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Haptoglobin Expression in Endometrioid Adenocarcinoma of the Uterus

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Objective: *Elevated serum haptoglobin (Hp) concentrations have been reported in patients with malignant diseases. We have shown that Hp is produced by and localizes only in the stroma and not the epithelium of endometriotic lesions, which share many characteristics of carcinoma. Furthermore, Hp mRNA and protein are found exclusively in the stroma of eutopic endometrium from women with endometriosis and not those without endometriosis. We hypothesized that characteristic patterns of* Hp *gene expression and protein localization in endometrioid adenocarcinoma of the uterus may provide insight into the clinical utility of Hp as a tumor marker or alternative therapeutic approach.* ***Methods:*** *Biopsies of endometrioid adenocarcinoma tumors of the uterus and their adjacent nonaffected endometrium were collected. Normal endometrium was collected from healthy women. Haptoglobin messenger RNA (mRNA) levels were quantified by quantitative polymerase chain reaction (Q-PCR). Haptoglobin protein cell-specific localization was identified by immunohistochemistry.* ***Results:*** *Haptoglobin mRNA levels were significantly greater (*$P < .005$*) in endometrioid adenocarcinoma and adjacent nonaffected endometrial tissues than normal endometrium. No correlation was found between Hp levels and cancer stage (*$P = .673$*) or grade (*$P = .739$*). Haptoglobin protein localized in both stromal and glandular epithelial cells of endometrioid adenocarcinoma and their adjacent nonaffected tissue but not in control endometrium.* ***Conclusions:*** *Our results have identified, for the first time, unique patterns of Hp mRNA expression and protein localization in the stromal and glandular epithelial cells of endometrioid adenocarcinoma of the uterus. We propose that this unique pattern of endometrioid adenocarcinoma Hp expression may be developed as a novel diagnostic marker. Modulation of Hp, with its immunomodulatory and angiogenic properties, may generate novel methods of prevention or treatment for endometrial cancer.*

Key words: Haptoglobin, endometrioid adenocarcinoma, endometrium, tumor marker.

INTRODUCTION

Endometrial cancer is the most common gynecologic malignancy in the United States with a projected 40 100 new cases and 7470 deaths in 2008. Approximately US$1.8 billion is spent yearly in the United States on treatment of this disease.[1] Worldwide each year, 142 000 women are diagnosed and 42 000 women die from this disease.[2]

Endometrial carcinoma is broadly categorized into several subtypes, the most common of which are papillary serous adenocarcinoma and endometrioid adenocarcinoma. Serous adenocarcinoma is nonestrogen-related, highly aggressive and has much lower cure rates than the more common endometrioid adenocarcinoma.[3,4] It constitutes only 10% of all endometrial carcinomas.[5] Endometrioid adenocarcinoma is estrogen related and characterized by endometrioid histology. Endometrioid cancers are most commonly low grade (well to moderately differentiated), early stage (confined to the uterus), indolent lesions often associated with endometrial hyperplasia. Endometrioid adenocarcinoma represents

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70% to 80% of newly diagnosed cases of endometrial carcinoma in the United States.

If endometrioid adenocarcinoma is detected early, it can be treated with surgical therapy alone and the long-term survival is excellent (85% at 5 years). However, if these tumors are not caught early and spread beyond the uterus, chemotherapy and/or radiation are necessary to control the disease and long-term prognosis decreases significantly.[6,7] Unfortunately, no reliable biochemical markers for early-stage disease detection exist to guide the management of this condition. The identification of endometrial cancer-specific biomarkers is critical to early detection and better monitoring of tumor progression.

Elevated serum haptoglobin (Hp) concentrations have been observed in many malignant diseases including cervical,[8,9] ovarian,[10] lung,[11] and breast[12] cancers. Some of these studies have suggested that serum Hp may be of potential clinical value in these patients. To our knowledge, there have been no reports on the expression of Hp in endometrial cancer.

Our prior research using a large variety of quantitative and qualitative molecular and proteomic approaches has shown that ectopic endometriotic lesions and eutopic endometrium, specifically the stromal but not epithelial cells, from women with endometriosis de novo synthesize and secrete endometriotic Hp.[13-20] Little or no Hp mRNA or protein was found in eutopic endometrium from women without endometriosis.[14,16,20] Endometriosis is a common gynecologic disorder that shares some properties with invasive carcinoma such as attachment and implantation. Therefore, we hypothesized that the pattern of *Hp* gene expression and protein localization in endometrioid carcinomas may be different from that of endometriotic lesions or eutopic endometrium from women with endometriosis or normal healthy endometrium and therefore may have potential diagnostic, prognostic, and/or therapeutic value in these patients. The objective of this research was to quantify Hp mRNA levels and identify sites of Hp protein localization in endometrioid adenocarcinoma of the uterus.

MATERIALS AND METHODS

Human Participants and Tissue Collection

Informed consent, as approved by the University of Missouri Institutional Review Board–Health Science Section, was obtained from each participant prior to any specimen collection. Specimens from the cancer group (tumors and their adjacent nonaffected endometrium) and control group were collected either by biopsy or by extraction following hysterectomy. Tissues were placed into cold sterile phosphate-buffered saline (PBS) and transported to the laboratory for processing. The study group consisted of control patients who were free of endometrial cancer (n = 6) and patients with clinical and histopathological diagnosis of endometrioid adenocarcinoma of the uterus (n = 16). Tumors were classified by stage (I and III) and grade (1-3) based on the International Federation of Gynecology and Obstetrics cancer classification (FIGO).

Tissue Processing

Each specimen was divided into 2 pieces. One half was snap frozen in liquid nitrogen then stored at −80°C for subsequent analysis of Hp mRNA levels by real-time (RT) quantitative polymerase chain reaction (Q-PCR). The remaining half was fixed in 10% neutral buffered formalin (Fisher Scientific, Pittsburgh, Pennsylvania) for 24 hours then rinsed with PBS prior to processing for routine staining with hematoxylin and eosin and for analysis of protein localization.

Haptoglobin Gene Expression

Using the RNeasy Mini Kit (Qiagen, Valencia, California) per manufacturer's instructions, RNA from endometrial tissue (10-20 μg) was extracted then treated with RNAse-free DNAse (Qiagen) to eliminate any possible genomic DNA. Purified RNA was quantified using the NanoDrop ND-1000 spectrophotometer (Wilmington, Delaware), and RNA quality was assessed by ethidium bromide–stained agarose gel electrophoresis. Complementary DNA (cDNA) was constructed from 100 ng mRNA using random primers and the First Strand cDNA Synthesis kit for RT-PCR (Avian Myeloblastosis Virus [AMV]; Roche, Indianapolis, Indiana) per manufacturer's instructions.

Haptoglobin mRNA levels were measured by performing duplex Q-PCR with a Cepheid Smart Cycler II (Cepheid, Sunnyvale, California). The duplex method allowed for the coamplification of the Hp mRNA with the endogenous gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in the same reaction tube. The duplex Q-PCR assay was optimized following a standard curve method where liver cDNA was used as control template in 10-fold serial dilutions.

The Hp primers and probe were custom designed by Kathy L. Sharpe-Timms in collaboration with Scott Rose, PhD, of Integrated DNA Technologies, Inc (Coralville, Iowa). The sequences were as follows:

Forward Primer 5′-GCAAGACCAACCAAGATG-3′
Reverse Primer 5′-CATCATTGCCTGAGTCCACT-3′
TaqMan MGB Probe 5′-/56-FAM/TGGGAGCTGTCATTGCCCTCCTGCT/3IAbFQ/-3′

Glyceraldehyde 3-phosphate dehydrogenase primers and probe were obtained from a human GAPDH kit (Applied Biosystems, Foster City, California; sequence not provided by the manufacturer). The following reagents were used for amplification in 25 μL final reaction volume: 0.5 Omni mix HS PCR master mix bead (Takara Bio Inc, Shiga, Japan), Hp primers (250 nmol/L) and probe (250 nmol/L), 1.25 μL human GAPDH endogenous control, 4 μL cDNA, and nucleotide-free water. Thermocycling conditions were as follows: 30 s/95°C + 35 cycles for 15 s/95°C + 1 min/60°C. Complementary DNA from human liver was used as positive control. Water was substituted for cDNA as a negative control. Relative levels of the *Hp* gene were calculated using the $2^{(-\Delta\Delta Ct)}$ method.[21]

Haptoglobin cDNA Sequencing

The Q-PCR products from liver, endometrial tumors, their adjacent nonaffected endometrium, and cancer-free endometrium plus the water negative control were electrophoresed through a 3% agarose gel that was subsequently stained with ethidium bromide and viewed under ultra violet (UV) light. To confirm that the Q-PCR product extracted from the agarose was Hp, the amplified band was extracted using QIAquick gel extraction kit (Qiagen) following manufacturer's instructions. The sequence of the Q-PCR product was determined by The DNA Core at The University of Missouri-Columbia. Sequences were evaluated for matching sequences using the online Basic Local Alignment Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov).

Immunohistochemistry

Immunolocalization of Hp was performed on 5-μm thick serial sections from the formalin-fixed, paraffin-embedded tissues. The sections were heated at 60°C for 30 minutes, deparaffinized in xylene for 10 minutes then dehydrated through a series of graded ethanol (100%, 95%, and 70%). Sections were then incubated in 1% hydrogen peroxide for 30 minutes to inhibit endogenous peroxidase followed by a 20-minute incubation in 1% normal horse sera (Vector Laboratories, Burlingham, California) to block nonspecific binding.

The following primary antibodies were applied for 1 hour at room temperature: polyclonal rabbit anti-human Hp (3 μg/mL; Dako, Carpinteria, California), monoclonal mouse anti-human cytokeratin 8, 18 to help confirm the identity of endometrial epithelial cells (10 μg/mL; Santa Cruz Biotechnology, Santa Cruz, California), and monoclonal mouse anti-human CD45 to help distinguish immune cells from endometrial cells (20 μg/mL, Santa Cruz Biotechnology). Phosphate-buffered saline was substituted for primary antibody as staining control. Additional semiadjacent tissue sections were stained with either the rabbit anti-human Hp as described or normal rabbit immunoglobulin G (IgG; 3 μg/mL) as a negative control for the Hp antibody immunostaining. Corresponding secondary antibodies (rabbit or mouse IgG, 7.5 μg/mL; Dako) were applied for 1 hour at room temperature. Antibody–antigen complex was detected using the Avidin Biotin Peroxidase Complex reagent (Vector Laboratories) for 30 minutes. Color reaction was developed with diaminobenzadine tetrahydrochloride substrate (Vector Laboratories) for 1 minute, and counterstaining was performed with Harris hematoxylin (Fisher Scientific) for 1 minute.

Statistical Analyses

Kruskal-Wallis one-way analysis of variance (ANOVA) was used to compare differences in the age between the cancer-free, endometrioid adenocarcinoma, and adjacent nonaffected groups. Haptoglobin expression data from the 3 tissue sources were log transformed to achieve normal distribution. To compare differences in Hp mRNA levels among tissue sources, one-way ANOVA with Duncan's post hoc testing methods for pairwise multiple comparisons was used with the following model: Hp = tissue source (endometrioid adenocarcinoma, adjacent nonaffected, and cancer-free tissue) × interaction. To compare differences in Hp mRNA levels between tumor stages and among tumor grades, two-way ANOVA was used with the following statistical model: Hp = cancer stage (stages I and III) × cancer grade (grades 1, 2, and 3) × interaction. All statistical analyses were performed using Sigma Stat software (Systat Software, Inc, Point

Table 1. Patient Profiles

	Number of Patients	Age[a] (Years) Median (25%, 75%)
Endometrioid adenocarcinoma		50 (50, 60)
Surgical stage		
I	12	
III	4	
Histologic grade		
G1	6	
G2	6	
G3	4	
Adjacent to tumor	10	53 (46, 62)
Cancer-free	6	48 (43, 50)

[a] Kruskal-Wallis one-way analysis of variance, ages are not significantly different among the 3 groups, $P = .087$.

Richmond, California). A P value of $<.05$ was considered significant.

RESULTS

Patient Profiles

Table 1 shows the patient profiles with the number and type of specimens, including stage and grade of the tumors when present. Patient ages did not differ between the groups.

Haptoglobin Gene Expression

Quantitative PCR assay validation. The duplex real-time Q-PCR assay was optimized following a standard curve method where liver cDNA was used as a control template in 10-fold serial dilutions. Using 4 μL cDNA, human haptoglobin primers and probe (250 nmol/L) and human GAPDH endogenous control primers and probe (1.25 nmol/L) yielded an efficient duplex assay where the slopes of were −3.179 and −3.292, respectively, which reflects the desired 3.3 value and the high efficiency of this duplex assay.

Quantification of Hp mRNA levels. As determined by Q-PCR, the relative Hp mRNA levels in endometrioid adenocarcinoma and adjacent nonaffected endometrium were significantly greater ($P < .005$) than that of the controls (Figure 1). No significant differences in relative Hp mRNA levels were detected between cancer stages ($P = .511$) or cancer grades ($P = .739$).

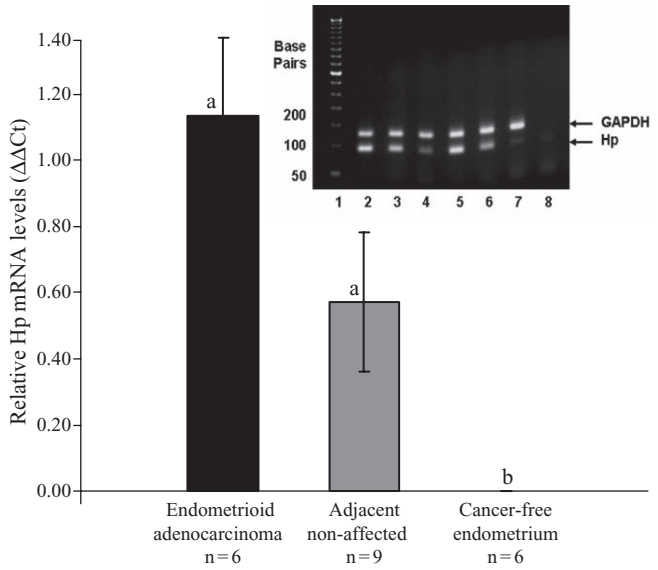

Figure 1. Representative haptoglobin (Hp) messenger RNA (mRNA) levels in endometrioid adenocarcinoma, their adjacent nonaffected endometrium, and normal cancer-free endometrial tissue. Differential Hp mRNA expression between samples was visualized by electrophoretic separation of real-time quantitative polymerase chain reaction (Q-PCR) products (inset). Lane 1 shows the DNA ladder indicating the number of base pairs. The 87-bp Hp real-time Q-PCR product was visualized in samples of liver (positive control, lane 2), endometrioid adenocarcinoma (lanes 3 and 5) and to a lesser extent in adjacent nonaffected endometrial tissues (lanes 4 and 6). (Lanes 3 and 4 and lanes 5 and 6 are matched tumor and adjacent tissue samples from two participants.) Trace amounts of the 87-bp band were present in cancer-free endometrium (lane 7), but no product was found in the water blank (negative control, lane 8). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) bands were found at 120 bp in all samples (lanes 2-7) but not in the water blank (lane 8). Significant differences identified by analysis of variance (ANOVA) and Duncan's all pairwise multiple comparison procedure (a, b; $P < .05$).

Electrophoretic separation of the Q-PCR products identified an 87 base pair band for the liver, endometrioid adenocarcinoma, and adjacent nonaffected endometrium, which was absent in the cancer-free endometrium or the blank negative control. Complementary DNA sequencing confirmed that the 87 base pair band was *Homo sapiens* Hp mRNA with a 100% maximal identity. The haptoglobin nucleotide sequence was deposited in the following Gene bank: http://www.ncbi.nlm.nih.gov with the accession number NM005143.2. The 120 base pair bands representing GAPDH were present in equivalent amounts in all samples, but absent for the blank negative control (Figure 1 inset).

Immunohistochemical localization of Hp. Localization of Hp protein was found in all stromal and many of the glandular

epithelial cells of the endometrioid adenocarcinoma and its adjacent nonaffected endometrial tissue. Little or no Hp localization was seen in and cell type of the cancer-free tissues (Figure 2).

DISCUSSION

For the first time to our knowledge, our research has shown that endometrioid adenocarcinoma of the uterus but not endometrial tissue from cancer-free patients has elevated levels of *Hp* gene transcripts. Haptoglobin protein localized in the tumors from patients with endometrioid adendocarcinoma of the uterus, but not in endometrium of cancer-free patients, correlating well with that of the gene expression. Surprisingly, Hp mRNA was elevated in the nonaffected endometrium adjacent to endometrioid adenocarcinoma tumors. This finding suggests pathophysiological activities exist in the normal looking tissues prior to obvious tumor development, which may contribute to the establishment of malignancy. These data also illustrate that healthy looking endometrial tissue from a uterus with endometrioid adenocarcinoma should not be used as control tissue in research studies.

The current study has shown significant amounts of Hp mRNA expression and protein localization in the stromal cells and some epithelial cells from endometriod adenocarcinoma tissues and its adjacent nonaffected endometrial tissue, regardless of menstrual cycle stage or postmenopausal status. To develop diagnostic markers or therapeutic approaches, Hp mRNA expression and protein localization in endometrioid adenocarcinoma must be different from other endometrial disorders.

Prior research in our laboratory found a distinct pattern of intense Hp protein immunostaining confined to the stromal component of endometriotic lesions,[20] shed endometrial tissue fragments,[19] and eutopic endometrium from women with endometriosis,[20] which was increased in the secretory stage of the menstrual cycle.[18,19] Only scant Hp protein localization clustered around the spiral arteries was seen in the endometrium from women without endometriosis, suggesting this Hp may have been of hepatic origin. We did not find epithelial cell Hp protein localization in any endometrial tissues studied from women with or without endometriosis. With the goal of identifying a rapid marker for endometrial cancer, quantification of Hp protein from isolated epithelial and stromal cells was not performed as it would involve more laborious methods.

We have also shown significant levels of Hp mRNA expression by the stromal component of endometriotic lesions, eutopic endometrium, and by isolated endometrial stromal cells but not epithelial cells from women with endometriosis and only trace amounts of Hp mRNA in the stromal component of eutopic endometrium from women without endometriosis.[14,16,19,20] These observations parallel the endometriosis-associated Hp immunolocalization data. The striking stromal cell-specific Hp mRNA expression and protein localization in eutopic endometrium from women with endometriosis and the near absence of Hp in normal endometrial tissues compared to the stromal and epithelial patterns of Hp in endometrioid adenocarcinoma of the uterus may be used to establish a rapid diagnostic tool to distinguish between these 2 diseases.

By contrast, Berkova and colleagues[22] reported that endometrial stromal and epithelial cells collected from normal participants undergoing tubal ligation contained Hp protein as detected by immunoblot analysis. Immunohistochemical analysis also showed localization of Hp in both endometrial epithelial and stromal cells, which was menstrual cycle stage specific. Haptoglobin was present in the greatest amounts in secretory-stage endometrium compared to proliferative stage endometrium, with more Hp staining in the epithelial and stromal cells in secretory stage compared to moderate staining of Hp protein in the stromal cells of proliferative endometrium. Yet, the source of the Hp in the Berkova study awaits further confirmation as neither Hp mRNA levels nor de novo synthesis and secretion of endometrial Hp were measured.

There are several explanations for these discrepancies in endometrial Hp protein staining patterns. One possibility may be differences in the epitopes recognized by Hp antibodies from different sources. Comparisons of the Hp staining patterns by the 2 Hp antibodies used in these studies in the same tissues are warranted.

Another possibility involves the source of the Hp found in the endometrium. Both laboratories made significant efforts to remove serum Hp from the tissues. Yet, despite dissection of endothelial vessels, washings, and preincubations, hepatic Hp from the serum may still be present in these well-vascularized tissues. Hence, Hp localized in normal endometrium may be of hepatic and not endometrial origin. An antibody made specifically against endometriotic/endometrial Hp, which does not recognize hepatic Hp, may provide resolution and be of clinical utility. We have reported endometriosis-associated Hp is differentially glycosylated compared to

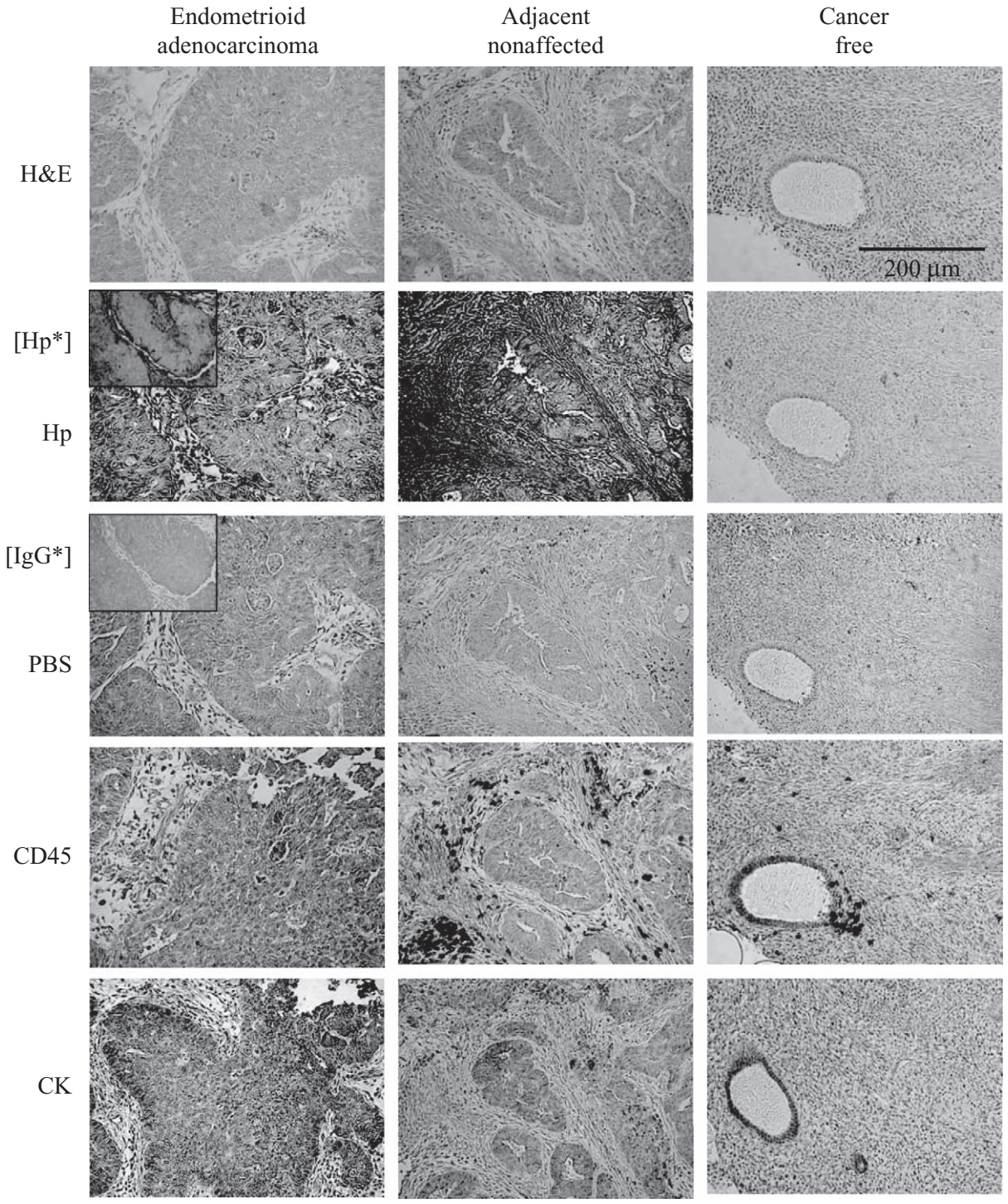

Figure 2. Immunolocalization of haptoglobin (Hp) protein in endometrioid adenocarcinoma tumors, adjacent nonaffected endometrium, and cancer-free endometrium in semiadjacent tissue sections. Hematoxylin and eosin (H&E) staining defines the histopathology of the tissues. Human Hp localized to primarily the stromal cells with random staining of the epithelial cells of the endometrioid adenocarcinoma and adjacent endometrium but was not found in the cancer-free endometrium. Phosphate-buffered saline (PBS) was used as a negative staining control. Additional semiadjacent tumor tissues sections were stained using the rabbit anti-human Hp antibody and normal rabbit immunoglobulin G (IgG) as additional negative antibody controls (insets in Hp and PBS endometrioid adenocarcinoma panels). Human CD45, a pan-leukocyte marker, was used to confirm that Hp was localizing in the stromal compartment and not resident immune cells in the tissues. Cytokeratin (CK) was used to confirm the location of epithelial cells.

hepatic Hp, a characteristic that may also be useful in distinguishing Hp from different sources.[23] Ultimately, for clinical utility it will be critical to distinguish between normal endometrium, endometriotic endometrium, endometrioid adenocarcinoma and other endometrial disorders, and even more precisely between the cell types associated with each disease.

We propose that in addition to or rather than a protein marker, the ability to assess Hp mRNA in these tissues and cell types may be the solution. Our Hp mRNA quantification and protein localization studies showing distinct patterns of Hp between endometrioid adenocarcinoma, endometrium from women with endometriosis, and normal endometrium from healthy women that may be the key to developing sensitive and specific diagnostic tools.

The elevation of Hp in endometrioid adenocarcinoma tumor tissue suggests that Hp may be involved in the pathogenesis of endometrial cancer. Studies have shown that Hp has angiogenic and immunomodulatory properties.[24,25] Haptoglobin enhances angiogenesis in in vivo models[26] and stimulates angiogenesis in patients with systemic vasculitis.[27] Others have shown that Hp is a natural inhibitor of collagen degradation and locally expressed by fibroblasts in arterial walls.[28] Haptoglobin therefore plays an important role in arterial restructuring, which can apply to other restructuring processes like angiogenesis.[29] We propose that Hp in endometrial carcinoma is involved in angiogenesis that leads to the establishment of tumors and malignancy.

It has also been reported that Hp is involved in immune suppression in cancer.[30] Samak and colleagues[31] suggested that Hp may act as a nonspecific blocking factor protecting tumors against the host's immunological attack. Oh et al[30] reported the presence of a unique epitope in a tumor-associated haptoglobin protein that manifests an immunosuppressive property in patients with ovarian, lung, and head cancer. Our research[17] has shown that Hp alters immune response and may be involved with the etiology and pathophysiology of endometriosis. We postulate that Hp may also participate in immune dysfunction in women with endometrioid adenocarcinoma.

Many studies have documented structural changes in Hp in patients with cancer compared to hepatic Hp. Hepatic Hp is a tetrameric glycoprotein composed of 2 α chains and 2 β chains linked by disulphide bonds. There are 3 major Hp phenotypes, Hp1-1, Hp2-2, and Hp2-1 that arise through the 2 different α chains, the β chains being identical.[32] Haptoglobin phenotypes as well as Hp carbohydrate profiles were reported to be different between disease-free patients and patients with ovarian, esophageal, gastric, and breast cancer.[33-38] Further studies of the phenotype and glycan profile of endometrioid adenocarcinoma Hp would add insight into its involvement in the angiogenic or immunosuppressive functions that may cause tumor establishment or malignancy.

To conclude, our research has identified Hp mRNA expression and protein localization in both the stromal and the epithelial cells from endometrioid adenocarcinoma and adjacent endometrial tissue in women with endometrial cancer. We propose that Hp has potential diagnostic and/or prognostic value and that modulation of Hp expression may lead to methods of prevention or treatment for endometrioid adenocarcinoma. Furthermore, Hp expression in endometrioid adenocarcinoma may be associated with localized angiogenesis and an altered immune response, which may lead to tumor establishment and malignancy. Additional studies on uterine Hp structure in patients with cancer may add some insight on the aberrant function of this protein.

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