

# Decreased apoptosis and sensitivity to macrophage mediated cytolysis of endometrial cells in endometriosis

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**Ectopic dissemination of endometrial cells and their subsequent implantation are the mechanisms involved in the development of endometriosis. While the process of dissemination appears to be a phenomenon common to all women, it is unknown what facilitates or prevents ectopic implantation of misplaced endometrial cells. Prior studies by our group and others suggest that cell-mediated immunity in patients with endometriosis is decreased. The present studies evaluated (i) peripheral blood monocyte (PBM) and peritoneal macrophage (PM) mediated cytolysis of autologous eutopic and ectopic endometrial cells and (ii) programmed cell death (apoptosis) in the eutopic and ectopic endometrium. PBM-mediated cytolysis was (mean  $\pm$  SD)  $23.1 \pm 13\%$  for the eutopic and  $7.8 \pm 7\%$  for the ectopic endometrium ( $P < 0.004$ ), while the corresponding percentages for PM-mediated cytolysis were  $5.4 \pm 7$  and  $0.3 \pm 1$  respectively ( $P < 0.04$ ). This indicates that PBM are much more effective than PM in inducing cytolysis of both eutopic and ectopic endometrium and that ectopic endometrial cells are significantly more resistant to both PBM- and PM-mediated cytolysis. The apoptosis was significantly decreased in the eutopic endometrium of women with endometriosis as compared to fertile controls ( $0.375 \pm 0.17$  versus  $1.57 \pm 0.3$ ,  $P < 0.0001$ ).**

**Furthermore, in matched samples apoptosis was significantly lower in the ectopic ( $0.149 \pm 0.075$ ) than eutopic ( $0.375 \pm 0.17$ ) endometrium ( $P < 0.001$ ). We conclude from these studies that the decrease in the capacity of monocytes to mediate cytolysis of the misplaced endometrial cells in the peritoneal locations and an increased resistance of these cells to apoptosis are fundamental to the aetiology and/or pathophysiology of endometriosis.**

*Key words:* apoptosis/cytolysis/endometriosis/endometrium/macrophages

## Introduction

Endometriosis is a disease characterized by ectopic growth and function of the endometrial cells, with associated clinical symptoms and findings. Numerous theories have been proposed to explain the aetiology and pathogenesis of this disease, but none have become generally accepted. It is well established that the dissemination of the endometrial cells during menses from the uterus and into ectopic locations is a common phenomenon, occurring probably in all women. It remains unknown, however, why misplaced endometrial cells implant only in ~10–15% of women, which represents the frequency of this disease in the general female population.

During the past two decades, several reports have been published by our group and other investigators which identified a decrease in the cell-mediated immunity in rhesus monkeys and in women with endometriosis (Dmowski *et al.*, 1981; Steele *et al.*, 1984; Oosterlynck *et al.*, 1991, 1992, 1993; Vigano *et al.*, 1991; Braun *et al.*, 1992; Kanzaki *et al.*, 1992). We also reported that monocytes/macrophages in women without endometriosis inhibit, but in women with endometriosis enhance the proliferation of endometrial cells *in vitro* (Braun *et al.*,

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1994a). Based on these data, we proposed that cells of the immune system, particularly monocytes/macrophages, can recognize misplaced endometrial cells and/or endometrial antigens as abnormal (Dmowski *et al.*, 1993, 1994). We postulated that macrophages, together with natural killer (NK) cells and cytotoxic T-lymphocytes (CTL) in healthy women, destroy misplaced endometrial cells through direct cytotoxicity, and suppress their proliferation, thereby preventing their ectopic implantation and development of endometriosis. We postulated further that in ~10–15% of women, because of an alteration at the endometrial cell and monocyte/macrophage level, interaction between these cells results in the abnormal proliferation and ectopic implantation of the misplaced endometrial cells *in vivo*, leading to endometriosis.

This hypothesis suggests the following possibilities: (i) monocytes/macrophages are principally responsible for the destruction of ectopic endometrial cells in normal fertile women; (ii) the development of endometriosis is facilitated by the failure of monocyte/macrophage systems to eliminate ectopic endometrial cells; (iii) defective elimination of ectopic endometrial cells reflects impaired or abnormal macrophage function and/or an inherent endometrial cell resistance to normal homeostatic mechanisms, including those mediated by cells of the immune system.

These possibilities were investigated in the present studies designed to compare the ability of peripheral blood monocytes (PBM) and peritoneal macrophages (PM) to mediate *in-vitro* cytolysis of autologous eutopic and ectopic endometrial cells in women with endometriosis, and to evaluate spontaneous apoptosis in eutopic and ectopic endometrial cells from women with endometriosis as compared to normal controls.

## Collection, isolation and analysis of monocytes and macrophages

### Study population

The subjects in these studies were women of reproductive age undergoing laparoscopy for suspected endometriosis. The presence and extent of the disease were determined laparoscopically and staging was performed according to the revised American Fertility Society (r-AFS) classification (1985). Controls were healthy, fertile women without evidence of endometriosis undergoing laparoscopic tubal sterilization. Peripheral blood and peritoneal fluid sampling and biopsies of the uterine endometrium were performed at the time of laparoscopy. In a subgroup of subjects who had exophytic endometriotic implants devoid

of fibrous tissue reaction, these endometriotic implants were removed with laparoscopic biopsy forceps.

### Collection and isolation of peripheral blood monocytes

Venous blood from subjects was aseptically drawn into sterile 10 ml vacutainer tubes containing 0.2 ml heparin at a concentration of 1000 U/ml. The blood was diluted 3:1 in Hank's balanced salt solution (HBSS), layered onto lymphocyte separation medium (LSM; Bionetics, Kensington, MD, USA) and centrifuged at 480 g for 30 min at room temperature to obtain a band of mononuclear cells. The mononuclear cells were collected, pooled and washed twice in HBSS prior to counting. The cell viability as determined by Trypan Blue was consistently >99.5%. Mononuclear phagocytes in an aliquot of the mononuclear cells were then quantified by latex ingestion, as we have described previously (Braun and Harris, 1981). The mononuclear cells were suspended in complete medium consisting of Roswell Park Memorial Institute (RPMI) 1640 medium (Whittaker Bioproducts, Walkersville, MD, USA), 10% heat-inactivated fetal bovine serum (FBS; Whittaker Bioproducts, Lot No. 0M0222), 50 IU/ml penicillin, 50 µg/ml streptomycin (Gibco, Grand Island, NY, USA) and L-glutamine (Sigma, St Louis, MO, USA) and the cell concentration adjusted to contain  $1.0 \times 10^6$  mononuclear phagocytes/ml. The cells were then dispensed into 96-well microtitre plates at a concentration of  $1 \times 10^5$  latex-ingesting cells/well in a final volume of 100 µl/well. The cells were allowed to adhere for 2 h at 37°C, 5% CO<sub>2</sub> and washed three times with RPMI 1640 before further manipulation.

### Collection and isolation of peritoneal macrophages

The peritoneal fluids collected sterilely at the time of laparoscopy were centrifuged and the cell pellet resuspended in RPMI medium (Whittaker Bioproducts), followed by centrifugation on LSM (Bionetics) 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 were then processed as described above.

### Assays for macrophage cytotoxicity and apoptosis

#### Endometrial cell preparation

All samples of eutopic and ectopic endometrium were divided into two parts. One part was placed in a formaldehyde solution for routine histological examination, and the other was placed in a normal saline for enzymatic digestion. This latter part was then subjected to a single 20 min cycle of digestion with a mixture of

collagenase (0.014%) and DNase (0.01%) in HBSS (Whittaker) at 37°C to prepare single cells (Braun *et al.*, 1994b). Following digestion, the endometrial cells were filtered through sterile mesh (3–163T Nitex Mesh; Martin Supply, Baltimore, MD, USA), collected by centrifugation and resuspended in McCoy's 5A medium (Whittaker) containing 10% FBS (Whittaker), 100 U/ml penicillin and 100 µg/ml 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 and thus the results represent the net cytolysis obtained with a mixture of both endometrial cell types.

#### Macrophage/cytotoxicity assay

Peripheral blood monocytes (PBM) and peritoneal macrophages (PM) were suspended at a concentration of  $1 \times 10^5$  in 100 µl of RPMI medium containing 10% FBS, 10 U/ml penicillin and 100 µg/ml streptomycin and allowed to adhere to microtitre wells for 18 h at 37°C. Adherent PBM and PM cultures were washed twice with RPMI medium +10% FBS, following which,  $5 \times 10^3$  <sup>51</sup>chromium-labelled autologous endometrial cells were added to each well. This number of target cells gave an effector/target cell (E/T) ratio of 20/1. Macrophages + target cells were incubated together at 37°C for 24 h prior to collection of supernatants with the Titertek system and quantitation of <sup>51</sup>chromium release by liquid scintillation counting. The percentage specific cytotoxicity produced by macrophages was calculated as follows:

$$\% \text{ specific cytotoxicity} = [(E - S)/(T - S)] \times 100$$

where *E* = cpm released from target cells in the presence of macrophages, *S* = cpm released from target cells in the absence of macrophages and *T* = total cpm released from

target cells following treatment with 2% sodium dodecyl sulphate.

#### Apoptosis assay

Single-cell suspensions of eutopic and ectopic endometria obtained with enzymatic digestion were each analysed for spontaneous apoptosis using the cell death detection ELISA kit (Boehringer Mannheim Corporation, Indianapolis, IN, USA). Briefly, cells were resuspended in ELISA buffer for 30 min at 4°C and centrifuged for 10 min at 20 000 *g*. Supernatants containing fragmented DNA were removed, diluted 1:10 in isotonic buffer and overlaid onto coated modules. Triplicate samples were then treated with anti-DNA peroxidase and developed with substrate. The resultant photometric data (in which increasing units of absorption correlate with the level of apoptosis) were measured using an ELISA plate reader.

### Cytolysis and apoptosis in eutopic and ectopic endometrial cells

#### Effectiveness of PBM and PM in the cytolysis of autologous eutopic endometrial cells

The results of this study demonstrated that PBM were significantly more cytolytic against autologous eutopic endometrial cells than PM in women with endometriosis (Table I). This was true when the entire population was considered, and when patients were considered based on the stage of the disease, or the phase of the menstrual cycle.

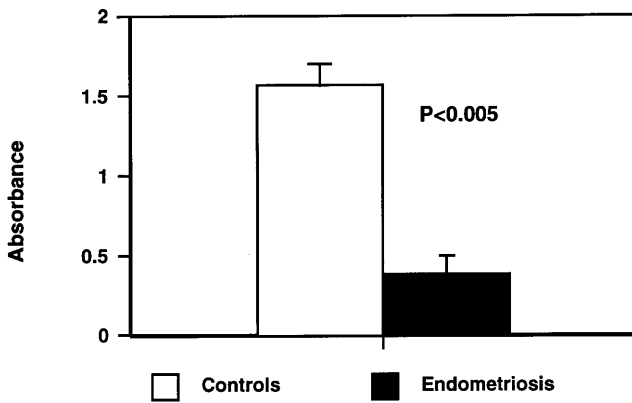
#### Sensitivity of ectopic versus eutopic endometrial cells to PBM- and PM-mediated cytolysis

The ectopic endometrial cells were significantly more resistant to cytolysis by either PBM or PM than eutopic endometrial cells from the same patients (Table II). Interestingly, PM had almost no cytolytic effect on the autologous ectopic endometrial cells.

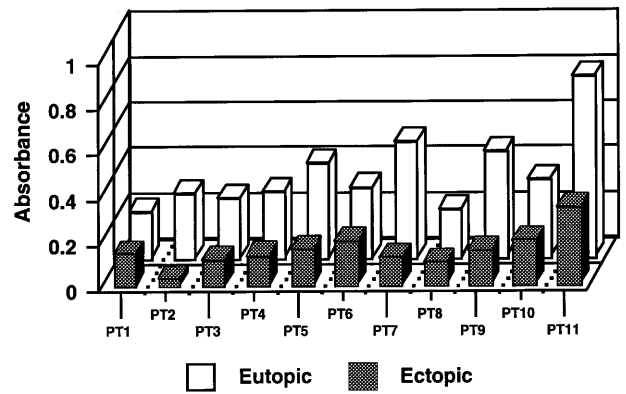
**Table I.** Cytolysis of eutopic endometrial cells by autologous peripheral blood monocytes (PBM) and peritoneal macrophages (PM) from women with endometriosis

Group	No. of patients	Cytotoxicity (mean % ± SD)		Significance <sup>a</sup>
		PBM	PM	
All patients	28	25.5 ± 18	12.3 ± 13	0.0006
Stage I/II	15	26.8 ± 15	11.7 ± 14	0.01
Stage III/IV	11	22.3 ± 21	10.0 ± 11	0.03
Proliferative	19	29.2 ± 20	13.5 ± 14	0.009
Secretory	9	15.2 ± 7	5.3 ± 10	0.03

<sup>a</sup>*P* values obtained from two-tailed, Student's *t*-test comparing PBM and PM.



**Figure 1.** Spontaneous apoptosis (mean  $\pm$  SD) in endometria from women with ( $n = 11$ ) and without ( $n = 6$ ) endometriosis.



**Figure 2.** Spontaneous apoptosis in paired samples of eutopic and ectopic endometrial cells.

**Table II.** Cytotoxicity of matched eutopic and ectopic endometrial cells by autologous peripheral blood monocytes (PBM) and peritoneal macrophages (PM) from women with endometriosis ( $n = 8$ )

Endometrial cell	Cytotoxicity (mean % $\pm$ SD)		Significance <sup>a</sup>
	PBM	PM	
Eutopic	23.1 $\pm$ 13	5.4 $\pm$ 7	0.03
Ectopic	7.8 $\pm$ 7	0.3 $\pm$ 1	0.03
Significance <sup>a</sup>	0.004	0.04	

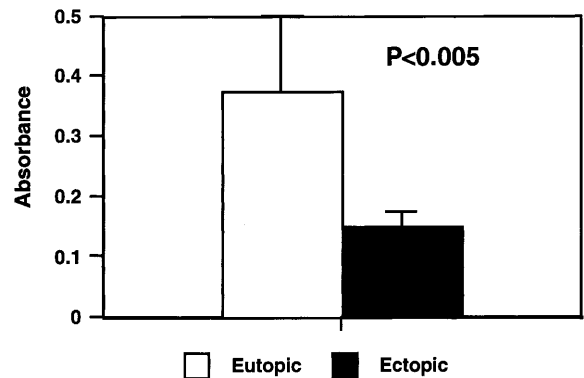
<sup>a</sup>*P* values obtained from paired Student's *t*-tests.

**Apoptosis of eutopic endometrial cells**

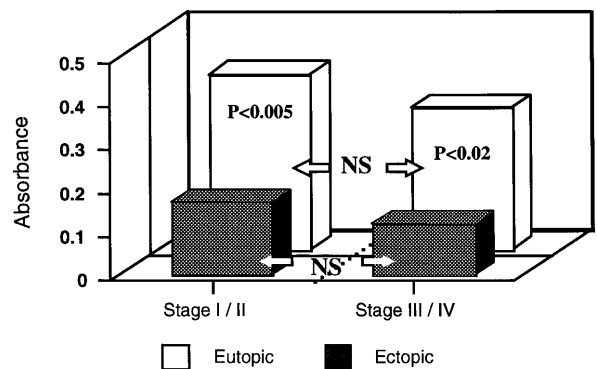
Spontaneous apoptosis in the uterine endometrium was significantly lower in women with endometriosis ( $n = 11$ ;  $0.375 \pm 0.17$ ) than in normal controls ( $n = 6$ ;  $1.57 \pm 0.3$ ,  $P < 0.0001$ ; Figure 1). The differences between spontaneous apoptosis of eutopic and ectopic endometrium were maintained when proliferative and secretory phases of the cycle were evaluated independently (data not shown).

**Rates of apoptosis in ectopic versus eutopic endometrium**

In matched samples of eutopic and ectopic endometrium (Figure 2), spontaneous apoptosis was significantly lower in the ectopic ( $0.149 \pm 0.075$ ) compared to eutopic endometrium ( $0.375 \pm 0.17$ ,  $n = 11$ ,  $P < 0.001$ ; Figure 3). When the patients were categorized according to the stage of endometriosis, the same pattern was still apparent. In advanced (stage III and IV) as compared to limited (stage I and II) endometriosis, spontaneous apoptosis was lower both in the eutopic ( $0.32 \pm .10$  versus  $0.4 \pm 0.2$ ) and ectopic ( $0.116 \pm 0.06$  versus  $0.160 \pm 0.07$ ) endometrium. However,



**Figure 3.** Spontaneous apoptosis (mean  $\pm$  SD) in eutopic and ectopic endometrial cells ( $n = 11$ ).



**Figure 4.** Spontaneous apoptosis (mean  $\pm$  SD) in eutopic and ectopic endometria, stages I/II ( $n = 7$ ) versus III/IV ( $n = 4$ ).

as shown in Figure 4, the differences did not reach statistical significance.

### The role of eutopic and ectopic endometrium in endometriosis

More than 70 years ago, John Sampson (1925) proposed that endometriosis develops as a result of the 'retrograde tubal regurgitation' of endometrial cells and tissue fragments followed by implantation of these cells and tissue fragments in the ectopic locations. This concept has subsequently received support from clinical observations and experimental studies and has become generally accepted as 'Sampson's theory' on histogenesis of endometriosis. According to this theory, it is logical to assume that two factors are required for the development of endometriosis: (i) dissemination of the endometrial cells from the uterus into ectopic locations and (ii) ectopic implantation of these cells. The first event has been demonstrated repeatedly over the years. Endometrial cells and tissue fragments have been identified in the lumen of the Fallopian tubes (Ridley, 1968), in pelvic vessels (Javert, 1949) and free floating in the peritoneal fluid (Koninckx *et al.*, 1980; Bartosik *et al.*, 1986; Kruitwagen *et al.*, 1991). Interestingly, however, the presence of the endometrial cells in the ectopic locations has not been synonymous with endometriosis, the disease. Endometrial cells have been identified in the peritoneal fluids around the time of menses with equal frequency in women with and without endometriosis (Koninckx *et al.*, 1980; Bartosik *et al.*, 1986; Kruitwagen *et al.*, 1991).

If dissemination of the endometrial cells into ectopic locations is a phenomenon common to all women, then implantation of these cells (or lack of it) must be crucial to the development of endometriosis. Our prior studies were focused on the role of the immune system and specifically on the role of cell-mediated immunity in the ectopic implantation of the endometrial cells or, alternatively, in the prevention of this event. The present studies extend our initial observations.

The experiments reported here suggest that survival of endometrial cells misplaced into ectopic locations may depend on two factors: differential cytolytic effects of the monocytes/macrophages in different locations, and the inherent ability of the endometrial cells to undergo programmed cell death or apoptosis. Our studies demonstrated that PBM were significantly more effective *in vitro* in the lysis of the endometrial cells than were PM from the same patients. If present *in vivo*, this phenomenon would explain why endometriotic lesions are far more common in the peritoneal cavity than in other parts of the body.

The differential cytolytic effect of PBM and PM was independent of the menstrual cycle, being evident in both

the proliferative and secretory phases. Furthermore, the greater cytolytic effect of PBM as compared to PM was independent of the r-AFS classification of the disease. When samples of eutopic and ectopic endometrial cells from the same patients were compared for sensitivity to *in-vitro* destruction by autologous PBM or PM, the same pattern of more effective cell lysis by PBM than PM was also apparent. However, these results also demonstrate that the ectopic endometrial cells are significantly more resistant to macrophage-mediated cytolysis than the eutopic endometrial cells from the same patients. The difference is quite striking if we consider that PBM-mediated lysis of eutopic endometrial cells was  $31 \pm 13\%$  while PM-mediated lysis of ectopic endometrial cells was practically non-existent ( $0.3 \pm 1\%$ ).

The increased resistance of the ectopic endometrial cells to both PBM- and PM-mediated cytolysis indicates a potential for the abnormal survival of these cells and suggests that ectopic endometrial cells may be resistant to normal growth regulatory mechanisms and resistant to the induction of programmed cell death or apoptosis. Indeed, a second series of experiments demonstrated that apoptosis in the eutopic endometrium was significantly decreased in women with endometriosis as compared to normal fertile controls. This difference exceeded the variability associated with the phase of the menstrual cycle and remained significant when proliferative and secretory phases were evaluated separately.

In women with endometriosis, when matched samples of the eutopic and ectopic endometrium were compared, apoptosis was lower in the ectopic endometrial cells in all patients and the differences were highly significant between the groups. These findings did not change when proliferative and secretory phases of the cycle, or when limited and advanced disease, were evaluated separately. Furthermore, it appeared that in both eutopic and ectopic endometrium, apoptosis was lower in advanced than in limited disease. The sample size, however, was small and the results did not reach statistical significance.

Programmed cell death can be induced by monocytes/macrophages or their secretory products such as tumour necrosis factor (TNF)- $\alpha$ . It is unclear, however, whether the two series of experiments performed here have a common foundation and whether the monocyte/macrophage-mediated cytolysis was expressed through apoptosis. It is well recognized that TNF- $\alpha$  receptor signalling via the sphingomyelin pathway can initiate a variety of cellular effects leading to cell proliferation in some cellular systems or apoptosis in others. It is possible that, in endometriosis, alteration in this pathway leads to cell proliferation, but in

women without endometriosis to apoptosis or programmed cell death.

It is relevant that the PM, while unable to mediate destruction of ectopic endometrial cells, can serve as a principal source of TNF- $\alpha$  and possibly other growth-enhancing signals (Rana *et al.*, 1996). While TNF- $\alpha$  is thought to be a primary local signal which initiates and modulates apoptosis during menstruation (Tabibzadeh 1996), dysregulation of endometrial signalling in response to TNF- $\alpha$  in women with endometriosis may produce cyclical enhancement of ectopic endometrial cell proliferation. The recent demonstration that PM from women with endometriosis also are resistant to apoptosis, based on their increased expression of the anti-apoptotic proteins Bcl-2 and Bax (McLaren *et al.*, 1997), raises the possibility that ectopic endometrium in women with endometriosis has acquired the capacity to exploit TNF- $\alpha$  as a growth-promoting factor in an environment which contains a constant source of this cytokine.

Both apoptosis and monocyte/macrophage mediated cytolysis were evaluated in this study in cell preparations consisting of all components of the endometrial tissue. No attempt was made to determine whether these events were occurring primarily in epithelial or in stromal cells, or perhaps in both. Future studies will address this issue.

In summary, these studies have demonstrated that women with endometriosis have both a defective capacity to mediate cytolysis in peritoneal locations and an increased resistance to programmed cell death on the part of ectopic endometrial cells. Taken together, the results of these studies suggest that defective cytolytic function by macrophages within the peritoneal cavity coupled with the inherent resistance of the endometrial cells to programmed cell death may be fundamental to the aetiology and/or pathophysiology of endometriosis.

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