# Time course of pelvic endometriotic lesion revascularization in a nude mouse model

*Julie Eggermont, M.D., Jacques Donnez, M.D., Ph.D., Françoise Casanas-Roux, Ph.D., Hélène Scholtes, M.D., and Anne Van Langendonckt, Ph.D.*

Department of Gynaecology, Université Catholique de Louvain, Brussels, Belgium

**Objective:** To assess the timing of endometrial lesion revascularization in a murine model.
**Design:** Prospective experimental study.
**Setting:** An academic research environment.
**Animal(s):** Twenty-six nude mice.
**Intervention(s):** Endometriosis was induced in mice by intraperitoneal deposition of human menstrual endometrium. Endometrial implants were recovered on days 1, 3, 5, 8, 10, 15, and 21 after implantation.
**Main Outcome Measure(s):** Sections from the endometrial implants were immunostained with species-specific antiplatelet endothelial cell adhesion molecule-1 (PECAM-1) antibodies and vessels of murine and human origin were counted.
**Result(s):** Endothelial cells of human origin in the implant progressively disappeared between day 3 and day 10. Seventy-eight percent of the vessel sections were positive for human PECAM-1 on day 5, 40.1% on day 8, and only 14.1% on day 10. However, there was a marked increase in murine PECAM-1-expressing vessels in endometrial stroma between day 5 (1.4%) and day 8 (68.0%), 10 (69.5%), and 15 (87.2%).
**Conclusion(s):** Our study demonstrates that PECAM-1 is a reliable endothelial cell marker to evaluate the role of angiogenesis in the nude mouse model. It also indicates that revascularization of human endometrial implants occurs between 5 and 8 days after implantation and involves the disappearance of native graft vessels, coinciding with the invasion of the interface and then the stroma by murine vessels. (Fertil Steril® 2005;84:492–9. ©2005 by American Society for Reproductive Medicine.)

**Key Words:** Angiogenesis, nude mouse, PECAM-1, pelvic endometriosis, vascularization

Pelvic endometriosis is characterized by the implantation and benign growth of endometrial tissue within the abdominal cavity. Most studies on peritoneal endometriosis are based on the implantation theory of Sampson (1), postulating that, during the menses, retrograde reflux of viable endometrial cells through the fallopian tubes may lead to the development of endometriotic lesions.

As observed in tumor growth, angiogenesis of the endometriotic implant appears to be essential to its survival and development (2). Numerous peritoneal blood vessels can be observed around active endometriotic lesions at laparoscopy and the implant itself is strongly vascularized, as shown histologically (3–15). Moreover, increased angiogenic activity has been demonstrated in peritoneal fluid (PF) from women with endometriosis (4, 6–8) and strong expression of angiogenic factors has been shown in active lesions (2, 5, 9, 10). Red lesions, which are thought to be the first stage of endometriotic lesion development (3), have a much higher proportion of immature, pericyte-free vessels than black lesions (11) and may be a good target for antiangiogenic therapies.

Further evidence of the importance of neoangiogenesis in the pathogenesis of endometriosis is provided by studies on animal models. Recent studies using a murine model of endometriosis have shown that intraperitoneal injection of angiostatic compounds or transient overexpression of the gene encoding angiostatin inhibit the growth of endometrial implants by disrupting their vascular supply (12–14). Experiments involving implantation of human endometrium into the pelvic cavity of immunodeficient mice have shown that endometriotic lesions derive their blood supply from the surrounding vascular network (15–17).

In the present study, platelet endothelial cell adhesion molecule-1 (PECAM-1) was used as a marker of vascular endothelium in endometriotic lesions.

PECAM-1 (CD31) is a cell adhesion molecule in the immunoglobulin supergene family that is expressed to a high level in endothelial cells and at a lower density in hemapoietic cells (18, 19). PECAM-1 has been implicated in a number of important biological processes including vascular development, angiogenesis, leukocyte transmigration, T-cell activation, and platelet aggregation, as reviewed by Cao et al. (18) and Jackson (19). Its expression has been demonstrated in the endometrium throughout the menstrual cycle

Received July 14, 2004; revised and accepted March 21, 2005.
Supported by grant 3.4552.00 from the Fonds National de la Recherche Scientifique de Belgique.
Reprint requests: Jacques Donnez, M.D., Ph.D., Department of Gynaecology, Université Catholique de Louvain, Cliniques Universitaires St Luc, Avenue Hippocrate 10, 1200 Brussels, Belgium (FAX: 32-2-764.95.07; E-mail: Donnez@gyne.ucl.ac.be).



0015-0282/05/$30.00
doi:10.1016/j.fertnstert.2005.03.034

(20), as well as in red and black pelvic endometriotic lesions collected from patients (21).

The aim of the present study was to follow the time course of neovascularization of endometriotic lesions, experimentally induced in nude mice and collected at different time intervals after implantation of endometrial fragments (1, 3, 5, 8, 10, 15, and 21 days). For this purpose, PECAM-1 expression was investigated by immunohistochemistry. The use of species-specific anti-PECAM-1 antibodies allowed us to distinguish between human and mouse endothelial cells.

## MATERIALS AND METHODS

### Collection of Human Menstrual Effluent

The use of human tissue for this study was approved by the Institutional Review Board of the Université Catholique de Louvain. Menstrual endometrium was obtained from 14 reproductive-aged women (27–44 years) without endometriosis who were undergoing surgery for benign conditions. All of them had regular menstrual cycles and none of them had used any form of hormonal treatment for at least 3 months before collection. Surgery was performed during menstruation (day 2 or 3). The effluent was recovered by aspiration with a syringe without any anticoagulants, placed in ice-cold sterile phosphate-buffered saline (PBS) solution, pH 7.4, and transported to the laboratory where specimens were cut into pieces not exceeding 1 $mm^3$. One part of the biopsy was fixed in 4% buffered formaldehyde, dehydrated, and embedded in paraffin. Sections were stained with Gomori's trichrome for histological confirmation of the menstrual phase.

### Transplantation Into Nude Mice

The guidelines for animal welfare were approved by the Committee on Animal Research of the Université Catholique de Louvain.

Twenty-six 8-week-old female nude mice (Swiss nu/nu) with a deficient T-lymphocyte system were used in this study. This murine model has been described in detail elsewhere by our group (22). During the experimental period, no hormonal therapy was administered to the mice. Three mice were housed per cage under laminar-flow (HEPA)-filtered hoods in rooms maintained at 28°C with a 12:12-hour light–dark cycle. All housing materials and food and water were autoclaved before use.

The mice were fed ad libidum on laboratory chow and acidified water.

The mice were anesthetized with an intraperitoneal injection of 0.07 mL of Imalgene 500 (Merial, Brussels, Belgium) and 0.16 mL of Rompun 2% (Bayer, Brussels, Belgium), 1/100 vol/vol.

A small incision, less than 3 mm, was made on the ventral midline, just caudal to the umbilicus, and four to five 1-$mm^3$ fragments of human menstrual endometrium were deposited into the lower part of the pelvic cavity without any suture or damage to the peritoneal layer. Opening the peritoneal cavity took less than 1 minute to limit the time of exposure to air. The cutis was sutured with a 4/0 nylon thread.

Implants showing macroscopic characteristics of endometriosis were removed at different time intervals after implantation, on days 1, 3, 5, 8, 10, 15, and 21. On day 1 after implantation, 10 mice were operated on to remove one or two implants. On day 3, one or two implants were recovered from the same 10 mice. On day 5, the 10 mice were killed and all the implants were removed. Another four mice were killed on day 8, 4 on day 10, 4 on day 15, and 4 on day 21. Two operators (J.E. and F.R.) examined their abdominal cavities under a dissecting microscope for the presence of implants.

In total, 78 implants were biopsied, fixed in 4% buffered formaldehyde, and embedded in paraffin. Four-micrometer sections were stained with Gomori's trichrome. Lesions showing the typical aspect of endometriosis, with glandular structures and stroma, were immunostained with anti-PECAM-1 antibodies.

### Immunohistochemistry

PECAM-1 labeling was performed with the immunoperoxidase technique and water bath unmasking at 96°C for 75 minutes.

Human endothelial cells were revealed using a primary mouse monoclonal antibody against human PECAM-1 at a dilution of 1/100 (DAKO, Glostrup, Denmark) and goat antimouse immunoglobulin conjugated to peroxidase as a secondary antibody.

To reveal mouse endothelial cells, a rat monoclonal antibody against mouse PECAM-1 was used at a dilution of 1/20 (BD Biosciences Pharmingen, San Diego, CA), followed by biotinated goat antirat IgG secondary antibodies. Primary antibodies were specific for the PECAM-1 protein, as checked by Western blot. One single band of 130 kDa was detected, corresponding to PECAM-1 (not shown).

3,3′-Diaminobenzidine (DAB) was used as a chromogen and nuclei were counterstained with hematoxylin.

### Counting of PECAM-1-Positive Vessel Sections in Lesions

Two serial sections per lesion were examined blindly by two operators (A.V.L. and H.S.). Double staining was not performed. One serial section was stained with antihuman antibody and the next with antimouse antibody. Video image analysis, routinely performed in our department, proved that vessel number did not differ between two consecutive 4-$\mu$m serial sections.

In sections immunostained with antihuman PECAM-1 antibodies, all the vessel and capillary sections, identified histologically, were counted and the number of PECAM-