Original Article

Angiogenesis Following Three-Dimensional Culture of Isolated Human Endometrial Stromal Cells

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Abstract

Background: Endometriosis is the presence of endometrial tissue outside of the uterine cavity and is the most common gynecologic disorder in women of reproductive age. We have preliminary evidence that in the presence of a 3-dimensional (3-D) fibrin matrix, human endometrial glands, stroma, and neovascularization can develop in vitro, mimicking the earliest stages of endometriosis. The aim of the present study was to determine if angiogenesis can be developed in a 3-D culture of human stromal cells in vitro.

Materials and Methods: This was an in vitro study of human endometrial biopsies in 3-D culture of fibrin matrix and conducted at a university affiliated infertility center. Biopsies were taken from ten normal ovulating women undergoing infertility treatment. The samples obtained from fundus of the uterine cavity were minced, stromal cells isolated and placed in a 3-D fibrin matrix culture system. Degree of proliferation of stromal cells, invasion of the fibrin matrix, gland formation, vessel sprouting and immunohistochemical characterization of cellular components were recorded.

Results: Three-dimensional culture of human stromal cells formed sheets of cells in the fibrin matrix. By 3-4 weeks, endothelial cell branching was observed and rudimentary capillary-like structures formed and endothelial cells confirmed by CD31 immunostaining.

Conclusion: These data show that stromal cells from endometrial explants can proliferate and invade a fibrin matrix in vitro generating new vessels. This procedure represents a controlled, quantifiable model for the study of angiogenesis during the menstrual cycle, and in conditions such as endometriosis and cancer.

Keywords: Endometriosis, Three-Dimensional Culture, Angiogenesis, Stromal Cells, CD31

Introduction

Endometriosis is the presence of endometrial glandular and stromal cells outside of the uterine cavity. This disease is found in about 10% of women of the reproductive age and is seen more frequently in women with infertility. Endometriosis can cause chronic pelvic pain, severe dysmenorrhea, and dyspareunia. Many women with endometriosis have no symptoms. In fact, they may first find out that they have endometriosis if they are not able to get pregnant. The most widely accepted hypothesis for the development of endometriosis is retrograde menstruation through the fallopian tubes into the peritoneal cavity. No medical therapy has been demonstrated to be effective in eradicating the disease or in preventing it without unacceptable side effect (1,2) and it depends on the extent of the disdisease, symptoms, and whether the patient wants to have children. Surgery continues to be the first-line of treatment to eradicate endometriotic lesions but symptoms return within 1 year in 47% of women who have had surgery (2).

The endometrium, which is situated in the lining of the uterus, is unique among adult tissues because it undergoes intense proliferation, secretion, regression, and regeneration during each menstrual cycle. The endometrium is composed of mesodermal-derived glandular and luminal epithelia that are supported by a basement membrane and connective tissue stroma. Basement membrane likely plays a key role in promoting an epithelial phenotype. In addition, stromal cells provide a regulatory role for growth and differentiation of the

overlying epithelium (3). The human endometrium develops new capillaries from existing microvessels, i.e. angiogenesis, which then undergo maturation and remodeling (4). Angiogenesis, the development of new capillaries from pre-existing blood vessels is an essential component of endometrial renewal. Angiogenesis occurs regularly in the endometrium throughout the reproductive life of females as part of the rapid growth and regression of this tissue that occurs during the menstrual cycle. Excessive endometrial angiogenesis is proposed as an important mechanism in the pathogenesis of endometriosis (5).

In our previous studies we showed that in vitro culture of human endometrial tissue in a 3-D fibrin matrix can proliferate and sprout new vessels (6,7). Moreover we have shown that glycodelin positive glandular epithelium and COX-2 positive new vessels were seen in cultures with angiogenesis-like structures (8). The aim of the present study was to determine whether stromal cells isolated from a fragment of human endometrium and cultured in a specific three-dimensional (3-D) in vitro fibrin matrix model, are capable of proliferation and the generation of new capillaries.

Materials and Methods
Patients
Endometrial biopsies were done in premenopausal patient's women referred to Toronto Centre for Advanced Reproductive Technology (TCART) for infertility treatment. The Research Ethics Committee of Mount Sinai Hospital in Toronto approved the performance of endometrial biopsy and the use of fragments of human endometrium as described. A written informed consent describing the procedures and aims of the study was obtained from each donor in compliance with regulations concerning the use of human tissues. Endometrial samples were collected from a total of ten normal ovulating women on cycle days19-24. The biopsies were obtained from the fundal region of the uterine cavity using an endometrial sampling device (Endocell; Wallach Surgical Devices Inc., Orange, CT, USA). In all patients, accurate menstrual dating was carried out according to the last menstrual period in the early proliferative phase of the cycle and appropriate histological dating of each biopsy confirmed the endometrium as proliferative.

Materials
Cell culture media, supplements and all other chemicals not listed in this section were obtained from Sigma Chemical Co. (Oakville, ON, Canada). Disposables for cell culture were supplied by Falcon (Becton Dickinson Labware, Franklin Lakes, NJ, USA). Target retrieval solution and monoclonal antibodies against CD31 were purchased from DAKO Diagnostic (Mississauga, ON, Canada). 4´, 6-diamidino- 2-phenylindole (DAPI) was obtained from Sigma Chemical Co., St. Louis, MO, USA.

Human endometrial stromal cells isolation and culture in 3-D fibrin matrix
The biopsy tissue was washed in Dulbecco's phosphate buffered saline (DPBS) contaning100X antibiotic solution (2%) (Penicillin, streptomycin, amphotericin), minced and treated with collagenase (2mg/ml in Hank's balanced salt solution, supplemented with 100X antibiotic solution) at 37°C for 60-90 min. Following tissue digestion, epithelial and stromal cells were separated using filtration through a100 um and 45 um strainer. The cells were then centrifuged at 1000g for 15 min and underwent ficoll purification. The cells were then washed with PBS several times. Isolated stromal cells were examined to be free from endothelial cells using CD31 staining. The cells were then mixed with 1 ml/well fibrinogen solution (3mg/ml in M199 culture medium), plated in culture dishes (24 wells) and 15 ul thrombin (50NIH unit/ml in 0.15M NaCL) was added. After gel formation, each well was covered with 1ml M199 supplemented with 5% fetal bovine serum (FBS), 0.1% e-amino-glutamine and 100 X antibiotic solutions. For each biopsy sample 6 wells were cultured. The cells were cultured at 37 °C in 5.5% CO_2 and 95% humidity up to 4-6 weeks. The medium was changed every 3 days and cultures were observed twice weekly with an inverted microscope for any visual evidence of angiogenesis.

Histology and immunohistochemistry
To document the cellular characteristics of the structures grown in the wells, the cultures were fixed in block in the fibrin clots in 10% formalin. Five -micron paraffin sections were stained with Hematoxylin-Eosin for histological examination and paraffin-embedded sections were stained with DAPI for nuclear identification. Briefly, slides immersed in target retrieval solution were autoclaved at 15psi/121°C for 20min, cooled down to 60°C, removed form the autoclave and rinsed with PBS at room temperature. After blocking nonspecific binding with 10% normal horse serum, mouse & human CD31 (endothelial cell marker) primary antibody (dilution 1:50) was applied. Also, some sections were treated with mouse IgG1 as a negative control for the first antibody (dilution 1:50).

Slides with sections of human placenta prepared in the same manner were used as positive control. Secondary antibody, donkey & mouse IgG, FITC 20 conjugated label was applied (dilution 1:200); on some sections the secondary antibody was omitted for negative control. Slides were examined on Zeiss Axioplan Photomicroscope equipped with epifluorescent ultraviolet light and corresponding excitation and barrier filters.

Results

The main cellular events observed during the first week of culture of stromal cells were formation of sheets of cells in the fibrin matrix (Fig 1).
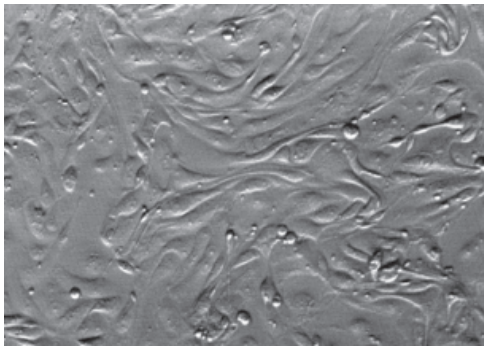

Fig 1: Stromal cells formed sheets of cells in the fibrin matrix

From the beginning of the third week, angiogenesis was observed as tube-like structures sprouting into the 3-D fibrin matrix (Fig 2).
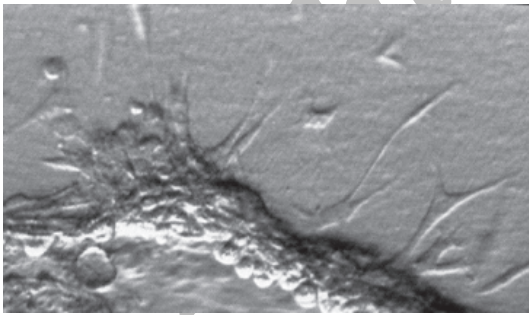

Fig 2: Tube-like structures sprouting into the fibrin matrix

On the 4th week of culture, a vessel outgrowth was observed arising from the endometrial stromal cells outgrowth in 43 of 60 wells (70.1%). Immunohistochemical staining of these structures was positive for markers of endothelial cells such as CD31 and Hematoxylin-Eosin staining showed endothelial cells in the satellite tissue. Unlike endometrial implants, isolated stromal cells in the 3-D culture did not form glandular structures.

Discussion

In the present study, we have utilized 3-D in vitro fibrin matrix for culturing isolated endometrial stromal cells. Using this model we have demonstrated three-dimensionally cell proliferation and invasion resulting in generation of new vessels. Vessel formation is plausible since stromal cells and endothelial cells are both of mesenchymal origin. The relationship between epithelial cells and stromal cells and their precursor mesenchymal cells is tissue specific and established during a critical period of development (9).

The pathogenesis of endometriosis remains poorly understood, mostly because the initial stages of the disease are neither detectable nor observable in humans. By the time endometriosis is brought to medical attention, the disease is already well established. The most widely accepted hypothesis for the development of endometriosis is retrograde menstruation through the fallopian tubes into the peritoneal cavity. Retrograde menstruation is a very common physiologic event in cycling women with patent tubes (10) which suggests that retrograde menstruation facilitates transport of endometrial tissue to the peritoneal cavity. However some other factor(s) render certain women susceptible to the implantation and growth of ectopic endometrium. The 3-D fibrin matrix reflects the environment established in the peritoneal cavity by retrograde menstruation where it is conceivable that fibrin adhesions and endometrial fragments coexist.

The fact that ectopic endometrial tissue requires development and activation of a vascular network (angiogenesis) for its early proliferation in the pelvis of women is currently well known (11). At the present time this vascular system is thought to originate from the peritoneum rather than from the endometrial tissue itself (6). Our results demonstrate that sprouting vessels consistent with new vessel formation can originate from endometrial tissue and indicate for the first time that the angiogenic capacity necessary for the initial growth of endometriotic implants are intrinsic to the endometrial fragment. This evidence leads to the hypothesis that the progression of pelvic endometriosis can be driven by the endometrium with an initial generation of vessels from the endometrial explants, and subsequent recruitment of a blood supply derived from peritoneal endothelial cells. Some investigators propose that the mesothelium acts as a barrier to attachment of ectopic endometrium, suggesting that exposure of the extracellular matrix through peritoneal damage is requisite to its implantation (12). On the contrary, Witz et al recently showed

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that both stromal and epithelial endometrial cells could adhere to intact mesothelium (13).

Conclusion

Based on our findings it seems that sloughed stromal cells as basic factor in angiogenesis reaching pelvic fibrin adhesions in a retrograde manner would be able to proliferate as the first step of endometriosis. Furthermore, this study in vitro model (3-D) allows the quantification of cell proliferation and angiogenesis from stromal cells. These two phenomena are considered crucial for the development of the angiogenesis in endometriotic lesions. Using stromal cells in in vitro studies, we can quantify the number of cells added to the dish and that represents a controlled, quantifiable model for the study of angiogenesis during the menstrual cycle, and in conditions such as endometriosis and cancer

Acknowledgments

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