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Experimental Endometriosis in Immunocompromised Mice Following Adoptive Transfer of Human Leukocytes

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Abstract

Objective: To develop a chimeric human/mouse model of experimental endometriosis for the examination of the role of human immune cells in this disease.

Design: Laboratory-based study.

Setting: University-affiliated medical center.

Patients: Healthy women undergoing volunteer endometrial biopsies and blood donation.

Interventions: None.

Main outcome measure(s): In vivo analysis of the impact of the adoptive transfer of human immune cells into immunocompromised mice receiving autologous human endometrium.

Results: Similar to our previous data using nude mice, human endometrial tissue fragments injected intraperitoneally in rag2 γ (c) mice readily established experimental disease. However, in the current study, we found a significant reduction in the severity of peritoneal disease in rag γ (c) mice readily established experimental disease. However, in the current study, we found a significant reduction in the severity of peritoneal disease in rag2 γ (c) mice receiving adoptive transfer of human immune cells compared to mice which did not receive immune cells. Interestingly, human immune cells readily track into the ectopic lesions established in mice.

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Capsule: Adoptive transfer of normal human immune cells impedes the development of experimental endometriosis in immunocompromised mice.

Conclusions: The ability of immune cells to limit intraperitoneal disease in mice suggests that a robust immune system is protective against the development of endometriosis.

Keywords

Endometriosis; immune cells; immunocompromised mice

INTRODUCTION

Eighty years ago, Sampson (1) theorized that endometriosis, the growth of endometrial tissue outside the uterus, occurs as a consequence of the mechanical transfer of endometrial tissue to the peritoneal cavity via retrograde menstruation. In order to better understand the mechanisms of ectopic endometrial attachment and survival, our laboratory established a nude mouse model of endometriosis in which exposed human endometrium, obtained during the proliferative phase of the menstrual cycle, is injected intraperitoneally into ovariectomized nude mice (2). Initially, we used this nude mouse model to investigate the impact of short-term in vitro steroid hormone exposures on the subsequent early invasive establishment of human tissue lesions within the peritoneal cavity of recipient mice (2,3). Additionally, this experimental model has been invaluable to identifying key factors expressed by either the invading tissue (human), the invaded peritoneal site (mouse) or both during the invasive process required for successful disease establishment (2-7). However, a significant limitation of our nude mouse model is the immunocompromised status of the host animal, making it difficult to examine the possible role of the immune system in the establishment of an endometriosis-like disease.

During menstruation, refluxed endometrial tissue is normally composed of apoptotic cells that are rapidly cleared by the immune system; however, in women with endometriosis, some studies have shown that immune surveillance is impaired and unable to adequately respond to displaced endometrium within the peritoneal cavity (8,9). A number of studies have demonstrated several specific alterations in immune cell function in women with endometriosis, including a decrease in natural killer (NK) cell cytotoxicity (10,11) and an enhanced activation state of monocytes and peritoneal macrophages (12-14). Whether or not these cell-specific immune alterations arise as a result of endometriosis or are, in fact, a precursor to the disease is not currently known. Certainly, ectopic attachment of endometrial tissue may be associated with an inflammatory response which may aid in the establishment and progression of this disease since cytokines, released by activated leukocytes, can dramatically affect endometrial cell proliferation, differentiation, apoptosis and angiogenesis (15,16). Equally important, steroids normally play a role in directing the migration and function of endometrial leukocytes; thus, a loss of normal progesterone action, identified in women with endometriosis (reviewed by 17), would likely result in alterations in immune cell function, activation and trafficking and additionally contribute to the establishment and progression of disease.

In order to address these issues, we have developed a new model system using rag2 γ (c) (Recombinant Activating Gene 2/common cytokine receptor γ chain (γ c) double null) mice. Rag2 γ (c) mice are more completely immunosuppressed compared to nude mice, demonstrating an absence of B cells, T cells and NK cell activity. Importantly, rag2 γ (c) mice do not demonstrate an age-related compensatory immunity, allowing for xenographic studies of much longer duration than that which can be conducted in nude mice. Finally, the severity and stability of the immune defects of the rag2 γ (c) animals also allows the introduction of elements of a human immune system (18,19). Previously, Greenberg and Slayden (20) reported successful development of an endometriotic-like disease in these animals following injection of human endometrial tissues. In the current study, we have expanded this model by additionally transferring human immune cells into mice prior to the introduction of autologous

human endometrial tissues. We have examined the impact of the presence and absence of human immune cells on the initial establishment and early growth of experimental endometriosis. Our results indicate that the presence of immune cells from women with no history of endometriosis impairs the growth of intraperitoneal experimental lesions, suggesting an important role of a robust immune system in preventing the development of this disease.

MATERIALS AND METHODS

Acquisition of human tissues

Endometrial tissues (n=8) were acquired by Pipelle® (Unimar, Inc; Wilton CT) biopsy during the proliferative phase (days 9-12) of the menstrual cycle from a healthy donor population (age 18-45) exhibiting normal menstrual cycles and no history of endometriosis. An endometrial thickness ≥ 9 mm (confirmed by vaginal ultrasound) and a serum progesterone level of ≤ 1.5 ng/mL were required for inclusion in this study. Individuals with a recent (< 3 months) history of hormone therapy (i.e., oral contraceptives) were excluded. Biopsies were washed in prewarmed, phenol-red free Dulbecco's Modified Eagles Medium/Ham's F-12 Medium (DME/F-12) (Sigma) to remove residual blood and mucous prior to culturing. Informed consent was obtained prior to biopsy and the use of human tissues was approved by Vanderbilt University's Institutional Review Board and Committee for the Protection of Human Subjects.

Organ Cultures of Human Tissues

Endometrial biopsies were dissected into small cubes ($\sim 1 \times 1$ mm³) and 8-10 pieces of tissue per treatment group were suspended in tissue culture inserts (Millipore; Bedford MA) as previously described (21). Organ cultures were maintained under serum-free conditions in DME/F-12 supplemented 1% Insulin-Transferrin-Selenium (ITS+; Collaborative Biomedical; Bedford MA) and 0.1% Excyte (Miles Scientific; Kankakee IL) and incubated at 37°C in a humidified chamber with 5% CO₂. All tissues were treated with 1 nM 17 β -estradiol (E, Sigma) and maintained in culture for 18-24 hours prior to injection into mice.

Isolation of Human Leukocytes from Peripheral Blood

Using methods similar to those described by others (22), human peripheral blood mononuclear cells plus neutrophils (collectively, leukocytes) were isolated from 50 mL donor blood samples. Specifically, whole blood was collected into heparin coated Vacutainer tubes (BD) using 19-gauge needles and then subjected to dextran sedimentation and density-gradient centrifugation on ficoll-hypaque for extraction of leukocytes. Isolated cells were counted in hemocytometer, labeled with PKH26 Red Fluorescent Cell Linker (PKH; Sigma) or with CarboxyFluorescein Succinimidyl Ester (CFSE; Invitrogen) as recommended by the manufacturer. Prior to injection into recipient mice, cells were washed in PBS and resuspended at 2.5×10^6 cells/100 μ l PBS.

Experimental Endometriosis

Rag2 γ (c) mice were previously developed by the National Institute of Allergy and Infectious Diseases (23). For our study, 5-week-old female mice were purchased from Taconic Farms, Inc (Germantown, NY) and allowed to acclimate for 1 week prior to initiation of any procedure. At 6 weeks of age, animals were subjected to ovariectomy under isoflurane (Henry Schein) anesthesia; castrated mice were immediately implanted with a subcutaneous slow-release silastic capsule containing 8 mg estrogen (in cholesterol). Prior to introduction into mice, proliferative phase human endometrium was established as organ cultures and maintained as described above. Twenty-four hours later, human tissues were washed in PBS and injected into mice intraperitoneally along the ventral midline just below the umbilicus. Some mice additionally received isolated and labeled human leukocytes (2.5×10^6 cells/100 μ l PBS each) via a similar ventral midline intraperitoneal injection 24 hrs prior to human tissue injection.

Prior to establishing experimental endometriosis in mice, we conducted a series of studies in which rag2 γ (c) mice (n=16) were subjected only to peritoneal injection of PKH labeled human immune cells obtained from four different human donors and sacrificed at various times after injection. Spleens, lymph nodes and peripheral blood were removed and analyzed by flow cytometry for the presence of human immune cells.

In the series of experimental endometriosis studies, four separate experiments were conducted using four different endometrial biopsies and autologous immune cells labeled with PKH as described above. Mice were injected with human immune cells or with vehicle alone prior to injection of human endometrial tissues. At necropsy, mice (n=27) were sacrificed 10-12 days after injection of human tissue by cervical dislocation under anesthesia and examined for the presence, number and size of lesions. Lesions were measured, photographed, removed and fixed for standard microscopy. Lesions were measured in two dimensions; the largest denoted “a” and the smaller denoted “b”. The total volume of lesions was calculated by standard methodology (24) using the formula: $V = a \times b^2 \times 0.5$

In the second series of experimental endometriosis studies, all mice (n=12) received both human endometrial tissues and autologous human immune cells labeled with CFSE as described in methods. Human cells and tissues were obtained from four different women. Mice were sacrificed 5 days after injection of human tissues and excised lesions were frozen for fluorescent microscopy in order to identify CFSE-labeled human immune cells.

All experiments described herein were approved by Vanderbilt University Institutional Animal Care and Use Committee in accordance with the Animal Welfare Act.

Identification of Human Immune Cells within Mouse Tissues

Mice received human immune cells which were labeled with PKH or CFSE prior to injection in order to facilitate identification of these cells in mice. Tissues removed from mice which received PKH labeled cells were subjected to flow cytometry. Frozen sections of experimental endometriosis from mice provided CFSE-labeled human immune cells were prepared for fluorescent microscopy. Specifically, tissues were fixed in acetone and coverslipped with ProLong AntiFade Gold with DAPI (Invitrogen) mounting reagent and viewed with a Zeiss AxioVert 200m Inverted Fluorescent Microscope.

Immunohistochemistry

Immunohistochemical staining of experimental lesions for von Willebrand Factor (vWF) was performed by standard methodology for formalin fixed, paraffin embedded tissues. Briefly, five micron sections were placed on charged slides and rehydrated. Endogenous peroxidase was neutralized with 0.03% hydrogen peroxide followed by a casein-based protein block (DakoCytomation, Carpinteria, CA) for nonspecific staining blocking. The sections were incubated with rabbit anti-human von Willebrand Factor (Dakocytomation, Carpinteria, CA) after antigen retrieval with proteinase K. The Dako Envision+ HRP/DAB System (DakoCytomation) was used to produce localized, visible staining. The slides were lightly counterstained with Mayer's hematoxylin, dehydrated and coverslipped.

Statistical Analysis of Experimental Endometriosis

Each mouse was assigned a lesion score based on the number and size of lesions. Comparisons between the means were performed using analysis of variance followed by post-hoc comparisons of individual means. In the absence of normality, non-parametric testing (Kruskal-Wallis) was used.

RESULTS

Impact of Immune Cells on the Presence of Human Lesions

In order to examine the possible role of immune cells on the establishment and early progression of experimental endometriosis, rag2 γ (c) mice were subjected to intraperitoneal injection of human endometrial tissues in the presence and absence of autologous human immune cells. Human endometrial tissues and leukocytes were obtained from healthy women and 2.5 million immune cells/mouse were injected intraperitoneally into ovariectomized and estrogenized mice 24 hours prior to injection of endometrial tissues, as described in methods. Mice were sacrificed 10-12 days after receiving human endometrial tissues. As shown in Table 1, 100% of mice receiving human endometrial tissues in the absence of human immune cells exhibited intraperitoneal sites of experimental disease. However, 56% of mice receiving endometrial tissues in the presence of autologous human immune cells were free of experimental disease at necropsy. Remaining mice from this group exhibited lesions which were significantly smaller and fewer in number compared to the control group. Taken together, these findings suggest that the presence of human immune cells impedes the establishment of experimental endometriosis in mice when the immune cells and endometrial tissue are acquired from healthy women.

Gross and Microscopic Analysis of Experimental Endometriosis

At necropsy, all mice were examined for the presence of endometriosis-like disease (Figure 1). Intraperitoneal lesions observed in mice receiving both human tissues and human immune cells were smaller and typically poorly vascularized compared to lesions in mice which did not receive immune cells. Microvessel density was assessed by immunohistochemical localization of von Willebrand Factor (vWF) in lesions established in the presence and absence of human immune cells. As shown in Figure 1E and 1F, the presence of normal human immune cells was associated with decreased microvessel density.

Identification of Human Immune Cells in Mice

Two methods were employed: pKH-labelled human immune cells were identified by flow cytometry and CFSE-labeled immune cells were detected by fluorescent microscopy.

Spleens, lymph nodes and peripheral blood were obtained from mice which received intraperitoneal injections of PKH-labeled human immune cells; animals were sacrificed at various times after injection and tissues were then examined by flow cytometry for the presence of human cells. Flow cytometry confirmed the presence of human immune cells in spleens, nodes and serum, with greatest numbers of cells detected in the spleen (Table 2). Although the percent of human immune cells declined over time, cells remain detectable in these tissues for at least 20 days (last tested time-point).

Following adoptive transfer of human immune cells and establishment of experimental endometriosis, flow cytometric analysis of PKH-labeled human immune cells were detected in the majority of endometriotic lesions. Notably, however, flow cytometry may only identify relatively large number of cells; fibrotic lesions containing fewer immune cells were therefore also evaluated microscopically, whereby PKH-labeled cell were detected in intraperitoneal lesions; under visible light PKH-labeled cells appeared blue (Figure 1A). Although pKH fluoresces red, it is a blue dye under visible light. Blue lesions were only observed in lesions of mice receiving pKH-labeled human immune cells and human endometrial tissues; thus, this observation suggests an accumulation of human immune cells within the endometriotic lesions. Additionally, this data supports an ability of intraperitoneally-injected immune cells to enter the general circulation as previously reported by other investigators (22).

In order to confirm the ability of human immune cells to track to the site of early endometrial invasion within the peritoneum, we also examined lesions which were established in the presence of CFSE-labeled immune cells by microscopy. Mice were sacrificed 5 days after human tissue injection (6 days after introduction of human immune cells). Lesions from these mice contained cells, which fluoresced green (Figure 2), indicating the presence of CFSE and thus the migration of human immune cells to sites of ectopic endometrial growth.

Discussion

The role of the immune system in the development and/or prevention of endometriosis remains poorly understood. Defects within this system have long been noted in women with endometriosis, but whether these defects are a cause of the disease or a result of inflammatory processes at ectopic sites is difficult to determine. Unraveling the relationship between the immune system and endometriosis could not only transform our understanding of endometriosis, but aid in the development of more targeted therapeutic approaches to this disease. We have established an experimental model system in which the role of immune cells can begin to be examined. Using a severely immunocompromised mouse strain, the *rag2 γ (c)* mouse, we have introduced human tissues after the adoptive transfer of autologous human immune cells.

Introduction of human immune cells into severely immunocompromised mice is not novel (19,22), and has proven useful in dissecting the role of the immune cell in numerous diseases including certain cancers and graft versus host disease. However, to our knowledge, this technique has not previously been applied to investigate the impact of immune cells on the development and progression of experimental endometriosis.

It has been suggested that all menstruating women have endometriosis, but that only some develop symptomatic disease (25). Unfortunately, what makes one woman, but not others, susceptible to symptomatic disease remains elusive. Our present (Figure 1) and previous (2) studies indicate human endometrium obtained from disease-free women readily establishes ectopic disease in immunocompromised mice, suggesting that eutopic endometrium is intrinsically capable of ectopic growth. In healthy, disease-free women, it is believed that prevention of endometriosis is dependent, at least in part, upon appropriate cell-mediated immunity which scavenges displaced endometrial tissues (8). Our data support this concept, since we demonstrated that intraperitoneal disease resulting from tissues obtained from disease-free donors can be inhibited by the presence of normal human immune cells. However, these same tissues grew unabated in mice, which did not receive immune cells, as observed in our previous studies as noted above. Thus, our current data using tissues and cells from disease-free women indicates a primary role for the immune system in preventing disease. There are likely multiple mechanisms for this effect, including the well known role of the immune system in clearing displaced endometrial cells (8). Additionally, we demonstrated a decrease in microvessel density (MVD) in endometriotic-like lesions, which developed in mice in the presence of normal human immune cells compared to lesions in mice which were not adoptively transferred with normal immune cells (Figure 1). Several studies demonstrate an increase in MVD in both eutopic and ectopic tissues from women with endometriosis (26,27). Angiogenesis is critical in order for ectopic tissues to survive and, not surprisingly, enhanced MVD is also a notable component of neoplastic tumors (28,29). Our data suggests that *normal* immune cells may act to impede vascular development of ectopic human tissues, likely playing a role in the reduction of implant load noted in these mice. For example, macrophages have been found to either promote tumor vascularization or promote wound healing depending on their state of activation (reviewed by 30). Thus, in women with endometriosis, in which peritoneal macrophages and monocytes are more abundant (31,32), and demonstrate increased expression of proinflammatory cytokines, angiogenesis and invasive activity may be

enhanced, resulting in survival of ectopic endometrial tissues rather than impairing these processes (33,34). Additionally, we and others have demonstrated a decreased endometrial sensitivity to progesterone in both the eutopic and ectopic endometrium of women with endometriosis (35-37). Progesterone has potent anti-inflammatory effects within the endometrium (38); a loss of normal progesterone response would likely lead to alterations in the microenvironment resulting in a more inflammatory-like phenotype. Specifically, in the normal mouse uterus, estrogen treatment is proinflammatory, promoting an influx of neutrophils and macrophages. Progesterone antagonizes this effect on immune cell migration, resulting in recruitment of fewer numbers of cells (39). However, in progesterone receptor knockout (PRKO) mice, progesterone is unable to impede immune cell recruitment and, importantly, neutrophils which traffick to PRKO uteri immediately become activated, further promoting an inflammatory environment (40). Thus, while the prevention of endometriosis appears to require an intact immune system, alterations to this system may actually promote development of the disease.

In our experimental model system, adoptive transfer of control human immune cells into rag2 γ (c) mice had a clear, beneficial impact against the development of experimental endometriosis. However, acquiring cells and tissues from women with active endometriosis for use in this model system should provide even greater insight into the failures of homeostatic mechanisms which allow for the development of this disease. Additionally, this model will allow the examination of the impact of specific populations of immune cells on development of experimental disease, which should greatly facilitate our understanding of the dual actions (beneficial and detrimental) of the immune system in endometriosis.

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Figure 1.

Gross (A-B) and microscopic (C-F; formalin-fixed) analysis of experimental endometriosis in *rag2γ(c)* mice. Proliferative phase human endometrium was injected intraperitoneally into mice in the presence (A, C, E) and absence (B, D, F) of human leukocytes. Sections shown in C and D were stained with hematoxylin and eosin. Sections shown in E and F were subjected to immunohistochemistry using an antibody to vWF. Magnification, A-B, 15x; C-F, 100x.

Figure 2.

Localization of human immune cells in frozen experimental lesions. **A.** Immunofluorescent imaging of CSFE-labeled human immune cells (green) within the human tissue growing in a mouse (human and mouse tissue stained with DAPI, fluoresces blue). **B.** Hematoxylin and eosin staining of a near sister section shown in A. In both frames, the dotted line marks the interface between the mouse (m) and human (h) tissues. Original magnification 200x.

Table 1

Experimental endometriosis in mice receiving human endometrial tissues in the presence or absence of human immune cells. Mice were sacrificed 10-12 days after receiving human endometrial tissues.

Treatment	Immune cells		P-value
	No	Yes	
Proportion of mice with lesions	9/9 (100%)	8/18 (44%)	0.016
Number of lesions per mouse	1.3±0.2	0.5±0.1	0.0003
Total volume of all lesions per animal (mm ³)	3.1±0.4	0.3±0.3	0.0001
Average volume of individual lesions (mm ³)	2.2±0.3	0.7±0.3	0.001

Table 2

Identification of PKH-labeled human leukocytes in blood and tissues obtained from rag2^γ(c) mice. Mice were sacrificed at various time-points after injection with human immune cells. Values represent the average percentage (±SEM) of human cells identified by flow cytometry from four separate experiments.

	4 hrs	72 hrs	5 days	10 days	20 days
Blood	1.5 ±0.3%	1.1 ±0.5%	0.6 ±0.6%	0.3 ±0.2%	0.1 ±0.2%
Spleen	5.2 ±1.5%	4.7 ±0.5%	2.7 ±0.2%	1.9 ±0.2%	1.2 ±1.0%
Node	4.8 ±0.3%	4.5 ±0.3%	2.2 ±0.2%	1.1 ±0.2%	0.8 ±0.1%