody of (C) secretory and (D) proliferative eutopic endometrium from women with endometriosis and (E) secretory and (F) proliferative eutopic endometrium from women without endometriosis. Like ENDO-I mRNA expression (A), ENDO-I protein (copper-brown immunohistochemical ENDO-I staining with blue haematoxylin counterstain) localized in the functionalis zone of the endometrial stroma, but not in the basalis region or the endometrial epithelial glands (levels of endometrial ENDO-I mRNA and protein expression are listed in Table I). f = endometrial functionalis; b = endometrial basalis; m = myometrium. Scale bar measurements are in µm.
with normal rabbit sera or phosphate-buffered saline solution substituted for primary antibody as negative controls for the immunohistochemical staining (not shown; absence of staining corresponded to endometrial glands which served as internal negative controls). Peroxidase activity was demonstrated by incubation with 3,3′-diaminobenzidine substrate, yielding a brown intracellular precipitate; this confirmed peroxidase staining. Sections were counterstained with haematoxylin.

**Data analysis**

Expression and localization differences were evaluated and scored on an inverted phase-contrast microscope by two different observers blinded to the tissue origin using the H-SCORE system (Lessey et al., 1994). The H-SCORE, representing levels of staining intensity and distribution, is calculated using the following equation:

\[ \text{H-SCORE} = \Sigma \text{Pi} (I + 1) \]

where I is the intensity of staining with a value of 1, 2 or 3 (weak, moderate or strong respectively), and Pi is the percentage of stained cells for each intensity, varying from 0 to 100%. Intra-observer variation was <5%, and inter-observer variation was <10%; data were averaged between observers. Photomicrographs were made using Kodak 400 ASA film (Eastman Kodak, Rochester, NY, USA).

In-situ hybridization and immunohistochemical H-SCORE data passed both normality testing \((P = 0.068, P = 0.511)\) respectively and equal variance testing \((P = 0.247, P = 0.103)\) respectively as determined using SigmaStat Statistical Software (Jandel Scientific Software, San Rafael, CA, USA). Differences in the mean level of ENDO-I mRNA expression between tissues (endometrium from controls, endometrium from women with endometriosis, and endometriotic lesions) during the stages of the menstrual cycle (proliferative and secretory cycle stages) were evaluated using two-way analysis of variance (ANOVA) using SigmaStat Statistical Software. The two-way ANOVA statistical model used was:

\[ \text{ENDO-I mRNA expression} = \text{tissue} \times \text{cycle stage} \times (\text{tissue} \times \text{cycle stage interaction}) \]

When F-testing indicated significance in all variables including tissue × cycle stage interaction, the effects of cycle stage on ENDO-I mRNA expression by the various tissues were evaluated using Tukey’s multiple comparison tests.

The same two-way ANOVA model was used to evaluate differences in the mean level of ENDO-I protein localization. As no statistical difference in ENDO-I protein localization was noted for the cycle stage variable \((P = 0.142)\), the data from the proliferative and secretory stages were pooled within each tissue type (endometrium from controls, endometrium from women with endometriosis, and endometriotic lesions) and a one-way ANOVA was performed. Tukey’s multiple comparison post-hoc analysis was subsequently used to evaluate determine differences in ENDO-I protein localization between the tissue types.

Differences in the proportions of eutopic endometrial tissues from women with or without endometriosis expressing ENDO-I mRNA or ENDO-I protein were evaluated using Fisher’s exact test for two-tailed probability.

**Results**

**Site-specific expression and localization of ENDO-I mRNA and protein**

When present, endometrial ENDO-I mRNA was expressed by the functionalis zone of the endometrial stroma, but not the

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**Table I.** The amount of ENDO-I mRNA as detected by in-situ hybridization and H-SCORE analysis (mean ± SEM)

<table>
<thead>
<tr>
<th>Cycle stage</th>
<th>Tissues</th>
<th>P (between tissues)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Endometrium</td>
<td>Endometriotic lesions</td>
</tr>
<tr>
<td>Control</td>
<td>0.64 ± 0.28&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.50 ± 0.29&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Secretory</td>
<td>0.17 ± 0.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.80 ± 0.37&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>P (between cycle days)</td>
<td>NS</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

<sup>a,b</sup>Values with different superscripts were significantly different as detected by Tukey’s multiple comparison post-hoc analyses.

NS = not significant.

**Table II.** The amount of ENDO-I protein as detected by immunohistochemistry and H-SCORE analysis (mean ± SEM)

<table>
<thead>
<tr>
<th>Cycle stage</th>
<th>Tissues</th>
<th>P (between tissues)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Endometrium</td>
<td>Endometriotic lesions</td>
</tr>
<tr>
<td>Control</td>
<td>0.79 ± 0.26</td>
<td>1.70 ± 0.54</td>
</tr>
<tr>
<td>Secretory</td>
<td>1.07 ± 0.25</td>
<td>2.21 ± 0.23</td>
</tr>
<tr>
<td>P (between cycle days)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Proliferative + secretory</td>
<td>0.93 ± 0.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.06 ± 0.23&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values with different superscripts were significantly different as detected by Tukey’s multiple comparison post-hoc analyses.

NS = not significant.
Evidence suggests that altered peritoneal immune phenomena and angiogenic activity may contribute to and/or permit establishment, growth or remodelling of ectopic endometrial tissue in the peritoneal cavity (for a review, see Ryan and Taylor, 1997). In view of recent data suggesting immunosuppressive and angiogenic activities of hepatic haptoglobin in association with certain disease states, the discovery of localized expression of extrahepatic haptoglobin (ENDO-I) by endometriotic lesions and eutopic endometrium from women with endometriosis in vivo is both exciting and provocative. It was shown recently (Berko et al., 1999) that cytokine-induced release of haptoglobin from human neutrophils subsequently increased the amount of haptoglobin at the sites of infection and led to modification of acute inflammatory response in situ. It is interesting to speculate that endometriosis-associated haptoglobin, END-I, may be interacting with peritoneal immune cells to alter immune function. There is a significant correlation between macrophage haptoglobin immunoreactivity and reduced adherence capacity of macrophages isolated from the peritoneal fluid of women with endometriosis as compared with peritoneal macrophages

Figure 2. Pattern of ENDO-I mRNA expression and protein localization in endometriotic lesions. (A, B) In-situ hybridization using an antisense probe demonstrates ENDO-I mRNA expression (dark purple staining) in the stroma of the endometriotic lesions, with preferential localization noted towards peripheral surfaces. (C–F) Immunohistochemical staining of additional semi-adjacent sections using the anti-haptoglobin antibody (intense copper-coloured immunohistochemical staining with blue haematoxylin counterstaining) showed that localization of ENDO-I protein in the stroma of the endometriotic lesions mirrored ENDO-I mRNA expression (A and B), with preferential localization noted towards peripheral surfaces. Endometrial glandular epithelium served as internal negative controls for ENDO-I immunostaining (levels of endometriotic lesion ENDO-I mRNA and protein expression are listed in Table II). Scale bar measurements are in µm.
from women without this disease (Sharpe-Timms et al., 2000a and unpublished data). This relates to prior reports of altered macrophage function in women with endometriosis (Halme et al., 1987; Dunselman et al., 1988; Taylor et al., 1997).

Stimulation of angiogenesis is another newly recognized biological function of haptoglobin. Using an established in vitro model of angiogenesis, haptoglobin was identified as an angiogenic factor in sera from patients with systemic vasculitis (Cid et al., 1993). Angiogenic activity is elevated in the peritoneal fluid of women with endometriosis (Oosterlynck et al., 1993; McLaren et al., 1995, 1996), yet the key mediators of angiogenesis and their sources in the peritoneal cavity of women with endometriosis are not clear. We postulate that ENDO-I may interact with peritoneal endothelial cells to promote neovascularization of the lesions in women with endometriosis. Indeed, endometriotic implants from rats with surgically induced disease (Vernon and Wilson, 1985) produce ENDO-I (Sharpe et al., 1993) and are highly vascularized within 40 days of implantation, with considerable amounts of neovascularization as early as 36 h post-implantation (K.E.Cox and K.L.Sharpe-Timms, unpublished observation). Ongoing investigations in our laboratory concern the angiogenic potential of ENDO-I and its role in neovascularization of endometriotic lesions.

These and other novel functions for haptoglobin have been inferred, in part, through differential glycosylation (van Dijk et al., 1994; Turner, 1995). Glycans can act as recognition and anti-recognition signals in immune function and glycan receptors (or membrane lectins) function in cell–cell recognition, adhesion, cell differentiation and therefore directly affect neighbouring cellular interactions (Montreuil, 1996; Biermann et al., 1997; Clark et al., 1997). Preliminary analysis of ENDO-I carbohydrate composition has revealed the presence of specific glycans (Sharpe-Timms et al., 2000b; Piva and Sharpe-Timms, 2000) that could provide a mechanism by which ENDO-I may misdirect local immune response, function in adhesion of endometrial cells in the peritoneal cavity, elicit differentiation of endothelial cells to promote neovascularization of the endometriotic lesions, and may explain why—although retrograde menstruation is nearly universal—only some women have implantation of ectopic endometrium in the peritoneal cavity.

In summary, these studies recapitulate our earlier in vitro investigations showing that endometriotic lesions produce significant amounts of ENDO-I (Sharpe and Vernon, 1993; Sharpe et al., 1993) and document that endometriotic lesions also produce ENDO-I in vivo (Sharpe-Timms et al., 1998; Piva and Sharpe-Timms, 1999). Furthermore, these studies provide novel evidence that endometrium from women with endometriosis expresses significantly more ENDO-I mRNA as compared with eutopic endometrium from women without this disease during the secretory stage of the menstrual cycle. This difference was not previously detected when reverse-transcription PCR (RT-PCR) was used to evaluate relative ENDO-I expression in similar tissues (Piva and Sharpe-Timms, 1999), and may be due to differences in methodology. Elevated amounts of ENDO-I protein localization observed in endometriotic lesions and eutopic endometrium from women with endometriosis, regardless of the menstrual cycle stage, may reflect the immunohistochemical detection of hepatic haptoglobin as well as ENDO-I. Endometriotic tissues or dysfunctional endometrial tissues might contain more blood due to increased capillary permeability, and therefore contain more haptoglobin. Yet overall, the fact that more ENDO-I is produced by, and localizes in, eutopic endometrium from women with endometriosis suggests that ENDO-I may be useful in the development of greatly needed, minimally invasive strategies to provide early detection of endometriosis and/or to facilitate the development of unprecedented therapeutic approaches for the management of this enigmatic disease. These developments could significantly reduce the substantial health burden attributed to this malady and increase the reproductive capacity of afflicted women.

Acknowledgements

This manuscript is dedicated to the memory of Dr Loren H.Hoffman, Vanderbilt University, for his enthusiasm for the study of extrahepatic haptoglobin in reproduction. The riboprobes used in these experiments were generous gifts from Gary Olson, Vanderbilt University, Nashville, TN, USA. This work was supported in part by the National Institutes of Health, Office of Research for Women’s Health HD 29026-05S1, TAP Holdings, Inc. and the University of Missouri Research Board, and was presented in part at the 55th Annual Meeting of the American Society for Reproductive Medicine in Toronto, Ontario, Canada.

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K.L.Sharpe-Timms et al.
Differential expression of endometriotic haptoglobin


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