

Cytolysis of eutopic and ectopic endometrial cells by peripheral blood monocytes and peritoneal macrophages in women with endometriosis

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Objective: To compare the ability of peripheral blood monocytes (PBM) and peritoneal macrophages (PM) to mediate the in vitro cytolysis of endometrial cells from eutopic and ectopic endometrium in women with endometriosis.

Design: Prospective study of immune function.

Setting: Institute for the Study and Treatment of Endometriosis and university-based research laboratories.

Patient(s): Twenty-four women with endometriosis (15 in stage I/II, 9 in stage III/IV) and 4 patients treated with GnRH agonists.

Intervention(s): Peritoneal fluid and peripheral blood were sampled and eutopic and ectopic endometrium were biopsied during diagnostic laparoscopy.

Main Outcome Measure(s): Lysis of autologous endometrial cells.

Result(s): Peripheral blood monocytes were significantly more cytolytic than peritoneal macrophages against autologous uterine endometrial cells. However, PBM and PM displayed a similar degree of cytolysis against a hepatoma cell line. Ectopic endometrial cells were significantly more resistant to cytolysis by autologous PBMC than were matched eutopic endometrial cells, and were completely resistant to cytolysis by autologous PM.

Conclusion(s): The reduced capacity of PM from women with endometriosis to mediate the destruction of endometrial cells coupled with the increased resistance of ectopic endometrial cells to macrophage-mediated cytolysis may facilitate the survival of these cells within the peritoneal cavity of women with endometriosis. (*Fertil Steril*® 1998;69:1103–8. ©1998 by American Society for Reproductive Medicine.)

Key Words: Endometriosis, macrophages, endometrial cells, cytolysis

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A satisfactory explanation for why endometriosis develops in some women remains elusive. Although dissemination of the endometrial cells into ectopic locations during menses appears to be a common phenomenon (1, 2), ectopic implantation of these cells takes place in only approximately 10%–15% of women (3).

The central concept guiding our thinking in this area has been the assumption that destruction and elimination of these ectopic endometrial cells within the peritoneal cavity is a fundamental requirement for the prevention of endometriosis (4). If that is true, then it follows that failure to eliminate ectopic endometrial

cells within the peritoneal cavity would enhance the probability that endometriosis will develop. Disturbances in normal homeostatic mechanisms within the peritoneal cavity have been advanced most often to explain the persistence of endometrial cells in women with endometriosis, with defective immune-mediated elimination of ectopic cells playing a prominent role in this scenario (5, 6).

An alternative explanation for the persistence of endometrial cells within the peritoneal cavity that could predispose some women to the development of endometriosis is the possibility that some endometrial cells may become resistant to normal growth-regulatory mecha-

nisms by resisting the induction of programmed cell death or apoptosis. Such cells would have a greater chance of surviving an otherwise hostile peritoneal environment but would still require the ability to implant and grow within the peritoneal cavity in a relatively autonomous fashion. Such growth could come about through the production of autocrine or paracrine growth-stimulating factors. This possibility recently has been suggested by studies that have demonstrated a differential capacity of monocytes or monocyte-derived cytokines to enhance or inhibit the proliferation of endometrial cells from women with endometriosis and healthy women, respectively (7).

In the present study, several of these concepts were investigated. Specifically, tests were performed to compare [1] the relative capacity of peripheral blood monocytes (PBM) and peritoneal macrophages (PM) from women with endometriosis to mediate the cytolysis of autologous eutopic endometrial cells (i.e., the differential immunologic capacities of systemic and regional immune cells to lyse endometrial targets); and [2] the relative sensitivity of eutopic and ectopic endometrial cells from the same patient to lysis by autologous PBM and PM. The results demonstrate that in women with endometriosis, the ability of monocytes and macrophages to mediate the cytolysis of endometrial cells varies with both the source of the macrophages and the source of the endometrial target cells.

MATERIALS AND METHODS

Study Population

The subjects in this study were women of reproductive age who were undergoing laparoscopy for suspected endometriosis. The extent of the disease was determined laparoscopically and staged according to the revised American Fertility Society classification (8). A total of 24 patients were tested at the time of initial diagnosis and staging, 15 of whom had stage I/II disease and 9 of whom had stage III/IV disease. In addition, 4 patients were tested after treatment with GnRH agonists (GnRH-a).

During laparoscopy in all subjects, peritoneal fluid was aspirated into a sterile container with 0.2 mL of heparin at a concentration of 1,000 U/mL, and an endometrial biopsy was performed with the use of a Novak curet. A part of each endometrial biopsy specimen then was placed in a sterile container with normal saline solution for the macrophage study and another was sent for routine histologic examination. In a subgroup of eight subjects, exophytic endometriotic implants devoid of fibrous tissue reaction were identifiable during laparoscopy. These implants were removed with the biopsy forceps and placed in normal saline solution.

A part of each specimen then was examined histologically and another was processed for the macrophage study. Peripheral blood sampling was performed in all subjects after the induction of anesthesia. The histologic examination of all

biopsy specimens of the endometriotic implants demonstrated endometrial glands and stroma without connective tissue elements. The endometrial biopsies demonstrated proliferative endometrium in 15 subjects and secretory endometrium in 9 subjects. The study was approved by the institutional review board at our center and the subjects gave their written informed consent.

Collection and Isolation of Peripheral Blood Monocytes

Venous blood from the subjects was drawn aseptically into sterile 10-mL Vacutainer tubes (Becton Dickinson, Bedford, MA) containing 0.2 mL of heparin at a concentration of 1,000 U/mL. The blood was diluted 3:1 in Hanks' balanced salt solution (HBSS; Whittaker Bioproducts, Walkersville, MD), layered onto lymphocyte separation medium (Bionetics, Kensington, MD), and centrifuged at $480 \times g$ for 30 minutes at room temperature to obtain a band of mononuclear cells. The mononuclear cells were collected, pooled, and washed twice in HBSS before counting. The cell viability as determined by Trypan blue staining was consistently $>99.5\%$.

Mononuclear phagocytes in an aliquot of the mononuclear cells then were quantified by latex ingestion, as described previously (9). The mononuclear cells were suspended in complete medium consisting of Roswell Park Memorial Institute (RPMI)-1640 medium (Whittaker Bioproducts), 10% heat-inactivated fetal calf serum (FCS, lot number OMO222; Whittaker Bioproducts), 50 IU/mL of penicillin, 50 $\mu\text{g}/\text{mL}$ of streptomycin (GIBCO, Grand Island, NY), and L-glutamine (Sigma, St. Louis, MO), and the cell concentration was adjusted to contain 1×10^6 mononuclear phagocytes per milliliter. The cells then were dispensed into 96-well microtiter plates at a concentration of 1×10^5 latex-ingesting cells per well in a final volume of 100 μL per well. The cells were allowed to adhere for 2 hours at 37°C in 5% CO_2 and then were washed three times with RPMI-1640 before further manipulation.

Collection and Isolation of Peritoneal Macrophages

The peritoneal fluid collected sterilely at the time of laparoscopy was centrifuged and the cell pellet was resuspended in RPMI medium, followed by centrifugation on lymphocyte separation medium at room temperature to obtain the mononuclear cell layer. The percentage of macrophages was estimated in an aliquot of mononuclear cells by latex ingestion and the cells then were processed as described previously.

Representative samples were examined microscopically and counted to ensure that differential adherence between samples was not a significant problem. However, to ensure that differential adherence could not contribute to or confound the interpretation of the results, the cytotoxicity assay used an effector/target cell ratio that ensured maximum cytotoxicity for all samples tested.

Endometrial Cell Preparation

The eutopic and ectopic endometrial tissues were subjected to a single 20-minute cycle of digestion with a mixture of collagenase (0.014%) and deoxyribonuclease (0.01%) in HBSS at 37°C to prepare single cells (7). After digestion, the endometrial cells were filtered through sterile mesh (3-163T Nitex Mesh; Martin Supply, Baltimore, MD), collected by centrifugation, and resuspended in McCoy's 5A medium (Whittaker Bioproducts) containing 10% FCS (Whittaker Bioproducts), 100 U/mL of penicillin, and 100 µg/mL of streptomycin. The cell suspensions contained both stromal cells and glandular epithelial cells based on morphology; no attempt was made to separate stromal cells from epithelial cells, so the results represent the net cytolysis obtained with a mixture of both endometrial cell types.

Macrophage Cytotoxicity Assay

We used a method that we described previously (10) to perform the macrophage cytotoxicity assay. Briefly, PBM and PM were suspended at a concentration of 1×10^5 in 100 µL of RPMI medium containing 10% FCS, 100 U/mL of penicillin, and 100 µg/mL of streptomycin and allowed to adhere to microtiter wells for 18 hours at 37°C. Adherent PBM and PM cultures were washed twice with RPMI medium containing 10% FCS, and then 5×10^3 51 chromium-labeled autologous endometrial cells were added to each well.

This number of target cells gave an effector/target cell ratio of 20/1. Macrophages and target cells were incubated together at 37°C for 24 hours before the collection of supernatants with the Titertek system (Skatron, Sterling, VA) and the quantitation of 51 chromium release by gamma counting. The percentage of specific cytotoxicity produced by the macrophages was calculated as follows:

$$\text{Percentage of specific cytotoxicity} = \frac{(E - S)}{(T - S)} \times 100$$

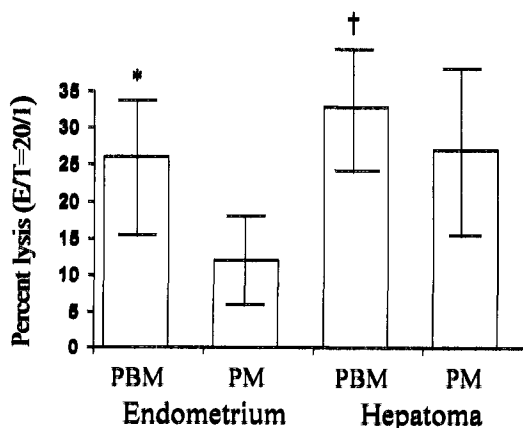
where E = counts per minute released from target cells in the presence of macrophages, S = counts per minute released from target cells in the absence of macrophages, and T = total counts per minute released from target cells after treatment with 2% sodium dodecyl sulfate.

Statistical Analysis

The data in this study were analyzed by paired, two-tailed *t*-tests and by Wilcoxon's rank sum test for nonparametric data. Differences between and among subgroups of patients were tested by analysis of variance (ANOVA).

FIGURE 1

Lysis of autologous eutopic endometrial cells and Chang hepatoma cells by peripheral blood monocytes (PBM) and peritoneal macrophages (PM) from women with endometriosis. The cytotoxicity assay was performed at an effector/target cell ratio of 20/1 (E/T = 20/1). Results are expressed as means \pm 1 SE. **P* = 0.001 (PBM versus PM by ANOVA). †*P* = not significant (PBM versus PM).



RESULTS

Cytolysis of Autologous Eutopic Endometrial Cells by Peripheral Blood Monocytes and Peritoneal Macrophages From Women With Endometriosis

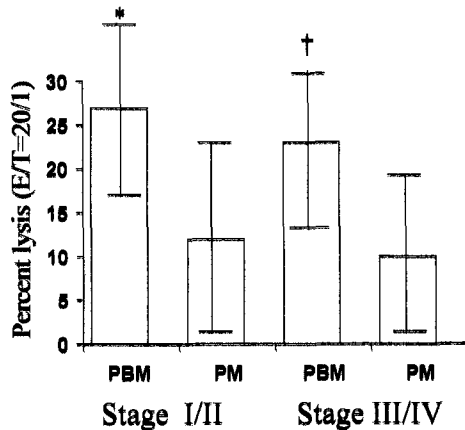
Peripheral blood monocytes were significantly more cytolytic against autologous eutopic endometrial cells than were PM from the same patient. This was true when the entire patient population was considered as a group (mean [\pm SD] percentage of cytotoxicity, 25.5% \pm 18% for PBM and 12.3% \pm 13% for PM; *P* = 0.001 by ANOVA (Fig. 1) and also was maintained when patients with limited disease (mean [\pm SD] percentage of cytotoxicity, 26.8% \pm 15% for PBM and 11.7% \pm 14% for PM; *P* = 0.007 by ANOVA) or advanced disease (mean [\pm SD] percentage of cytotoxicity, 22.3% \pm 21% for PBM and 10% \pm 11% for PM; *P* = 0.04 by ANOVA) were considered separately (Fig. 2).

The same relation also was found when patients in the proliferative (*P* = 0.004 by ANOVA) or secretory (*P* = 0.03 by ANOVA) phase of the menstrual cycle were considered separately (Fig. 3). Finally, this relation also was observed in four patients who were tested while they were receiving therapy with GnRH-a for 1 month (*n* = 1), 3 months (*n* = 2), or 6 months (*n* = 1) (Fig. 4).

It is of interest, however, that this differential cytotoxic activity of PBM and PM was not observed when the same immune cells were tested for their cytolytic activity against a reference, natural killer (NK) cell-insensitive target cell line, the Chang hepatoma. In these tests, the

FIGURE 2

Lysis of eutopic endometrial cells by autologous peripheral blood monocytes (PBM) and peritoneal macrophages (PM) from women with either stage I/II ($n = 15$) or stage III/IV ($n = 11$) endometriosis. The cytotoxicity assay was performed at an effector/target cell ratio of 20/1 (E/T = 20/1). Results are expressed as means \pm 1 SE. * $P = 0.007$ (PBM versus PM by ANOVA). † $P = 0.03$ (PBM versus PM).



cytolytic activity of PBM and PM was equivalent (i.e., it did not differ significantly on analysis by paired t -tests or a rank sum test). The mean (\pm SD) percentage of cytotoxicity was 32.8% \pm 19% for PBM and 26.9% \pm 15% for PM (Fig. 1).

FIGURE 3

Lysis of eutopic endometrial cells by autologous peripheral blood monocytes (PBM) and peritoneal macrophages (PM) from women with endometriosis tested during the proliferative ($n = 19$) or the secretory ($n = 9$) phase of the menstrual cycle. The cytotoxicity assay was performed at an effector/target cell ratio of 20/1 (E/T = 20/1). Results are expressed as means \pm 1 SE. * $P = 0.004$ (PBM versus PM by ANOVA). † $P = 0.04$ (PBM versus PM).

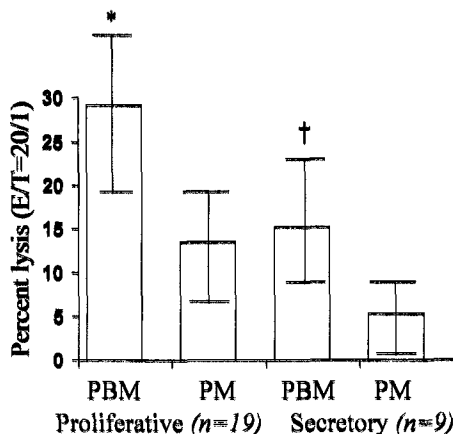
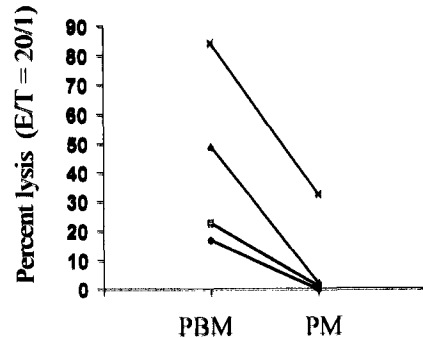


FIGURE 4

Lysis of eutopic endometrial cells by autologous peripheral blood monocytes (PBM) and peritoneal macrophages (PM) from four patients tested during treatment with GnRH-a. The cytotoxicity assay was performed at an effector/target cell ratio of 20/1 (E/T = 20/1); individual results for matched PBM and PM specimens are depicted.



Cytolysis of Autologous Endometrial Cells From Eutopic and Ectopic Sites by Peripheral Blood Monocytes and PM

In a subset of patients ($n = 8$), it was possible to obtain pure ectopic endometrial cells from peritoneal implants identified at laparoscopy. This permitted a direct comparison of the ability of PBM and PM from these women to mediate the in vitro cytolysis of autologous endometrial cells from separate anatomic sites. The results for this subgroup of patients (Fig. 5) showed that PBM are superior to PM in mediating cytolysis against both eutopic and ectopic endometrial cells. The mean (\pm SD) percentage of cytotoxicity was 23.1% \pm 13% for PBM and 5.4% \pm 7% for PM ($P = 0.03$) against the eutopic targets and 7.8% \pm 7% for PBM and 0.25% \pm 0.7% for PM ($P = 0.03$) against the ectopic targets.

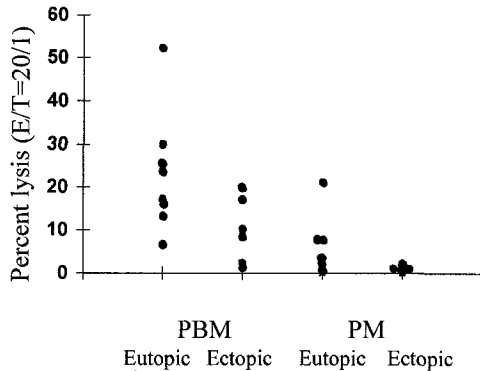
It also can be appreciated from these results that there was a statistically significant decrease in the sensitivity of the ectopic endometrial cells to cytolysis by macrophages compared with the matched eutopic endometrial cells. This was true for both PBM ($P = 0.004$, eutopic versus ectopic) and PM ($P = 0.04$, eutopic versus ectopic) as the effector population. There was no difference in the sensitivity of ectopic endometrial cells to lysis by PBM or PM when patients in the proliferative and secretory phases of the menstrual cycle were considered separately.

DISCUSSION

The results of this study demonstrate that the capacity of macrophages from patients with endometriosis to lyse autologous endometrial cells varies with both the source of the macrophages and the source of the endometrial cells. Thus, PBM were significantly more effective at mediating the in vitro destruction of endometrial cells than were PM from the

FIGURE 5

Lysis of eutopic and ectopic endometrial cells by autologous peripheral blood monocytes (PBM) and peritoneal macrophages (PM) from women with endometriosis. The cytotoxicity assay was performed at an effector/target cell ratio of 20/1 (E/T = 20/1). The individual results for eight patients are depicted.



same patients. Further, ectopic endometrial cells obtained from the peritoneal cavity of patients with endometriosis were significantly more resistant to macrophage-mediated cytotoxicity than were eutopic endometrial cells obtained from the same patients.

These relations were seen in women with both limited and advanced disease and during both the proliferative and secretory phases of the menstrual cycle. These same effects also were seen in patients undergoing treatment with GnRH-a. Taken together, these results suggest that the changes described herein are a hallmark of endometriosis.

Based on the assumption that normal homeostasis within the peritoneal cavity of menstruating women requires macrophages to participate in the destruction of ectopic endometrial cells, the results of the present study suggest that the impaired ability of PM to destroy endometrial cells and the inherent resistance of ectopic cells to immune-mediated destruction are fundamental to the etiology and/or pathophysiology of endometriosis.

If endometriosis develops when macrophage systems fail to eliminate ectopic endometrial cells, then it is important to elucidate the mechanism responsible for such failure. In the present investigation, we postulated that such failure could come about as a result of either defective immunologic capacity on the part of macrophages or an inherent resistance of ectopic endometrial cells to immunologically mediated destruction. The results obtained in this study suggest that both phenomena can occur in women with endometriosis. Thus, PM consistently were found to be less cytolytic than were circulating monocytes from the same patients. This suggests that conditions within the peritoneal cavity of women with endometriosis may inhibit macrophage-mediated cytolytic functions.

Similar deficits have been demonstrated for lymphocytes and NK cells from women with endometriosis (4, 6). In addition, studies that document increased levels of inflammatory and/or immunoregulatory cytokines in peritoneal fluid from these women (11, 12, 13), some of which may be produced by PM themselves (14), provide at least one explanation for the impaired PM cytotoxicity demonstrated in the present investigation.

Nevertheless, it also is important to recognize that the deficiency of PM in mediating endometrial cell destruction was not observed when a different target cell was used. When an NK cell-insensitive hepatoma cell line was used, PM were found to mediate substantial levels of cytotoxicity that were equivalent to those mediated by circulating monocytes from the same subjects. These results also are supported by our previous study (10), which demonstrated that monocytes and macrophages from patients with endometriosis have an increased capacity to lyse NK cell-insensitive target cells compared with the activity of macrophages from control subjects.

This finding suggests that conditions inherent in the peritoneal cavity of women with endometriosis that favor endometrial cell survival are relatively specific for endometrial cells and are not the result of generalized immunosuppression. Different mechanisms for the cytotoxicity of endometrial cells and of the hepatoma cell line also could explain the greater capacity of PBM than PM to lyse endometrial targets but not the hepatoma cell line.

Perhaps the most unexpected finding in this study was the demonstration that endometrial cells from ectopic implants are substantially more resistant to macrophage-mediated cytotoxicity than are eutopic endometrial cells from the same subject. In fact, when autologous PM were used as the effectors in these studies, the ectopic endometrial cells demonstrated virtually complete resistance to cytotoxicity.

The explanation for this difference in sensitivity is unknown and may reflect differences in the relative ratio of glandular and stromal cells in ectopic versus eutopic cells. Although our working hypothesis is that an intrinsic resistance to immune-mediated apoptosis is a fundamental property of the endometrial cells from women with endometriosis, direct support for this hypothesis will require a determination of the relative sensitivity of glandular and stromal cells from eutopic and ectopic endometrium to immune-mediated cytotoxicity.

If additional studies demonstrate resistance of endometrial cell subtypes to macrophage-mediated cytotoxicity, this could reflect an abnormal response of the resistant cells to the apoptotic signaling molecules expressed on the surface of activated immune cells. Although molecules such as FAS, tumor necrosis factor, or other members of the tumor necrosis factor superfamily of molecules normally activate the apoptotic program in susceptible cells (15, 16), tumor necro-

sis factor and related molecules also can provide proliferative or differentiation signals to cells that have lost the ability to undergo programmed cell death (17).

This mechanism appears to explain the resistance of certain tumor cells to immune-mediated programmed cell death (18), and it could operate in subsets of endometrial cells from women with endometriosis. Such a mechanism also would explain our demonstration that PBM or monocyte-derived cytokines enhance eutopic endometrial cell proliferation in women with endometriosis but suppress endometrial cell proliferation in healthy women (7). Our recent demonstration that endometrial cells from women with endometriosis are relatively resistant to spontaneous apoptosis compared with endometrial cells from healthy women also supports this hypothesis (19), although further studies are needed to determine whether there is differential sensitivity of endometrial cells to macrophage-mediated programmed cell death as well.

On the basis of the results of this study, we speculate that endometriosis may depend, in part, on the ability of ectopic endometrial cells to resist macrophage-mediated cytolysis and/or induction of apoptosis in peritoneal environments. This study showed that specific factors intrinsic to the macrophages within the peritoneal environment, coupled with specific characteristics of endometrial cells within that same environment, may favor the establishment of endometriosis in some women. If this is so, then it also can be speculated that altering the cytolytic activity of peritoneal macrophages or the sensitivity of ectopic endometrial implants to macrophage-mediated cytolysis through biologic manipulations may provide new approaches to disease management.

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