Histocompatibility leukocyte antigen-G is not expressed by endometriosis or endometrial tissue

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Objective: The immunological mechanisms that support persistence and proliferation of ectopic endometrial implants within the peritoneal cavity of women with endometriosis are unknown. Inhibition of natural killer (NK) and cytotoxic T-cell function has been proposed as a mechanism. We tested the hypothesis that expression of a nonclassical major histocompatibility antigen, HLA-G, might explain the local immunosuppression associated with ectopic endometrium.

Design: Nested case-control study of women with and without laparoscopic evidence of endometriosis.

Setting: Reproductive endocrinology clinic at a university hospital.

Patient(s): Peritoneal fluid specimens from 10 women with revised AFS stage I–IV endometriosis and from 10 age-matched normal controls without laparoscopic evidence of endometriosis were tested for the presence of HLA-G protein. Endometriosis and normal endometrial biopsies from four patients were used to prepare stromal cell cultures directly evaluated for HLA-G protein.

Intervention(s): None.

Main Outcome Measure(s): The expression of HLA-G in peritoneal fluid, tissue, and cell cultures was determined by immunoblotting with a specific monoclonal antibody.

Result(s): HLA-G protein was not detectable in peritoneal fluid specimens of endometriosis patients or controls. Moreover, ectopic and normal endometrial tissues and stromal cells did not express HLA-G.

Conclusion(s): Immune cell inhibition in endometriosis must be mediated by factors other than HLA-G.

Key Words: Endometriosis, HLA-G, natural killer cells, cytotoxic T cells

Endometriosis is a common but complex gynecological syndrome of unknown pathogenesis. For years, an immunological etiology has been conjectured, based on evidence of increased concentrations of activated peritoneal macrophages, T cells, and polyclonal B cells. The persistence of islands of displaced endometrial tissue fragments within the pelvic cavity suggests that immune recognition and killing of ectopic cells is impaired in women with endometriosis. Because retrograde menstruation is a nearly universal phenomenon, one of the most plausible mechanisms for endometriosis implant formation is defective peritoneal immunological surveillance.

Natural killer (NK) cells are the effector cells that usually recognize and destroy tumor cells, virus-infected host cells, and transplanted foreign cell lines. Oosterlynck and colleagues were the first to demonstrate decreased NK activity and cytotoxicity against autologous endometrial cells in women with endometriosis, which correlated with the stage of disease (1). The same group subsequently showed that peritoneal fluid from women with endometriosis contained significantly greater NK cell suppressive activity than peritoneal fluid from fertile controls. Other investigators confirmed these findings in serum and pelvic fluid of endometriosis patients and have reported similar deficiencies in cytotoxic T-cell function.

A novel protein with immune cell inhibitory activity is the nonclassical major histocompatibility complex class I molecule, human
leukocyte antigen G (HLA-G). HLA-G is an inhibitory ligand selectively expressed on cytotrophoblasts at the fetomaternal interface, where it has been postulated to play an important role in maternal tolerance of the semiallogeneic fetus. Because expression of classical MHC class I molecules previously had been implicated in the susceptibility of endometrial cells to NK cell lysis (2), we postulated that expression of HLA-G by endometriosis lesions might explain both their persistence within the peritoneal cavity and the observed inhibition of pelvic fluid NK and cytotoxic T-cell activity in these patients.

**MATERIALS AND METHODS**

**Subjects**

Normally menstruating women undergoing laparoscopy for various indications, including evaluation of infertility, pelvic pain, pelvic mass, or elective tubal sterilization, were recruited. Women taking oral contraceptives or GnRH analogs within 3 months before operation were excluded. Informed consent was obtained for all subjects under a protocol approved by the Committee on Human Research at the University of California, San Francisco. The presence or absence of endometriosis was identified independent of fertility status at the time of operation by systematic observation of the pelvis. Endometriosis was confirmed histologically. Twelve women with active endometriosis lesions were assigned to the endometriosis group; all revised American Fertility Society stages (I–IV) were represented. Twelve control subjects were age-matched women undergoing laparoscopy for tubal sterilization in whom no visible evidence of pelvic pathology was found. All subjects underwent laparoscopy and endometrial or endometriosis biopsy during the proliferative phase of their menstrual cycles.

**Sample Preparation**

Peritoneal implants from two women with endometriosis and endometrioma biopsies from two normal controls were used to prepare tissue homogenates. Protein lysates were prepared by homogenization in lysis buffer (20 mM Tris, pH 7.4, 1% Triton, 20% glycerol, 2 mM PMSF, 2 mM DTT, 5 mM EDTA, 0.5 µg/mL leupeptin, 1 µg/mL pepstatin) and centrifugation at 15,000 × g.

**Cell Preparation and Culture**

Two endometriosis and two normal subjects provided specimens for the preparation of stromal cell cultures. The techniques for isolation and culture of human endometrial and endometriosis stromal cells have been discussed in detail elsewhere (3). Briefly, biopsies from endometriosis implants and the control endometrium were rinsed, and endometrioma cyst linings were dissected free from cyst walls. The specimens were minced, digested with collagenase, and then serially filtered through narrow-gauge sieves with apertures of 38–105 µm to trap the glandular epithelium. Stromal cells were plated and allowed to adhere to plastic cell culture dishes for 30 minutes, at which time contaminating blood cells and tissue debris were rinsed free. Cultures were allowed to proliferate in minimum essential medium (MEM)-α, supplemented with 10% fetal calf serum (FCS), nucleosides, and non-essential amino acids. All experiments were performed with confluent cells at passage two, within 12 days of initial isolation in 2.5% FCS. In some experiments, endometriosis and normal endometrial stromal cells were treated with TNF-α (100 ng/mL) and IFN-γ (100 ng/mL) for 48 hours before cell lysis. These doses previously had been shown to maximally activate endometrial stromal cell Regulated on Activation Normal T cell Expressed and Secreted (RANTES) production (3). Culture supernatants and cellular lysates were evaluated to ascertain whether HLA-G was present in either soluble or cell surface-associated fractions.

**HLA-G Immunoblotting**

Peritoneal fluid specimens from 10 women with endometriosis and 10 matched normal controls were collected at the time of laparoscopy and frozen at −70°C. Tissue, stromal cell, and peritoneal fluid protein preparations were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were blotted to nitrocellulose paper. Western blotting for HLA-G was performed using monoclonal antibodies raised against a synthetic peptide corresponding to amino acids 61–83 of the α1 domain of HLA-G (EEETRTNTKAHTDRMRNLQTLRG) coupled to keyhole limpet hemocyanin (4). Human JEG-3 choriocarcinoma cells, previously established to express HLA-G, were used as a positive control. Ten to 40 µg of protein from peritoneal fluid, tissue, and cell lysates was loaded per lane in the PAGE gels.

**Data Analysis and Controls**

HLA-G protein bands were compared with JEG-3 cell standards run simultaneously on the SDS-PAGE gels. To verify that peritoneal fluid did not contain factors that mask detection of HLA-G, the former was spiked with JEG-3 cell lysates and subjected to Western blotting. Recovery of 90%–100% of the signal was noted under these conditions.

**RESULTS**

**HLA-G Expression in Peritoneal Fluid**

Using the Western blotting assay, which is sensitive to 50 ng of protein, we were unable to detect HLA-G in specimens of peritoneal fluid from endometriosis or normal subjects (Figure 1A and B, lanes 1 to 10, respectively). By contrast, JEG-3 cell lysates (lane J) demonstrated the expected 37-kD immunopositive band. Twenty (C1) and 40 µg (C2) of peritoneal fluid protein was spiked with JEG-3 cell lysates. Recovery of 90%–100% of the signal was noted under these conditions.
HLA-G Expression in Endometriosis and Normal Endometrial Tissues

Neither endometriosis-derived nor normal endometrium biopsies contained detectable HLA-G protein. By contrast, Western blots of the positive controls yielded detectable HLA-G protein (data not shown).

HLA-G Expression in Endometriosis and Normal Endometrium Stromal Cells

We hypothesized that HLA-G might only be transiently up-regulated in cytokine stimulated endometrial cells. To test this hypothesis, we prepared cultures of endometriosis-derived and normal endometrial stromal cells that previously have been characterized extensively. Both cell types express functional estrogen α and β, progesterone as well as epidermal growth factor (EGF) receptors, verifying their endometrial phenotype. Under basal culture conditions, neither endometriosis nor normal endometrial stromal cells expressed HLA-G protein. Even after incubation with TNF-α and IFN-γ, the cells failed to express detectable HLA-G protein. These negative findings were obtained for both cell-associated and secreted proteins (data not shown).

DISCUSSION

In recent years, investigators have uncovered multiple connections between endometriosis and the immune system. Activated macrophages are found in greater numbers in the peritoneal cavity of women with endometriosis. Peritoneal T cells also are reported to be more highly concentrated, together with an elevated CD8:CD4 ratio in the circulation of women with endometriosis. Moreover, some researchers have reported higher titers of circulating autoantibodies in patients with the disease and have speculated that this finding may be related to increased polyclonal B-cell activation.

Despite these indicators of immune hyperresponsiveness, evidence of deficient peritoneal immune cell activation also has been presented. Cytotoxic T cell function is diminished in peritoneal fluid from women with endometriosis, and NK cell activity is inhibited 69% in these subjects, relative to normal controls (1). It has been suggested that NK cells are the first line of defense against the peritoneal implantation of ectopic endometrial cells. Circulating mediators of NK cell suppression in endometriosis have been sought by several
groups of investigators. One cytokine with NK cell inhibitory activity, interleukin-10, was noted to be elevated in the peritoneal fluid of women with endometriosis and its levels correlated with impaired immunological function in vitro.

Our studies of HLA-G expression in endometriosis were undertaken based on the observations that HLA-G functions as an NK cell inhibitory ligand in another endometrial process, namely trophoblast implantation. The failure of endometrial or endometriosis tissues to express HLA-G protein, and our inability to detect HLA-G in human peritoneal fluid, does not support this hypothesis but is consistent with the markedly restricted expression of this protein. Using highly sensitive RT-PCR assays, HLA-G mRNA transcripts have been detected in extravillous trophoblasts and choriocarcinoma cells (4), spermatozoa, and the anterior chamber of the eye. However, with the possible exception of rare cells in the human thymus, HLA-G protein expression has never been verified in any tissue except the placenta (4). It remains plausible that other nonclassical HLA proteins (e.g., HLA-E), not detected by our monoclonal antibody, could play an immunomodulatory role.

As our understanding of the inhibitory regulation of peritoneal immune cell activity grows, other candidates for the immunosuppressive mediator or mediators associated with endometriosis will be sought. This pursuit is likely to yield potential diagnostic markers and therapeutic agents for future investigations into the pathogenesis of endometriosis.

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References