AN EXPERIMENTAL MODEL FOR THE ENDOMETRIOSIS IN ATHYMIC MICE

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1. ABSTRACT

Endometriosis is an adhesion disorder characterized by the presence of endometrial tissue in ectopic sites outside the uterus. The disease is associated with dysmenorrhea, pelvic pain and infertility. Although endometriosis is the most common gynecologic disorder, relatively little is known regarding its etiology, pathogenesis and the course of the disease. This situation is primarily due to the absence of experimental systems to examine the mechanism of endometrial cell adhesion, role of inflammatory cells and the interactions of epithelial, and stromal cells with the peritoneum and ovarian tissue leading to the development of this disorder. Dissociated human endometrial cells were suspended in peritoneal fluids of individuals with and without endometriosis and were injected into the peritoneal cavity of athymic mice. This led to development of ectopic adhesions of endometrial cells at the peritoneal and ovarian surfaces. Endometrial cells which were marked with fluorescent lipophylic dyes, prior to intraperitoneal injection, could be visualized without surgery at such sites. The studies demonstrate a model for endometriosis in athymic mice.

2. INTRODUCTION

Endometriosis is an enigmatic disease, This disorder usually occurs in reproductive women aged 15 to 55 years and is characterized by the growth of functional endometrial tissue (glands, stroma or both) at ectopic sites. Endometriosis is a common cause of gynecologic morbidity. Some showed that the incidence of endometriosis to be as high as 50 percent in patients who undergo laparotomy for conditions un related to the endometrium (1). Although there is extensive literature on this disease, little definitive information is available on its etiology, pathogenesis and the mechanisms mediating the pain, dysmenorrhea and infertility (2). Paucity of scientific knowledge of this disease are related to the lack of carefully controlled investigations and lack of appropriate models for study of endometriosis and events leading to it.

Endometriosis has been reported to occur only in humans and subhuman primates. Attempts to develop animal models for this disease have primarily focused on surgical transplantation of endometrial tissue to peritoneal cavity in various animals (3-6). Trauma, inflammatory responses and adhesions - processes which may have a significant role in the pathogenesis of endometriosis - are induced by surgery and hence confound the investigations using these models. We have succeeded in generating ectopic endometrial adhesions in athymic mice following intraperitoneal injection of dissociated human endometrial glands and stroma mixed with peritoneal fluids from patients with and without endometriosis. In addition, fluorescent-tagging of the endometrial tissues with the lipophylic dye, DiO, prior to their intraperitoneal injection, allowed gross visualization of tissues adherent to peritoneal and ovarian surfaces. The fluorescent, ectopic endometrial tissue, adherent to the peritoneal surfaces, could be seen in intact animals without surgery.

3. MATERIALS AND METHODS

Endometrial tissues were obtained from women in their reproductive years (aged 25-45), with non-endometrial problems were utilized in these studies. Peritoneal fluids were collected as part of the routine work-up of infertile patients and the presence or absence of endometriosis in these individuals were noted during laparoscopy. Peritoneal fluids were noted during laparoscopy. The peritoneal fluids were routinely centrifuged at 1500 RPM for 10 minutes to remove cell debris. The supernatants were stored at 4^{o}C until use.

3.1. Isolation of endometrial tissue into glands and stromal cells and their intraperitoneal injection into athymic mice

Endometrial tissues were digested with 0.25% collagenase (Type 1) mixed with Ham's F-10 medium containing serum in a shaker water bath (37 ^{o} C, 90-120

Table 1. Experimental endometriosis in athymic mice

Experimental Conditions	Number of animals	Occurrence of endometriosis	Sites of adhesions
Peritoneal fluid of endometriosis patients	10	6	Peritoneum, ovary, uterus
Peritoneal fluid of non-endometriosis patients	10	3	Injection site
Trauma	5	5	Trauma site
hPBL	2	2	Fat around spleen&pancreas

stroke/min.) for 1 hr according to the procedure previously described (7). Without isolating the individual cell laparoscopy. The peritoneal fluids were routinely centrifuged at 1500 RPM for 10 minutes to remove cell debris. The tissues were removed and examined within one day to 8 weeks after intraperitoneal injection of tissues.

3.1. Dissociation of endometrial tissue into glands and stromal cells

Endometrial tissues were digested with 0.25% collagenase (Type 1) mixed with Ham's F-10 medium containing serum in a shaker water bath (37 ^{o} C, 90-120 stroke/min.) for 1 hr according to the procedure previously developed by us (7). Without isolating the individual cell population (glands and stroma), the washed preparations were suspended in peritoneal fluids or Ham's F-10 medium and used in these studies. Dissociated glands and stroma were sedimented, washed and mixed with peritoneal fluid (0.3 ml) from either endometriosis or non-endometriosis individuals or $5X10^{6}$ isolated human PBLs (hPBL) in saline (0.3 ml) (table 1). PBLs were isolated from the buffy coat after centrifuging blood over Ficol-Hypaque. Tissues were injected i.p. to athymic female mice. Trauma was induced by pinching the skin around the peritoneum and forcefully rubbing it together with the end of the curved scissors six times. Animals were sacrificed after 1 day to several weeks and examined for endometriosis. Suspicious peritoneal sites, uterine horns, ovary and fat around pancreas and spleen were fixed in formalin and were embedded in paraffin. H&E stained tissue sections were examined for the presence of endometrial glands and stroma.

3.2. Labeling of endometriaI cell preparations with fluorescent, lipophyilic dye, DiO

Thc endometrial cells were labeled by incubation in a medium supplemented with DiO (3,3'-dioctadccyloxacarbocyanine (Molecular Probes, Eugene, OR). To label the cells, a stock solution of 0.1% (w/v) was prepared by dissolving DiO crystals in a 1:9 mixture of DMSO and absolute ethanol. This solution was sonicated to dissolve all DiO crystals and then was added to culture medium containing endometrial cell suspension to a final concentration of 20 μg/ml. After 8-18 h incubation at 37 ^{o} C incubator, the labeled cells were washed in several changes of fresh medium. Cells were viewed by fluorescence microscopy to ensure labeling.

DiO-labeled endometrial cell preparations were suspended in saline and 0.05 ml of pelleted cells in 0.3 ml volume per animal was injected intraperitoneally into groups of female, 6-8 week old, athymic mice. The mice were subcutaneously implanted with 17-β estradiol pellets (Innovative Research of America, Sarasota, FL) designed to maintain a serum concentration of 200-300 pg/ml for 60 days.

3.3. Fluorescence, surgery and processing of tissues

Visualization of fluorescently labeled tissues prior to or following laparotomy was done using a self-assembled system comprised of a blue light source of a dissecting microscope fitted with a 480 nm filter which proved optimal. The adherence of human tissues to peritoneal surface was identified by gross examination. Such tissues were excised and frozen in OCT embedding medium and stored in liquid nitrogen. However, the fluorescently tagged tissues were identified by exciting them to fluoresce. Four to 6 micron cryostat sections of these tissues were used for visualizing the fluorescently labeled endometrial cells and for histochemical examination following hematoxylin-eosin (H&E) staining. The human origin of the implanted tissues was established using the Hoechst stain #33258 according the method of Cunha *et al* (8).

4. RESULTS

Two assumptions formed the basis of our investigations: 1) The peritoneal milieu must be conductive to the adhesion and implantation of the endometrial cells which gain access to peritoneal sites by retrograde menstruation and 2) the peritoneal fluid of endometriosis patients may contain factor(s) that can alter the adhesive properties of endometrium. Based on these assumptions, we predicated that a suspension of dissociated endometrial cells (which resemble the retrograde menstrual endometrium) in the peritoneal fluids obtained from endometriosis patients, and placed in the peritoneal cavity of athymic female mice may enhance the adhesion of the endometrium. As predicted, adhesions, characteristic of endometriosis (pink and blue nodules) were grossly visible at 4 to 8 weeks on the peritoneal and ovarian surfaces. Gross appearance of a blue endometriosis implant is shown in figure 1. Histologic examination of the H&E stained sections of the endometriotic nodules on the peritoneal and ovarian surfaces revealed the presence of endometrial glands and stromal cells, characteristic of endometriosis (figure 2-3).

Secretory changes with stromal decidualization and hypersecretory endometrium with glands showing Aria-Stella reaction were seen in the peritoneal implants of some specimens (figure 3). Whereas in other experiments, proliferative type of endometriosis with several mitotic figures were observed. Endometriotic foci occurred in several different regions including skeletal muscle (figure 4) and fatty tissue around abdominal organs such as liver. The endometriotic foci contained endometrial glands, stroma or both. In addition, lymphocyte infiltration similar to that observed in normal human endometrium was also evident within the implanted endometrial tissues (figure 2).

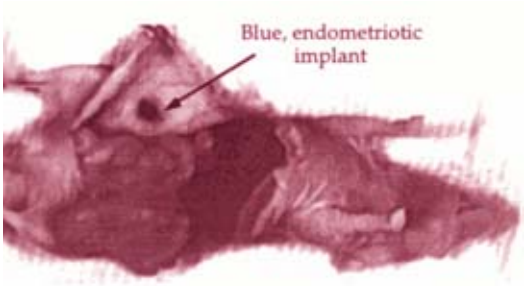

Figure 1. Endometriosis in nude mouse. A blue endometriotic nodule (arrow) is seen on the peritoneal surface in a nude mouse injected i.p. with dissociated endometrial cells and peritoneal fluid of an endometriosis patient.

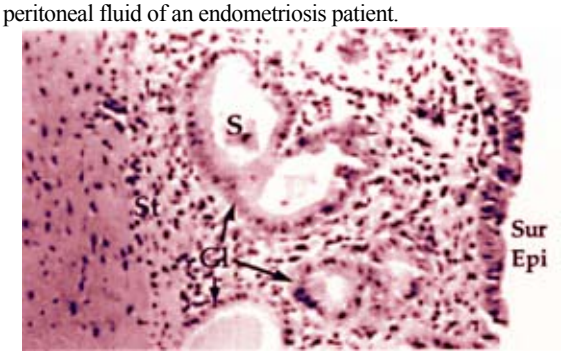

Figure 2. Hematoxylin-eosin stained section of endometrial implant in the ovary. S: Secretory products Gl: gland St: Stromal cells; Sur Epi: surface epithelium.

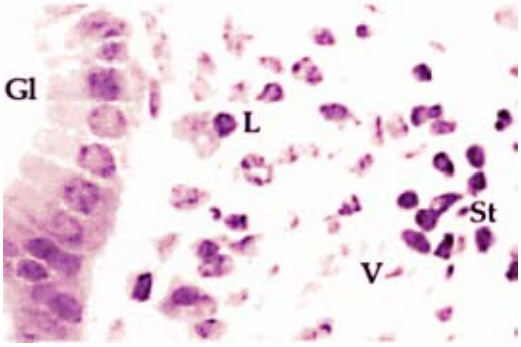

Figure 3. Hematoxylin-eosin stained section showing glandular (GI) and stromal (St) cells of the endometrial implant at the peritoneal site. Lymphoid (L) infiltration is evident. V:vessel.

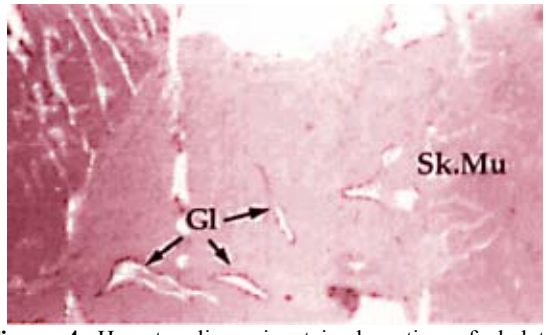

Figure 4. Hematoxylin-eosin stained section of skeletal muscle (Sk. Mu) showing endometriotic foci of endometrial glands (Gl).

In control animals injected with endometrial cells mixed with peritoneal fluid from non-endometriosis subjects endometrial implants were observed only in the skin at the injection site. Peritoneal implants were not observed in these control animals.

Several experimental manipulations, known to induce inflammatory changes within the peritoneum, such as mechanical trauma, or intraperitoneal injection of human peripheral blood lymphocytes were done. These were effective in eliciting adhesions of endometrial cells to the peritoneum (table 1). Trauma and intraperitoneal injection of human peripheral blood lymphocytes appeared to be most effective.

Co-injection of human peripheral blood leukocytes with endometrial cell preparation resulted in adhesions in the fat surrounding the spleen and pancreas. Peritoneal adhesion of endometrial cells consistently and predictably occurred following the induction of mechanical trauma at the trauma site.

In the above studies, only grossly visible nodules were examined. In order to make identification of the endometriotic foci easier, the endometrial tissues were labeled prior to injection. Dissociated endometrial cells were tagged with the lipophylic fluorescent dye, DiO. Small fluorescent nodules of human endometrial cells could be easily visualized following the intraperitoneal injection of DiO-tagged cell preparations (figure 5). Contiguous cryostat sections of the excised fluorescent tissues were examined by fluorescence microscopy and by hematoxylin and eosin staining (figure 6). Little, if any, fluorescence was seen in the mouse tissue, while the DiO tagged human endometrial cells exhibited a green fluorescence (figure 6A). Fluorescent endometrial tissues were detected in the H&E stained sections (figure 6B). Human and mouse tissues were differentiated by Hoechst staining. The punctate blue fluorescence, characteristic of mouse cells differentiated these cells from the uniformly stained human cells (figure 6C).

5. DISCUSSION

Only human and subhuman primates are known to develop endometriosis and currently there is no ideal animal model for studying endometriosis. Autologous transplantation of endometrial tissue into the peritoneal sites has been attempted in rabbits, rhesus monkeys as an *in vivo* model of endometriosis (3-6). Although these experimental systems are useful in examining the consequence of ectopic endometrium on fertility and related problems, their utility in the study of the pathogenesis of endometriosis is quite limited. Trauma, inflammatory responses and adhesions are processes common to endometriosis and the healing wound. Therefore, a non-surgical model system is essential for understanding the sequence of events which mediate the ectopic adhesions of endometrium. Athymic mice, which are deficient in the T-lymphocytes have proven ideal for maintaining a variety of human tissues *in vivo* (9). We and

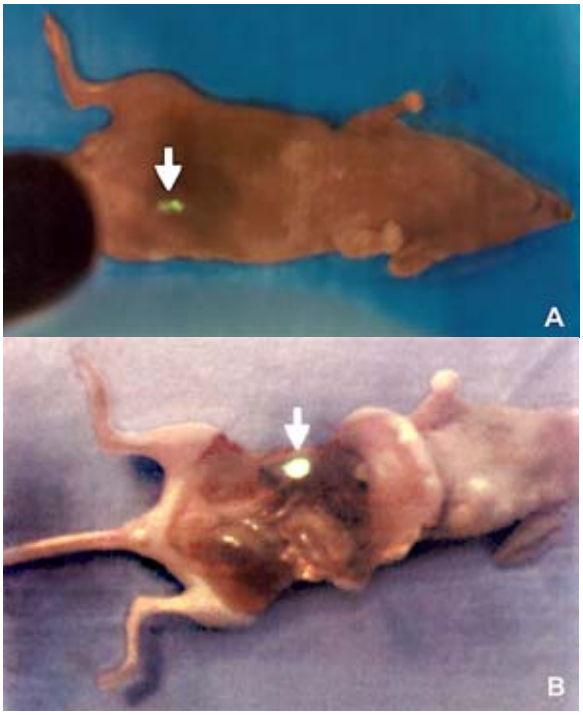

Figure 5. Visualization of DiO-tagged human endometrial tissue. The light source of a dissecting microscope was fitted with a 480 mm filter resulting in the emission of 500 mm blue light. Such light source was placed over the abdomen. Green fluorescence was captured using an orange filter attached to a Nikon camera. DiO-tagged tissues could be visualized by their green fluorescence from outside (A: white arrow) or after opening the abdomen (B: white arrow).

others have previously reported on the successful transplantation of normal and neoplastic human endometrium (10-12) Such tissues have proven successful in determining the effects of steroid hormones and other agents on the growth, maintenance and modulation of morphologic and biochemical functions of endometrial tissue, in vivo (10-12). Studies on the morphologic effects of various steroid hormones in eutopic and ectopic endometria transplanted subcutaneously or intraperitoneally to athymic mice have also been reported (13-15). In the present study, we successfully mimicked the formation of ectopic adhesions of endometrium on peritoneal and ovarian surfaces in athymic mice. The underlying premise of these studies is that upon transportation of the endometrial tissue to the peritoneum, the local environment may regulate the adhesion of endometrial tissue components to intraperitoneal sites. The basis for the notion that retrograde menstrual flow into peritoneum occurs in endometriosis, derives from the transplantation theory of Sampson (16). This idea is widely accepted and is well supported by the available evidence. According to this theory, pieces of endometrial tissue shed during menstruation are transported through the Fallopian tubes in a retrograde fashion to the peritoneal cavity where they can implant. Reverse flow of menstrual fluid and endometrial cells through the Fallopian tubes into the peritoneal cavity during menstruation appears to be a common phenomenon in normally menstruating women. Spontaneous endometriosis has been found to be more common in baboons with recurrent retrograde menstruation (17). In addition, a relationship seems to exist between the duration and amount of menstrual discharge and development of endometriosis in women (18). Presumably, not all women exhibit such a reverse flow in each menstrual cycle. Since only a fraction of women during the reproductive years suffer from endometriosis, it is reasonable to assume that peritoneal environment must be conducive to implantation of the endometrial cells and the development of endometriosis. Based on these assumptions, we surmised that intraperitoneal deposition of dissociated endometrial tissue, suspended in the peritoneal fluid of endometriosis patients, may closely resemble the peritoneal micro-milieu of endometriosis patients. It is reasonable to assume that peritoneal fluids of endometriosis patients may contain factor(s) that play a role in enhancing tissue adhesion.

Several investigators have suggested that inflammatory responses within the peritoneal cavity may lead to the adhesion and implantation of endometrial cells at peritoneal sites (19). Inflammatory cytokines mediate the inflammatory response and the presence of bioactive interleukins, IL-1 and IL-2, and TNF-α and interferon-γ in the peritoneal fluid has been reported (20-21). Therefore, lymphoid cells, their products may augment the development of endometriosis. Consistent with this hypothesis, the co-injection or endometrial tissue along with peritoneal fluid of endometriosis patients or PBLs led to the development of endometriosis in the peritoneal cavity of the athymic mice. Ectopic endometrial adhesions, identical to those seen in endometriosis patients were consistently observed in our experimental model. Presence of endomoetrioma in and around scar of Cesarian section and abdominal incision sites have been widely reported (22-24). Consistent with this finding, we could observe implanted endometrial tissue around the site of truama. Control studies, where dissociated endometrial cells were suspended in the peritoneal fluid of women without endometriosis failed to show similar ectopic adhesions. In some control animals, endometrial adhesions were barely detectable grossly at the injection site and could be visualized only upon histologic examination of tissues derived from the injection site.

During the development of this experimental animal system, it became evident that only large, grossly visible adhesions could be grossly identified. Smaller nodules of human endometrial cells on the peritoneal surfaces were not detectable. Tagging the endometrial cells with the fluorescent dye, DiO, allowed their detection and identification. DiO is a highly fluorescent, stable and membrane specific dye. This lipophylic carbocyanine dye is not metabolized or exchanged between cells and has been successfully used in tracking various cells. This tagging technique allows follow-up of the fluorescent tissues over time in the same animal, making it possible to track such tissues grossly and without a need for surgery.

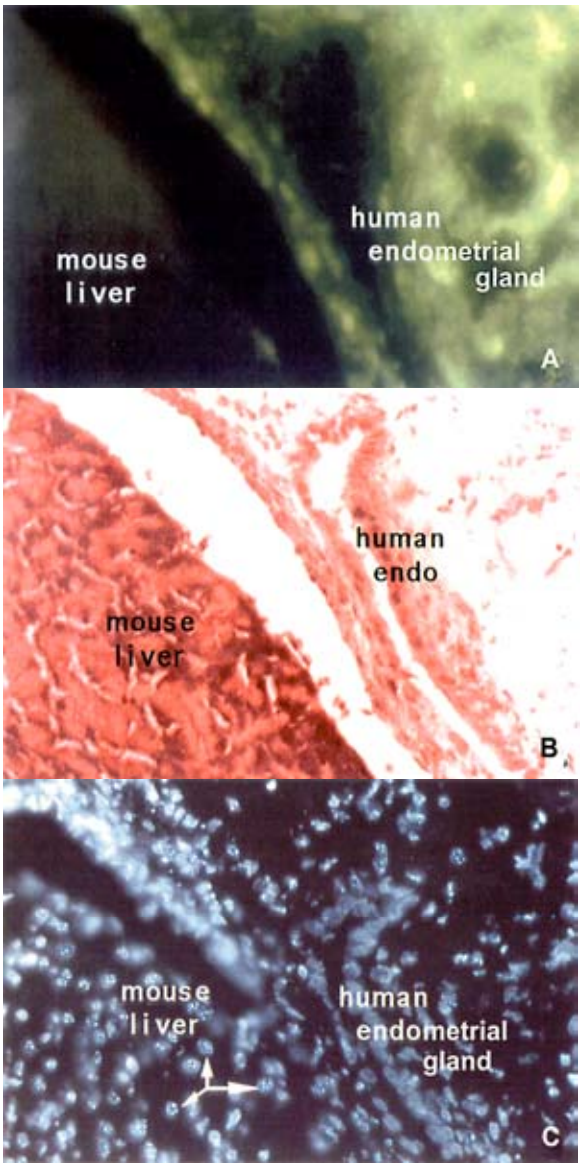

Figure 6. A: A fluorescently labeled tissue tagged with DiO injected to the athymic nude mice was removed and examined in a cryostate section under fluorescent microscope. A Leitz fluorescent microscope equipped with a fluorescein-rhodamine filter combination was used to excite the fluorescently labeled human endometrium. The mouse liver on the left-hand side of this tissue section is devoid of fluorescence and is barely visible. Human endometrium exhibits intense fluorescence B: Hematoxylin-and eosin staining of a section from the same tissue reveals the focus to consist of an endometrial gland and surrounding stroma (Human endo). C: Identification of human endometrial cells by Hoechst staining. A contiguous section to the one shown in figure 2B was stained with Hoechst #33258 dye according to the method of Cunha *et al* (6). While the epithelial cells of human endometrial gland show a uniform blue fluorescence, the mouse cells show a characteristic intense punctate blue fluorescence under ultraviolet light which is characteristic of mouse cells (arrows).

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