Role of major histocompatibility complex class I expression and natural killer-like T cells in the genetic control of endometriosis*

Claudia Semino, Ph.D.; Antonio Semino, M.D.; Gabriella Pietra, Ph.D.; Maria Cristina Mingari, Ph.D.; Sergio Barocci, Ph.D.; Pier Luigi Venturini, M.D.; Nicola Ragni, M.D.; Giovanni Melioli, M.D.

Centro di Biotecnologie Avanzate, Istituto Nazionale per la Ricerca sul Cancro; Ospedale S. Martino; and Istituto di Ginecologia ed Ostetricia, Università di Genova, Genova, Italy

Objective: To evaluate whether the expression of human leukocyte antigen (HLA) class I on eutopic and ectopic endometrial cells modify the susceptibility to lysis mediated by lymphocytes.

Design: Evaluation of T lymphocyte cytotoxic activity and HLA class I expression on endometrial cells.

Setting: Subjects were recruited at laparoscopy.

Patients: Patients with endometriosis (n = 7). Healthy women as controls (n = 10).

Main Outcome Measures: Human leukocyte antigen class I molecule analysis of endometrial cells was carried out by immunofluorescence and flow cytometry. Phenotyping of T lymphocytes was performed to analyze T-cell subsets. Cytotoxicity was performed to determine cytolytic activity against endometrial cells.

Results: In vitro culture of endometrial cells down-regulates the expression of HLA class I molecules and enhances the susceptibility to lysis mediated by natural killer (NK)-like T lymphocytes. Cytolytic T-cell clones, expressing the CD94 antigen, are inhibited by the HLA-B7 allele on endometrial cells. Ectopic endometrial cells modulate the expression of HLA class I molecules.

Conclusions: The resistance to lysis of endometrial cells is related to expression of surface HLA class I molecules, which send a negative signal for lysis mediated by NK-like T lymphocytes. The HLA-B7 allele inhibits the cytotoxic activity, suggesting that the growth of ectopic endometrial cells might be under a genetic control.

Key Words: Endometriosis, MHC class I molecule expression, NK-like T lymphocytes mediated cytotoxicity

In women of reproductive age, the incidence of retrograde menstruation has been estimated at 90%, although only between 10% and 15% of premenopausal women are thought to suffer from endometriosis (1). In another study, endometriosis was diagnosed in up to 77% and 82% of patients suffering from infertility and pelvic pain, respectively (2). This matter raises the question of what factor might favor the development of endometriosis. A number of clinical and experimental evidences indicates that human endometriosis has, at least in part, an immunologic pathogenesis. Several authors have described changes in both humoral and cellular immunoresponse. Mathur et al. (3) showed increased levels of...
autoantibodies (immunoglobulin [Ig] G, IgA) to endometrium and ovarian tissues, including granulosa and theca cells, in the sera of patients with endometriosis but not in normal controls. Other authors found the presence of a high number of autoantibodies in endometriosis patients (4), suggesting a polyclonal B lymphocyte activation. This finding could support the hypothesis that endometriosis could be an autoimmune disease (4). Along this line, several authors have demonstrated a decreased T-cell response in women with endometriosis (5). In the past, it has been shown that both the humoral and cellular immunoresponse are implicated (6). More recently, it has been suggested that a peculiar defect of the natural killer (NK) cells activity in the recognition and lysis of endometrial cells is one of the crucial points in the pathogenesis of endometriosis (7, 8). In this context, we, along with other authors, were able to demonstrate that the incubation of autologous T cells with high doses of recombinant interleukin 2 (rIL-2) was able to correct this immunodeficiency, reconstituting the ability of lysing autologous cultured endometrial cells in patients with endometriosis (9, 10). This finding suggested the possibility of an immunologic treatment of endometriosis using in vitro activated T lymphocytes and rIL-2 infused intraperitoneally. However, from a clinical point of view, the identification of the T-cell subset responsible for the capacity of lysing autologous endometrial cells is crucial to define the strategy for the preparation of protocols of adoptive immunotherapy.

In this article, we further analyze the fine cellular mechanisms involved in the lysis of autologous endometrial cells and we demonstrate that lysis is mediated by NK-like T lymphocytes under the control of major histocompatibility complex (MHC) class I molecules expressed on endometrial cells. In addition, we show that the number of MHC class I molecules expressed on ectopic endometrial cells is highly variable. These results indicate that the susceptibility to lysis can be controlled by some endometrial cell-specific mechanism. Finally, in a selected number of cases, we describe that the consensus to or the protection from lysis is mediated by the expression of a peculiar allele of human leukocyte antigens (HLA)-B, thus suggesting a genetic control in the pathogenesis of endometriosis.

MATERIALS AND METHODS

Subjects

Seven patients undergoing diagnostic laparoscopy because of pain and/or infertility were found to have endometriosis at different stages. Collection of eutopic endometrial cells was performed routinely in the midluteal phase. Another 10 women, in which endometriosis was laparoscopically ruled out, served as control group. During the procedure, eutopic endometrium was sampled by dilation and curettage. Of these samples, three were collected at day 10 (patients 1, 2, and 3), whereas samples from patients 4 and 5 were collected at day 20. Contemporary peripheral blood was drawn in all patients. In addition, in another group of five endometriosis patients, a biopsy of ectopic endometrium was obtained. At the same time, peripheral blood was drawn. None of these patients has been treated with hormone preparations in the 60 days preceding surgery. Informed consent was obtained from all patients before enrollment into the study.

Culture of Endometrial Cells

Tissue specimens were placed in sterile conditions in RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with antibiotics and immediately processed in laboratory. The tissue was dissected mechanically into small pieces and then dissociated enzymatically for 2 hours at 37°C. Cell suspension was washed twice in fresh medium and seeded in two to four plastic flasks at a concentration of 5 x 10^6 cells/mL in complete medium as previously described (10). No gonadal steroid hormones was added during the culture. Culture growth was monitored using the CAM 5.2 monoclonal antibody (mAb; Becton-Dickinson, San Jose, CA) to enumerate the epithelial cells in the flasks. Cultures were used when the percentage of epithelial cells was >80%.

Isolation and Culture of Peripheral Blood Lymphocytes

Peripheral blood mononuclear cells were isolated using a discontinuous gradient of Ficoll-Paque as described (10). To evaluate T lymphocyte function, cultures were then depleted of platelets and monocytes using plastic adherence (45 minutes at 37°C in RPMI 1640 additionated with 20% fetal calf serum [FCS; GIBCO, Milano, Italy]). Highly purified lymphocytes were then used immediately for further experiments or cultured in vitro after activation with phytohemagglutinin (PHA, 1% vol/vol; Difco, Milano, Italy) and rIL-2 (500 IU/mL Proleukin; Eurocetus, Amsterdam, The Netherlands) in RPMI 1640 additionated with 10% FCS.

Isolation of T-cell Subpopulations

T-cell subpopulations were positively isolated from other peripheral blood lymphocytes using magnetic immunobeads (Immunotech, Luminy, France). Briefly, 50 μL of washed beads coated with anti-
mouse mAb were incubated with a proper dilution of mAb specific for the population to be sorted. After further washings, magnetic beads were added to 10^7 purified mononuclear cells in PBS with 30% FCS. Cells were gently mixed, incubated for 15 minutes, and separated using a cobalt-Samarium magnet (Immunotech). Cells were then washed twice and cultured as described below.

**Isolation and In Vitro Expansion of T-Cell Clones Derived From Healthy Donors and Patients With Endometriosis**

T-cell clones were derived as follows: limiting number of purified lymphocytes (ranging from 8 to 0.5 cell/well) were plated in microwells on a feeder layer of allogeneic irradiated peripheral blood mononuclear cells in RPMI 1640 additioned with 1% PHA, 500 IU rIL-2, 10% FCS, and 20% AIM-V (GIBCO) as described (11). After 2 to 3 weeks, proliferating wells were scored visually, and clones were further expanded in the presence of rIL-2. A complete phenotypic and functional characterization of these clones was performed at 4 to 6 weeks of culture.

**Phenotypic Analysis of T Lymphocytes and Endometrial Cells**

A panel of mAbs, directed against human lymphoid cell surface molecules (namely, CD2, CD3, CD4, CD7, CD8, CD16, CD19, CD20, CD56, and CD57) was obtained by Coulter Scientific (Milano, Italy). Anti-CD54 (intercellular adhesion molecule I) and anti-CD58 (lymphocyte function-associated antigen 3) mAbs were obtained from Immunotech. Indirect immunofluorescence was performed using a fluorescein isothiocyanate conjugate (FITC)-labeled goat anti-mouse IgG antiseraum (Coulter Scientific). Analyses were carried out by flow cytometry (Coulter EPICS Elite, Milano, Italy). To study the presence of NK-cell putative receptors, anti-p53 (GL183 and EB6) (12) and anti-p43 (anti-CD94) (13) mAbs were used. To evaluate the expression of both MHC class I and MHC class II molecules on endometrial cell surface, the W6/32 mAb, directed against a framework determinant of MHC class I (14) and the D1/12 anti-MHC class II mAb, were used (15). Finally, to better characterize the MHC class I alleles, the BB7.2 (anti-HLA-A2 [16]), the GAP-A3 (anti-HLA-A3, ATCC HB122), the 131 (anti-HLA-A1, A3, A11, A24 [12]), and the BB7.6 (anti-HLA-B7 and B40, ATCC HB105) mAbs were used. Analyses were carried out by indirect immunofluorescence and flow cytometry. For the quantitative analysis of the expression of surface molecules on endometrial cells, cell size, and number of molecules expressed on the cell surface were calculated using different standard beads with known diameter and number of FITC equivalent (Coulter Scientific). The molar ratio protein:FITC was calculated according to Wells et al. (17).

Further HLA typing was performed serologically according to the standard National Institutes of Health microlymphocytotoxicity technique (18).

**Analysis of the Cytolytic Activity**

The cytolytic activity of fresh and cultured peripheral blood T lymphocytes, as well as of T lymphocyte subpopulations and clones, was studied as described (10). Briefly, 1 x 10^6 endometrial cells were labeled with 100 μCi of ^51^Cr for 1 hour and then extensively washed and incubated at different effector:target ratios (ranging from 50:1 to 1.5:1) for 4 hours in V-bottomed microplates. Supernatants were then collected, and the percentage of lysis was calculated as follows: (counts per minute [CPM] of the experiment - CPM of spontaneous release)/CPM of the total release - CPM of spontaneous release) x 100 as described (10). In addition, to evaluate the relevance of the expression of MHC class I molecules on target cells, cultured endometrial cells were pretreated for 48 hours with 500 ng of γ-interferon and then labeled with ^51^Cr assay.

**RESULTS**

**Down-Regulation of the Expression of MHC Class I Molecules in In Vitro Cultured Endometrial Cells**

Endometrial cells, freshly isolated from normal women or endometriosis patients, have a high number of MHC class I molecules (median value 92,000, ranging from 54,000 to 136,000 molecules per cell in 5 different experiments). When these cells are cultured in vitro for 2 weeks, a significant reduction (median value 57,000 molecules per cell, ranging from 13,000 to 105,000) of the expression of MHC-class I molecules was observed. Interestingly, other surface molecules, such as CD58 and CD54, resulted unmodified by the long-term culture (not shown).

**Reconstitution of the Number of MHC Class I Molecules in In Vitro Cultured Endometrial Cells After Treatment With γ-Interferon**

The treatment with α- or γ-interferon is well known to enhance the expression of MHC class I and II on cancer cells (19). When cultured endometrial cells were incubated with γ-interferon, a clear rising of MHC class I surface molecules, from 57,000 molecules per cell (median value, range 13,000 to 105,000) to 92,000 molecules per cell (median value,
range 31,000 to 162,000) was observed. These modifications corresponded to 80% median enhancement of the number of MHC class I molecules on the surface of cultured endometrial cells after treatment with γ-interferon.

Enhanced Susceptibility to Lysis of In Vitro Cultured Endometrial Cells Mediated by Cytolytic T Lymphocytes

As demonstrated in a previous report (10), in vitro activated and rIL-2 expanded T lymphocytes are able to lyse autologous endometrial cells in a short-term cytolytic assay. In this article, we compared the susceptibility to lysis of cultured endometrial cells with that of fresh uncultured endometrial cells. As shown in Figure 1, fresh uncultured endometrial cells were more resistant to lysis than endometrial cells cultured for 14 days. The calculation of the number of MHC class I molecules on the surface of cells used for the cytolytic assays demonstrated that fresh cells have a median number of MHC molecules ranging from 54,000 to 136,000 molecules per cells and cultured endometrial cells have a reduced number of molecules (from 13,000 to 105,000) on the surface. On the contrary, other surface molecules involved in the mechanisms of cell-to-cell contact and lysis, such as CD58 and CD54, remained unchanged. These findings suggest that the susceptibility to lysis of endometrial cells could be caused by the reduction of a putative "protective" signal mediated by MHC class I molecules.

Reconstitution of the Resistance to Lysis of In Vitro Cultured Endometrial Cells After Treatment With γ-Interferon

Based on the evidence that the treatment with γ-interferon is able to enhance the expression of MHC class I molecules on cultured endometrial cells, γ-interferon-treated 51Cr-labeled in vitro cultured endometrial cells were incubated with autologous activated T lymphocytes in a 4-hour cytotoxic assay. Figure 2 shows the results of the assay: all samples of cultured endometrial cells had a variable susceptibility to lysis but after treatment with γ-interferon, the susceptibility was clearly reduced, thus suggesting that the enhancement of MHC class I molecules, induced by γ-interferon, reconstitute the resistance to lysis of endometrial cells.

Analysis of the Activity of T-Cell Clones on Autologous Endometrial Cells

To analyze the frequency of lymphocytes capable of recognize and lyse autologous endometrial cells, we derived 89 T-cell clones from peripheral blood of both normal subjects (37 clones) and endometriosis patients (52 clones). Twelve of 89 clones were able to lyse autologous endometrial cells, independently from the surface phenotype (7 were CD4+ and 5 were CD8+).

Identification of the Effector T-Cell Population Involved in the Lysis of Endometrial Cells

The relatively high number (>13%) of T-cell clones able to lyse autologous endometrial cells clearly indicates that some mechanism of recognition, different from those mediated by the CD3/T-cell receptor complex, was involved in the phenomenon. Indeed, it is common notion that 1 of 10,000/1 of 100,000 lymphocyte is specifically alloreactive, whereas NK cells, constitutively lacking T-cell receptor–like structures, have a very limited repertoire of allospecificities (20). Furthermore, it is well known that both NK cells and NK-like T cells are able to lyse efficiently both autologous and allogeneic target cells upon activation (21). Thus, an NK-like activity, mediated by T cells, seemed to be implicated in the lysis of endometrial cells. To confirm this hypothesis, we
analyzed the capability of T-cell clones, reactive against autologous endometrial cells, to lyse allogeneic endometrial cells. The large majority of these T-cell clones efficiently lysed at least one out of three different cultures of allogeneic endometrial cells, demonstrating that the mechanism is not MHC restricted. This confirmed the NK-like nature of T-cell clones characterized by the capability of lysing autologous endometrial cells. Then, we analyzed the presence of GL183, EB6, and CD94, three surface antigens described on NK cells, on T-cell populations and clones. Although both GL183 and EB6 resulted virtually absent in the population of T cells expressing the capability of lysing endometrial cells, the CD94 surface antigen was expressed on a variable proportion (ranging from 5% to 27% in different donors) of highly purified CD8+ lymphocytes. Interestingly, CD94 were absent in CD4+ populations. To evaluate the lytic machinery directed against autologous and allogeneic endometrial cells, CD8+CD94+ lymphocytes were separated from CD8+CD94− cells using microbeads, and the two populations were tested against autologous in vitro cultured endometrial cells. $^{51}$Cr-labeled endometrial cells were lysed prevalently by the CD8+CD94+ subset (Fig. 3). Interestingly, target cells, pretreated with γ-interferon, resulted resistant to lysis.

**Identification of the Presence of the Natural Ligand of CD94 as a Protective Signal on Endometrial Cells**

It has been demonstrated that CD94 is the ligand of HLA-B7 molecule (3). For this, we analyzed the expression of this molecule on effector cells used in previous experiments. Human leukocyte antigen-B7 was detected in one out of four donors and was present in freshly isolated and γ-interferon-treated endometrial cells, whereas it was significantly down-regulated in endometrial cells cultured for >2 weeks.
Thus, we evaluated whether the presence of HLA-B7 molecules could send a negative signal to cytolytic effector cells, expressing the CD94 surface antigen. CD94+ clones were tested against both cultured endometrial cells (expressing low level of HLA-B7) and γ-interferon-treated endometrial cells (which up-regulated HLA-B7), labeled with 51Cr. Figure 4 shows the results of the experiment. To define the role of HLA-B7 molecule, anti-HLA-B7 mAb (13) was added to the cytolytic test and it was shown to restore the lyse of γ-interferon treated endometrial cells.

**Analysis of the Expression of HLA Class I Molecules on Endometriotic Cells Freshly Isolated From Endometriosis Lesion in the Peritoneum**

The clear evidence that endometrial cells can be efficiently lysed by autologous and allogeneic MHC unrestricted lymphocytes suggests that in vivo endometrial cells, expressing a high number of MHC class I molecules on their surface, give a protective signal to autologous lymphoid effector cells. However, little is known about the expression of MHC molecules on ectopic endometrial cells. For this, we obtained five samples of cells collected on endometriotic lesions in course of diagnostic laparoscopy. The phenotypic analysis of these samples, performed in parallel with the analysis of endometrial cells, showed that a variable number of HLA class I molecules (ranging from a reduction of 15% to an enhancement of 30% compared with endometrial cells collected contemporary in the uterine mucosa) was detectable. Interestingly, in two samples (patients 2 and 3, collected at day 10) a reduced expression of HLA class I was detected. In another sample, collected at day 10 (patient 1), only a slight (+3%) enhancement of MHC class I expression was observed. Finally, in two patients (4 and 5, collected at day 20) an increased expression of MHC class I molecules was observed.

**DISCUSSION**

The immunological pathogenesis of endometriosis has been suggested in the past years and at present, a number of clinical and experimental data indicate that both the humoral and the cellular arm of the immune system are involved in this disease.

We previously have demonstrated that the immune defect of endometriosis patients, found to be partially unable to lyse autologous endometrial cells, could be circumvented by the treatment of effector cells with high doses of rIL-2 (10). Now, in this article, we define the cellular properties of the effector lymphocytes and we show that cultured endometrial cells, expressing low levels of MHC class I, could be lysed more efficiently than freshly isolated cells, expressing a “normal” level of HLA molecules. More interestingly, we detected that ectopic endometrial cells may modulate the expression of MHC molecules, thus suggesting that in vitro cultures of endometrial cells could be a reliable model for the study of susceptibility to lysis of ectopic cells.

The enhanced susceptibility to lysis, induced by long-term culture in vitro, is clearly associated with the down-regulation of MHC class I molecules. In addition, the treatment with γ-interferon, which up-regulates the expression of MHC class I molecules, completely restored the resistance to lysis. The clonal analysis of lymphoid population involved in this phenomenon clearly indicated that at least one of seven clones is able to recognize and lyse autolo-
rious and allogeneic endometrial cells. This frequency, comparable with that of T cells expressing the capability of lysing the K562 NK cell target, is higher than expected (1:10,000 to 1:100,000) if the lysis would be under MHC restriction. In addition, the capability of lysing allogeneic endometrial cells (without a presensitization) further confirms the involvement of a nonspecific mechanism. For this, the presence of molecules, such as GL183, EB6, and CD94 (which has been extensively described to be involved in the mechanism of protection of lysis in the NK cell subset) was investigated. Although GL183 and EB6 resulted virtually absent in the T-cell population, CD94 was found at a relatively high frequency in the subset of cells able to lyse endometrial cell, whose MHC class I was down-regulated. Furthermore, the potentiation of the expression of HLA-B7, the natural ligand of CD94 detectable in approximately 6% of the white population, completely restored the resistance to lysis. This indicates that in our experiments, the lysis is partially under genetic control and is mediated by a particular allele of HLA. Obviously, a wider analysis, including donor and patients with different alleles, could demonstrate the presence of other genetically defined ligand-anti-ligand complex.

The absence of correlation between a given HLA phenotype and development of endometriosis has been described in the past (22). Our findings, however, indicate that the modulation of a given antigen, expressed on the surface of endometrial cells, modifies the susceptibility to lysis mediated by a particular subset of T lymphocytes. This seems to be a physiological mechanism of control that could be altered on the surface of ectopic endometrial cells, independently from the HLA phenotype.

More interestingly, the calculation of the number of MHC class I molecules on ectopic endometrial cells clearly (even if indirectly) indicates that not all of these cells are susceptible to lysis. The reason why ectopic endometrial cells down-regulate or up-regulate the expression of the protective molecules is unknown, and both local cytokine-mediated or hormonal-controlled mechanisms can be hypothesized. Along this line, it should be noted that in three patients, in which ectopic endometrial cells were collected during the progestinic phase of the cycle, the number of MHC class I molecules was clearly higher in ectopic than eutopic cells. On the contrary, in another two patients, collected at day 10, the expression of histocompatibility antigens was lower in ectopic cells. This finding suggests a possible role of sexual hormones in the modulation of some surface molecules on ectopic endometrial cells. Nevertheless, at present, because of the great variability of MHC expression in vivo, it is unknown whether differences observed unrelated to the modified expression of MHC class I on ectopic cells or, on the contrary, to the altered expression on eutopic cells. Further studies on a larger cohort of patients could shed some light on this finding. It is of note that ectopic endometrial cells cannot be lysed efficiently even if they are susceptible to lysis, because in vivo, the number of activated cytotoxic lymphocytes in the peritoneal cavity is very low (23). On the contrary, an immunotherapeutic approach, which provides the infusion of autologous activated T cells, may be found effective when the down-regulation of MHC class I molecules could be demonstrated ex vivo or some drug will be able to reduce the expression of MHC molecules on ectopic endometrial cells.

Acknowledgments. Anti-p53 (GL183 and EB6) and anti-p43 (anti-CD94) (13) mAbs were kindly provided by A. Moretta, M.D., and y-interferon was kindly provided by V. Cantone, Ph.D., Rous sel Pharma, Milano, Italy.

REFERENCES

12. Ciccone E, Pende D, Viale O, Di Donato C, Oreno AM, Biassoni R, et al. Involvement of HLA class I alleles in natural killer (NK) cell specific functions: expression of HLA-CW3 confers selective protection from lysis by alloreactive NK