Review Article

Immunopathogenesis of Pelvic Endo metriosis: Role of Hepatocyte Growth Factor, Macrophages and Ovarian Steroids

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Running Head: Role of immune system and ovarian steroids in endometriosis.

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1. Abstract

Endometriosis, a chronic disease characterized by endometrial tissue located outside of the uterine cavity and is associated with chronic pelvic pain and infertility. However, an in-depth understanding of the pathophysiology of endometriosis is still elusive. It is generally believed that besides ovarian steroid hormones, the growth of endometriosis can be regulated by innate immune system in pelvic microenvironment by their interaction with endometrial cells and immune cells. We conducted a series of studies in perspectives of pelvic inflammation that is triggered primarily by bacterial endotoxin (lipopolysacccharide, LPS) and is mediated by toll-like receptor 4 (TLR4) and showed their involvement in the development of pelvic endometriosis. As a cellular component of innate immune system, macrophages were found to play a central role in inducing pelvic inflammatory reaction. We further reported here that peritoneal macrophages retain receptors encoding for estrogen and progesterone and ovarian steroids also participate in producing an inflammatory response in pelvic cavity and involved in the growth of endometriosis either alone or in combination with hepatocyte growth factor (HGF). As a pleiotropic growth factor, HGF retains multifunctional role in

endometriosis. We describe here the individual and step-wise role of HGF, macrophages and ovarian steroid hormones and their orchestrated involvement in the immunopathogenesis of pelvic endometriosis.

Key Words: pelvic endometriosis, hepatocyte growth factor, macrophages, toll-like receptor 4, ovarian steroids, inflammation

2. Introduction

Endometriosis, the presence of functional endometrium outside of the uterine cavity, is a common disease, causing abdominal pain, dysmenorrhea, dyspareunia and infertility in about 10% of the female population.[1] Besides metaplastic transformation of endometrial and peritoneal mesothelial cells, the transplantation, implantation and growth of exfoliated menstrual debris on the peritoneal and ovarian surfaces are the widely accepted mechanisms of endometriosis.[2-5] A number of literatures have already demonstrated the potential role of ovarian steroid hormones in the regeneration of endometrium after menstruation and the growth of endometriosis.[6,7] However, as a

non-self lesion in pelvic environment, the growth or persistence of endometriosis can also be regulated by innate immune system. The mitogenesis or angiogenesis of eutopic and ectopic endometrium possibly involves an extensive interplay between endometrial cells, inflammatory cells, ovarian hormones, soluble factors and the extracellular matrix.[8]

As a cell component of innate immune system, peritoneal fluid (PF) and intact tissue derived from women with endometriosis have been shown to contain higher numbers of activated macrophages.[9-11] than that found in women without endometriosis. This results in the secretion of higher concentrations of growth factors including hepatocyte growth factor (HGF) and other cytokines in PF as produced by the stimulated-Mϕ in these patients.[12-14] This indicates that the growth or persistence of endometriosis is a normal inflammatory response and this was established by the accumulation of inflammatory cells in grafted endometriotic lesions in mouse endometriosis model.[15]

Since mesenchymal cells retain estrogen receptor, production of different cytokines by endometrial stromal cells and its modulation by estrogen has been demonstrated.[16] Considering that infiltrated Mϕ is one of the cell components of

endometriotic lesion in pelvic environment, reports describing expression of steroid receptors by Mϕ and the secretion of different macromolecules in response to steroid hormones are scanty. Therefore, in this review article, we discussed the orchestrated role of HGF, innate immune system and ovarian steroid hormones in inducing pelvic inflammation and consequent development of pelvic endometriosis.

3. Pathogenesis and natural course of pelvic endometriosis (Figure 1)

3.1. Common concepts in the pathogenesis of endometriosis

In addition to transplantation and implantation theory of Sampson[4] and coelomic metaplasia theory of Meyer[17], immuno-surveillance of the refluxed endometrial cells is another attractive theory for the development of pelvic endometriosis, The immune tolerance or immune defect theory could be responsible for a deficiency in the rejection of the autologous cells derived from the eutopic endometrium in the peritoneal cavity after menstrual reflux. This rejection in the clearance of endometrial cells could be contributed by a dysfunctional immune response in the pelvic cavity.[18-20]

According to the retrograde menstruation theory, endometrial fragments flow back through the fallopian tubes, reach the peritoneal cavity, attach on the pelvic

mesothelium, invade the peritoneum and develop into endometriotic lesions.[4] Limited information still exists regarding early endometrial-peritoneal attachment and invasion in the development of endometriosis and is derived mainly from in vitro studies.[21-23] After overcoming a phase of immune tolerance, a key step in the development of early endometriosis is the ability of endometrial cells to adhere to mesothelium and invade the extracellular matrix. These effects are contributed by a number of intercellular adhesion molecules (ICAM) and sub-cellular matrix degrading metalloproteinases (MMPs).[24-29] The expressions of these ICAMs such as integrins and E-cadherin and MMPs are already detected in cells derived from menstrual effluent, endometrium, peritoneal fluid, peritoneum, and endometriosis.[30,31]

3.2. Role of integrins and E-cadherins in endometriosis

Integrins and E-cadherin are proteins known to mediate adhesion of cells to neighboring cells or to extracellular matrix[32] play an important role in this process. The expressions of integrins and E-cadherins in both mesothelial and in endometrial cells have been demonstrated in women with endometriosis.[23] The $alpha_V beta3$ integrins transmit signals to the cytoskeletal structures of cells and usually mediate the expression

of fibronectin and vibronectin[22], and have been localized in endometriotic lesions and endometrium in women with and without endometriosis during both follicular or secretory phases.[26] However, agents blocking alpha$_V$beta3 integrin activity only minimally reduce the adhesion of menstrual endometrium to extracellular matrix in vitro.[33] On the other hand CD44, a key receptor for hyaluronic acid, has been demonstrated in endometrial cells and pretreatment of mesothelial cells by hyaluronidase diminishes the binding of endometrial cells to mesothelium.[34] These data suggest that the hyaluronic acid–CD44 binding may have a role in the initial attachment of endometrium to peritoneal mesothelial cells. The cellular mechanisms of ectopic endometrial growth involve invasive events similar to metastatic neoplasms that require extracellular matrix degradation.[27,35] Invasion of endometrial cells into the mesothelium occurs after initial attachment to the peritoneal wall and is favored by the endometrial expression of matrix metalloproteinases (MMPs)[28] that remodel the mesothelial lining of the peritoneum. There is substantial evidence that ectopic endometrium has the capability to invade the surrounding tissue. Viable endometrial cells from human endometriotic biopsies but not from human endometrial biopsies are invasive in an *in vitro* collagen invasion assay.[36]

3.3. Role of heme metabolism in endometriosis

A potential implication of hemoglobin in the pathogenesis of peritoneal endometriosis has been recently reported.[37] A simple hypothesis is that hemoglobin being released into the peritoneal cavity after red blood cell lysis may activate cell adhesion molecules, induce cytokine production, cell proliferation and the process of neovascularization. Degradation of hemoglobin yields biologically active molecules, heme and its products of oxidative cleavage by heme oxygeneses (HO) such as iron, carbon monoxide, biliverdin and bilirubin. Accumulation of heme in the peritoneal cavity might have a number of deleterious effects including induction of oxidative stress, stimulation of cell adhesion, and cytokine production by macrophages. All these biological events are finally involved in the pathophysiology of endometriosis. In fact, higher levels of hemoglobin were found in the peritoneal fluid of women with endometriosis. There was no concomitant increase in bilirubin concentrations in the peritoneal fluid and HO-1 was poorly expressed in peritoneal mesothelium and macrophages.[37] In contrast, HO-1 and HO-2 were strongly expressed in ectopic endometrium, especially in red lesions. These results suggest that heme may be involved

in the pathogenesis of endometriosis. The HO system, although expressed, might be insufficient to detoxify heme in women with endometriosis.

The lesions of early endometriosis are either transparent or translucent because they still lack formation of vasculatures around them. We named these early lesions as non-opaque lesions[38] because these lesions contain either of watery, serous or mucinous secretion and there is no collection of blood in the stroma by histology (Figure **2 A, B**). Once cellular attachment and invasion of endometrial cells are established, the subsequent growth or maintenance of endometriotic lesions is maintained by promotion of mitogenesis and angiogenesis with the continuation of menstrual cycle. The growth promoting effect of endometriosis is contributed by an orchestrated action of estrogen and other inflammatory or proinflammatory mediators. Over proliferation of micro-vessels in the growing endometriotic lesion causes oozing of blood in the stroma of these lesions and appear as blood-filled opaque red lesions by laparoscope (Figure **2 C, D**).[38] With the progression of time, there is deoxygenetion process from hemoglobin to metheomoglobin or hemosiderin leading to color changes of these opaque red lesions to black lesion or related lesions. In this stage, collection of blood in the stroma disappears (Figure 2 **E, F**).

Black lesion again changes to white lesion due to collection of bilirubin or biliverdin and collection of fibrous tissue. In this stage, gland gradually becomes smaller and stroma sometimes disappears due to deposition of fibrous tissue. Finally old lesions disappear and there is new focus of endometriosis due to continuation of menstrual reflux.[38] These sequential events indicate that once exfoliated, the endometrium enters into the pelvic cavity, becomes attached to the mesothelial layer and then a process of angiogenesis, heme metabolism and fibrosis ensue to maintain the natural course of endometriosis. We still don't know why these sequential events occur to change the color appearance of peritoneal lesions even there is constant and variable degree of inflammatory reaction in pelvic cavity. We speculate that the process of initiation, progression and maintenance might be different among them. The sequential events in the natural course of pelvic endometriosis are illustrated in Figure **1**.

4. Role of hepatocyte growth factor (HGF) in endometriosis

4.1. Biology of HGF and its receptor, c-Met

Hepatocyte growth factor (HGF) was first discovered as a mitogen for adult hepatocytes[39,40] and is identical to scatter factor.[41] HGF is a heparin-binding

glycoprotein that consists of a 60-kDa α-chain and a 30-kDa β-chain linked by disulfide bonds.[42] Several lines of evidence have implied that HGF behaves like a pleiotropic (multi-functional) growth factor and has mitogenic (cell proliferation), motogenic (cell migration), and morphogenic (change in cell type) functions in vitro on various epithelial cells derived from rodents and humans.[43,44] The HGF receptor is the c-met proto-oncogene product (c-Met) and is a transmembrane tyrosine kinase, a 190-kDa glycoprotein consisting of a 145-kDa membrane-spanning β-chain and a 50-kDa α-chain.[45]

The presence of c-Met on human endometrial epithelial cells and endothelial cells has been reported.[46] The synthesis of HGF by mesenchymal cells, coupled with demonstrated effects on epithelial and endothelial cells, suggests a paracrine mode of action.[47] HGF is considered to be a principal mediator of the mesenchymal-epithelial/endothelial interactions that contribute to embryogenesis, organ regeneration, wound healing, and angiogenesis.[48,49] Furthermore, HGF has been shown to stimulate the proliferation, migration and morphogenesis of endometrial epithelial cells.[2] Although production of HGF by alveolar macrophages and hepatic kuffper cells

has been reported.[50-52] information regarding production of HGF by peritoneal macrophages is limited. Since macrophages are the potent components of innate immune system, it is reasonable to speculate that these inflammatory cells might produce HGF in pelvic environment. Considering all these observations, we postulated that HGF and c-Met expression could be relevant to the pathophysiology of endometriosis.

4.2. HGF concentrations in different body fluids

Elevations in the peritoneal fluid (PF) concentrations of HGF, vascular endothelial growth factor (VEGF) and interleukin-6 (IL-6) in advanced endometriosis have been reported.[53-55] Previous report demonstrated that PF concentrations of HGF in women with advanced endometriosis (stage II-IV) was significantly higher than those from women without endometriosis and found no difference from women with early endometriosis (stage I-II). [53] The report from our laboratory demonstrated that changes in the PF levels of HGF have an association with estradiol and both of them are elevated in the early stage of endometriosis, especially in those women with endometriosis harboring dominant distribution of highly active and blood-filled (opaque lesions) red peritoneal lesions.[14,38]

The revised American Society of Reproductive Medicine (revised-ASRM) classification describes the morphologic appearance of endometriosis for color recognition of peritoneal lesions and a scoring protocol for evaluating the advancement of endometriosis.[56] This scoring system of revised-ASRM classification mostly includes the coexistence of fibrosis in the peritoneum or the presence of ovarian endometrioma of various sizes. However, this classification still leaves the question of debate about the importance of fibrous extension of disease or tissue activity of endometriosis and regarding their association with infertility. We demonstrated for the first time that the different pelvic endometriotic lesions that are included within the same red morphologic group of the revised-ASRM classification have different biological and tissue activities. Among these lesions, opaque red lesions displayed the highest activity in women with endometriosis. This was established by the measurement of different cytokines and chemokines in the PF and immunoreaction of HGF and proliferating cell nuclear antigen (PCNA) in intact tissue.[38] A recent study demonstrated that women with early or advanced endometriosis as measured by r-ASRM scoring system are not associated with an increase in either serum or PF concentrations of HGF. Rather HGF levels in serum and

PF were significantly increased in women harboring blood-filled red peritoneal lesions and may be clinically useful to predict the activity of pelvic endometriosis.[57]

Since retrograde reflux of menstrual blood is one of the causes of endometriosis and a higher level of HGF was detected during menstrual phase, we recently measured HGF concentrations in PF and in corresponding menstrual fluid and serum of women with and without endometriosis. A higher level of HGF in seminal fluid has been reported which is possibly involved in the migration of sperm after sexual intercourse.[58,59] We found a significantly higher concentration of HGF in the menstrual fluid and PF of women with endometriosis when compared with that in women without endometriosis (Figure **3**). Menstrual fluid level of HGF in women with endometriosis was similar to that of seminal fluid. Among the three body fluids, HGF level was the highest in the menstrual fluid, intermediate in peritoneal fluid and lowest in the serum of women with endometriosis (Figure **3**). This indicates that reflux of a portion of menstrual blood in every cycle may be responsible for a substantial accumulation of HGF in PF in addition to local production of HGF by endometriotic cells and immune cells.

4.3. HGF as mitogenic and angiogenic factor in endometriosis

In addition to an increase in the immunoexpression of HGF, c-Met and VEGF in eutopic and ectopic endometrium, a parallel increase in proliferating activity and micro-vessel density was also found in both the eutopic endometrium and red pigmented lesions.[60] A significant correlation between the quantitative histogram (Q-H) scores of HGF/c-Met and PCNA was observed in both the glandular epithelium and the stroma. Like the increased mitogenic activity, HGF also revealed marked angiogenic activity as demonstrated by the significant correlation of HGF expression with total micro-vessel counts.[60,61] These mitogenic and angiogenic activities of HGF were observed for both the eutopic and ectopic endometrium of women with endometriosis and were found to be stronger when compared with tissues derived from women without endometriosis.

4.4. HGF as a scattering factor in endometriosis

Hepatocyte growth factor has been reported to scatter malignant cells and is possibly involved in the hematogenous or lymphogenous dissemination of cancer cells.[62-64] We also reported that a variable dose of HGF (50-100ng/mL) was also able to scatter epithelial cells and stromal cells derived from the eutopic endometria of women with and without endometriosis. Epithelial cells were easier to scatter and stromal cells

were found to scatter only in a collagen non-coating culture plate (Figure **4 A, B**). This scattering effect in endometrium indicates that higher levels of HGF in the menstrual blood could be responsible for the scattering of cells after endometrial break down and may cause retrograde regurgitation of endometrial cells during menstruation.

4.5. HGF as motogenic (migration) factor in endometriosis

The motogenic effect of HGF on cultured endometrial epithelial cells was determined with Boyden's chamber assay. As shown in Figure **4 (C, D**), addition of 50 ng/mL and 100 ng/mL of HGF were able to migrate the epithelial cells and migrated cells were significantly higher when compared with HGF non-treated cells. No difference was observed in the migration of cells between 50ng/mL and 100ng.mL of HGF. This indicates that after initial endometrial-peritoneal mesothelial attachment, locally produced HGF may be involved in the invasion of endometriotic cells deep into the peritoneum.

4.6. HGF as morphogenic factor in endometriosis

Though coelomic metaplasia theory is attractive, a conclusive proof that peritoneal epithelium can undergo spontaneous or induced metaplasia to form

endometriosis-like lesion is lacking. It has been reported that endometrial epithelial cells can transform into a gland-like structure under the influence of HGF.[2] Another report demonstrated that HGF could alter peritoneal mesothelial cells to hemispherical cells.[62] Both of these studies emphasized the ability of HGF to induce change in cell morphology interchangeably between endometrial epithelial cells and peritoneal mesothelial cells. These results suggest that HGF functions as a mediator of morphological transformation between mesothelial cells and endometrial epithelial cells, both of which are derived from the same embryological origin.

We demonstrated that HGF might be involved in cellular changes to the peritoneal mesothelium adjacent to pelvic endometriosis.[3] We reported an array of changes occurring with pelvic peritoneum from normal flat, through cuboidal and finally to columnar cells in the peritoneum adjacent to red lesions of pelvic endometriosis.[3] HGF was clearly immunolocalized in cuboidal or flat mesothelial cells adjacent to red lesions in significantly stronger intensity when compared with corresponding cells adjacent to black peritoneal lesions. The intensity of HGF immunostaining in the cudoidal or columnar cells surrounding the red lesions was correlated well with clustered

accumulation of macrophages recognized among these cells.[3] The result of this study is the direct evidence to indicate that the central feature of peritoneal endometriosis is a combined effect of different phases of inflammatory reactions which are elicited by transplantation of endometrial tissue fragments and subsequent transformation of mesothelial cells into endometrioid structure via the action of HGF. The multifunctional role of HGF in the development of endometriosis is shown in Figure **5**.

5. Role of Mϕ in pelvic inflammation and growth of endometriosis

5.1. Infiltration of Mϕ in eutopic and ectopic endometrium

The retrograde reflux of menstrual debris into the pelvic cavity can induce an inflammatory response and release different chemo-attractant proteins, which in turn recruit peripheral blood mononuclear cells (PBMCs) into the peritoneal environment. These PBMCs time-dependently mature into macrophages.[9,12] The matured macrophages (Mϕ) could be harbored either in intact tissue or in the peritoneal fluid. These Mϕ can induce an inflammatory response by their accumulation or can produce pro-inflammatory mediators in response to any exogenous or endogenous stimuli. As a

component of the innate immune system, these activated Mϕ with their liberated cytokines and growth factors are suitable for the growth of endometriosis.[9,12] Although a link between the severity of endometriosis and macrophage activation has been reported.[65] information regarding the tissue infiltration of these inflammatory cells and their relationship with the revised-ASRM staging and morphologic appearance of endometriosis is limited.

Our recent study[11] demonstrated that tissue infiltration of Mϕ in the eutopic and ectopic endometrium in women with stage I-II endometriosis was significantly higher than with stage III-IV endometriosis or in control women. Red peritoneal lesions and their adjacent peritoneum had the greatest Mϕ concentration, compared with black or white lesions. These inflammatory cells showed a higher distribution in the secretory phase of the menstrual cycle. These results indicate that early endometriosis with red peritoneal lesions induces a higher inflammatory response in the pelvic cavity than advanced endometriosis by the increased recruitment and accumulation of Mϕ in these tissues and this inflammation may be extended to the peritoneum around these lesions.

The different cytokines that regulate HGF and other glycoprotein expression

are supplied by the infiltrated Mϕ in PF, endometrial tissues, and adjacent peritoneum.[11,66-68] However, the relationship between Mϕ infiltration and HGF expression or angiogenesis in endometrial tissues is unknown. We demonstrated a significant correlation between Mϕ density in the eutopic endometrium and corresponding red lesions and immunoreaction of HGF or between Mϕ accumulation and micro-vessel density in these tissues. No relationship was found between them in control women or in other peritoneal lesions. A substantial amount of HGF was also produced by the isolated basal Mϕ from women with endometriosis.

The inflammatory reactions in the eutopic and ectopic endometrium suggest that the growth of endometriosis does not depend on the fibrotic extension of disease; rather, it depends on the tissue activity of endometriosis. We presume that extension of disease could be related to pelvic pain, but higher activity of endometriosis associated with abundant recruitment and infiltration of Mϕ could be related to infertility. Our results agree with those of Donnez et al.,[69,70] who also reported that the increased tissue activity of endometriosis is associated with an increased pelvic inflammatory response. Our findings of tissue inflammatory reaction corresponded to similar reaction in PF. In

fact, PF of women with early endometriosis and those containing red lesions in the pelvic cavity harbored abundant Mϕ in PF when compared with that of control women, advanced endometriosis or other pigmented lesions or chocolate cysts.[71]

5.2. Production of HGF by Mϕ

The production of HGF by peritoneal Mϕ was confirmed at the gene and protein level in the isolated Mϕ and in intact tissue derived from women with and without endometriosis. We demonstrated transcriptional activity of HGF and its receptor, c-Met from the isolated peritoneal fluid Mϕ.[71] The mRNA expression of HGF was found to be significantly higher in women with endometriosis than those without endometriosis. No difference in the expression of c-Met mRNA nor phases of menstrual cycle was seen between these two groups of women (Figure **6 A**). The tissue localization of HGF was demonstrated in the same position as CD68-immunoreactive Mϕ in the serial section of intact tissues derived from the eutopic endometria of women with endometriosis (Figure **6 B, C**). This indicates that HGF is being synthesized and secreted by the infiltrated Mϕ of intact tissue and PF.

5.3. Role of endotoxin or lipopolysaccharide (LPS) in Mϕ-mediated

production of HGF and other macromolecules

We examined the ability of PF Mϕ to synthesize HGF and other macromlecules in basal conditions and after treatment with lipopolysaccharide (LPS), a bacterial endotoxin derived from the cell wall extract of Gram-negative bacteria. Since our initial study[72] demonstrated that PF of women with endometriosis contains a higher concentration of LPS (endotoxin) than that of those without endometriosis, we speculated that LPS could be a primary inflammatory mediator of Mϕ stimulation in pelvic microenvironment. In fact, activated Mϕ synthesize and secrete variable amount of different secondary inflammatory mediators such as IL-1, IL-6, IL-10, TNFα and other growth factors in response to LPS.[9,12,72]

We found that the production of HGF, VEGF, IL-6, and TNFα by the LPS-treated Mϕ was more remarkable in women with stage I-II endometriosis than those with stage III-IV endometriosis or control women (Figure **7**). In fact, a 3-fold and a 2-fold increase in the production of HGF by Mϕ was observed after LPS treatment in women with stage I-II endometriosis and stage III-IV endometriosis, respectively, when compared with non-treated Mϕ. However, only a 1.5-fold increase in the production of

HGF was observed in the control women after LPS treatment compared with no treatment. This was also confirmed at the gene level. A 3-, 5- and 4-fold increase in the expression of HGF mRNA in Mϕ was found in women with endometriosis at 1, 5, and 10 ng/mL of LPS treatment, respectively.[73] In contrast, a 1.5- to 2-fold increase in the relative expression of HGF mRNA was found in women without endometriosis.

5.4. TLR4-mediated cytokine production and growth of endometrial cells

The major component of the outer membrane of Gram-negative bacteria, endotoxin or lipopolysaccharide (LPS), is recognized by toll-like receptor 4 (TLR4) in association with other accessory molecules.[74-79] We examined the protein and gene expression of TLR4 in Mϕ, in the glandular epithelial cells and stromal cells derived from the eutopic and ectopic endometrium of women with or without endometriosis. We detected both the gene and protein expression of TLR4 in isolated Mϕ, stromal cells and epithelial cells and also in eutopic and ectopic endometria derived from women with and without endometriosis.[80-82]

In an attempt to examine that the stimulating effect of LPS in the production of all these macromolecules is mediated by TLR, we pre-treated Mϕ with antibody against

TLR4, then again treated them with LPS. We found that the levels of a number of cytokines and growth factors were significantly decreased in comparison with cells without blocking TLR4.[80,81] This was confirmed at both protein and gene levels. The pretreatment of glandular epithelial cells and stromal cells, derived from eutopic and ectopic endometria, with anti-TLR4 antibody was able to significantly suppress the growth of these cells when compared with cells without anti-TLR4 antibody treatment. These results indicated that LPS-mediated inflammatory reaction and growth of endometriotic cells is mediated by TLR4. We recommend that targeting TLR4 could be involved in decreasing pelvic inflammation and growth of endometriosis.[83]

6. Bacterial endotoxin (LPS) concentrations in differ ent body fluids

Since LPS or bacterial endotoxin acts as a primary inflammatory mediator and consequently stimulate immune cells for the production of secondary inflammatory mediators such as cytokines, chemokines, growth factors[9] but we don't know exactly the presence of endotoxin in pelvic environment of women with or without endometriosis. We measured endotoxin levels in the menstrual blood and corresponding peritoneal fluid

of women with and without endometriosis. We found that the concentration of bacterial endotoxin is 2-4 fold higher in the menstrual fluid when compared with that in peritoneal fluid and serum. The endotoxin level in menstrual fluid was also significantly higher in women with endometriosis than that of women without endometriosis. When we distributed endotoxin level in the peritoneal fluid according to menstrual cycle, we found the highest endotoxin level during the menstrual phase and persistence of a small amount of endotoxin during the proliferative phase or secretory phase of the menstrual cycle. This indicates that menstrual blood of women with endometriosis is highly enriched with bacterial endotoxin followed by the presence of a modest amount in the peritoneal fluid. But we do not know the exact source of endotoxin in menstrual blood in women with and without endometriosis.

There is a possibility that the lower genital tract of women with or without endometriosis is contaminated with a number of normal bacterial florae including *Escherichia coli* (*E.coli*). Therefore, we speculated that there might be an ascending migration of *E.coli* from the vaginal lumen up into the uterine cavity that causes contamination of menstrual blood and resulting in the subsequent release of its cell wall

extract, endotoxin, into menstrual blood and back to the peritoneal fluid. In fact, we found that a significantly increased colony formation of *E.coli* in the menstrual blood of women with endometriosis than that in the menstrual blood of women without endometriosis.[80]

7. Role of ovarian steroid hormones in endometriosis

The specific relation between ovarian steroid hormones and the development or maintenance of endometriosis remains unclear, although endometriosis occurs almost exclusively in menstruating women. Breast cancer, endometrial cancer, endometriosis, adenomyosis, and leiomyomas are diseases that are believed to grow in an estrogen-dependent fashion.[84] They commonly contain estrogen receptors (ERs), progesterone receptors (PRs) and androgen receptors. In past decades, the presence of ERs and PRs has been documented in both human endometrium and typical endometriosis using radioligand binding assay.[85-87] or more recently, using immunohistochemistry.[88,89] Using the latter method, investigators have researched whether the levels of expression of ER and PR in typical endometriosis, as defined by glandular structures and stroma, parallel those of the endometrium and whether typical endometriosis is under the same cyclic ovarian control as endometrium. Nisolle et al.[90]

reported that the contents of ERs and PRs in typical endometriosis lesions were lower than those of normal endometrium, but the menstrual cyclic changes were observed on the endometriosis in synchrony with the endometrium. On the other hand, other observers found no cyclic variation in typical endometriosis.[91,92] In addition, a marked heterogeneity in the expression of ERs and PRs in typical endometriosis from different patients was noticed; the contents of ERs and PRs differed between typical endometriosis and eutopic endometrium from the same patients.[91,93,94] The responsiveness of endometriosis tissue to ovarian steroid hormones also differed between samples of the same patients.[92,93] Thus, there is no consensus among investigators on the response of typical endometriosis to ovarian steroid hormones.

We have already learned that endometrial cancer, endometriosis, adenomyosis and leiomyomas contain not only ER but also aromatase, an enzyme that catalyzes the conversion of androgens to estrogens, suggesting that local estrogen production may increase the estrogen concentration.[84,95] Together with the circulating estrogen, this stimulates the growth of tissue mediated by the ER. Because of very low levels of aromatase activity and small tissue volume, it had been difficult to detect aromatase

activity in endometriotic implants. Reverse-transcription polymerase chain reactions enabled the detection of mRNA and the protein of aromatse P450 (P450arom), the major component of aromatase in such tissues. In addition, endometriotic tissue contains 17β -hydroxysteroid dehydrogenase type 1, an enzyme that converts estrone (E1) to the more potent 17β-estradiol (E2), whereas they lack 17β-HSD type 2, an enzyme responsible for the inactivation of E2 to E1, resulting in raising the local estrogen activity level.[84,96]

A number of literatures have already demonstrated the potential role of ovarian steroid hormones in the regeneration of endometrium after menstruation and the growth of endometriosis.[97,98] However, as a non-self lesion in pelvic environment, the growth or persistence of endometriosis can also be regulated by innate immune system. It has also been reported that the female sex hormones estradiol and progesterone modify the risk of uterine infection.[98,99] In fact, the association between ovarian steroid hormones and innate immune system is not well described.

7.1. Immunological role of estrogen and progesterone

In recent years increasing attention has been paid to innate immunity as the primary defense system against pathogens. *Escherichia coli (E.coli)* are the most

commonly isolated pathogenic bacteria from clinical uterine diseases in cattle and also in human vaginal cavity.[100,101] The ascending migration of *E.coli* towards endometrial cavity is possible that may cause contamination of endometrium. In bovine uterine lumen, there are high concentrations of the pathogenic ligand of *E.coli* known as bacterial endotoxin or LPS. The endometrium provides a barrier against infection and an opportunity to detect these bacteria by innate immune receptors.

The endometrium is regulated by changing concentrations of the female sex hormones, E2 and P during the ovarian cycle, and these steroids also have a profound effect on infections.[82,101] For example, in humans, rodents and cattle, progesterone suppresses uterine immune function by decreasing the proliferative capacity of lymphocytes as demonstrated earlier in vitro,[102] thereby increasing the susceptibility to bacterial infection.[99] Conversely, E2 may play a role in the recruitment of immune cells, as more macrophages are present in the endometrium when E2 concentrations are high in rodents.[101] However, study regarding TLR expression and its involvement in the control of steroid hormone function is still unknown. The role of E2 in infection is dependent on the species, tissue and concentration of the sex hormone. Estrogen is generally thought to

confer protection against infection.[99]

Besides potential role in bacterial infections, sex steroid hormones are known as modulators of the immune system and may influence development and course of a variety of autoimmune disorders.[103-105] A rapid decline of female sex hormone production after menopause may thus affect various immune parameters. Indeed, it has been reported that menopause may be associated with systemic and local changes in T and B cell subpopulation and function[106,107] as well as immunoregulatory cytokine production.[108,109] Immune responses may also be affected by exogenous hormone supplementation. Experimental in vivo administration of estrogens in mice has been reported to down-regulate NK cell cytotoxicity[110] and to be associated with viral infection or enhanced tumor growth.[111,112] NK cell activity in women taking oral contraceptives is significantly lower compared to non-users.[113,114] Considering the effect of HRT on NK cell-mediated cytotoxicity, there exist only few and inconsistent reports with variable effects on NK cells.[107,115] A recent clinical study demonstrated that HRT is associated with a significant decrease of NK cell cytotoxicity and a variable change of Th1 and Th2 cytokines. These changes in individual patients did not correlate

with changes of serum sex hormone levels.[116] These findings imply that estrogen/progesterone HRT may affect cell-mediated immunity and may be a potential factor influencing development of autoimmune disorders or neoplastic diseases.

Generally, sex steroid hormones are implicated in the immune response, with estrogens as enhancers at least of the humoral immunity and androgens and progesterone as natural immunosuppressors. The role of estrogen in humoral immunity has been supported by the detection of higher levels of E2 in synovial fluid and serum of patients with rheumatoid arthritis,[117,118] which is most probably due to an increase in local aromatase activity. It has also been demonstrated that ovarian steroids, E2 and P, equally participate in humoral immune responses by modulating asymmetric antibody synthesis.[119] In fact, sex steroids are potentially capable of driving the balance toward a cell-mediated (Th1) or humoral (Th2) response. It has been argued that imbalance in favor of a Th1 response is fostered by low levels of estrogen and prolactin, whereas, Th2 response is promoted by high levels of estrogen and testosterone.[120]

Despite the widespread associations between immunology and steroid hormones, the study of systemic interactions remains limited. Many diverse facts are still

accumulating on the interactions between endocrine and immune systems in the human endometrium. Understanding the molecular pathways in endocrine immune interactions in the human endometrium and in different endocrine diseases is crucial to better clarify events such as menstrual bleeding, tissue repair and regeneration, inflammation, angiogenesis, blastocyst implantation, and progression of pregnancy. These events require a balanced regulation of endometrial differentiation, proliferation, cell survival, immune cell recruitment, apoptosis, and angiogenesis by ovarian steroids. Recently, a novel concept of endometrial dissemination has been described as a result of a neuroendocrine-immune disequilibrium in response to high levels of perceived stress caused by cardinal clinical symptoms or tissue reaction of endometriosis.[121,122]

7.2. Estrogen (E2) and progesterone (P) levels in different body fluids

We already reported increased immunoexpressions of estrogen and progesterone receptors in eutopic endometrium and ectopic endometrial lesions from our laboratory.[6] However, information regarding concentrations of estrogen (E2) and progesterone (P) in the peritoneal fluid (PF) and other body fluids of women with and without endometriosis is scarce. It could be possible that ovarian steroids act directly or

indirectly via the production of different macromolecules in the growth or maintenance of endometriosis. Therefore, we measured E2 and P levels in the PF, serum and menstrual fluid of a group of women with and without endometriosis by a modified immulyze-enzyme amplified luminescence system and as described previously.[123] Estrogen and P concentrations in the seminal fluid derived from healthy volunteers were used as a positive control. We also investigated the changes of E2 and P in the PF according to revised ASRM staging and morphologic appearances of endometriosis.

We found the highest concentration of E2 and P in the PF, intermediate levels in the menstrual fluid and the lowest levels in the serum (Figure **8**). Both E2 and P levels in these body fluids were higher in women with endometriosis than that in control women, irrespective of menstrual cycle, revised-ASRM staging and color appearance. Although, no significant difference was observed in E2 and P levels between these two groups of women, when we distributed the patients in a different revised-ASRM staging, PF levels of E2 in women with stage I-II endometriosis were found to be significantly higher than in women with stage III-IV endometriosis or without endometriosis.[14] An increase tendency in P concentration was observed between them. When we distributed

the patients into different morphologic appearances of endometriosis, PF levels of E2 and P were found to be significantly higher in women containing dominant red peritoneal lesions when compared with women containing other peritoneal lesions or women without endometriosis.[14] Estradiol (E2) level in the PF of women with endometriosis in the proliferative phase was higher than that of women without endometriosis. Women with endometriosis also displayed higher P levels in the secretory phase than those in the proliferative phase. No difference was observed in serum levels E2 or P according to revised-ASRM staging or color appearances of endometriosis.

7.3. Expression of E2 receptor (ER) and P receptor (PR) in endometriosis

The expressions of estrogen receptor (ER) and progesterone receptor in the eutopic and ectopic endometria are well described.[85-94] Aside from typical endometriosis, there are atypical varieties of endometriosis characterized by a lack of glandular epithelial structures or stromal cells in non-pigmented lesions,[124,125] inclusion cyst, endosalpingiosis, reactive mesothelium, and columnar epithelial cells in the pelvic peritonea. Reports on the expression of ERs and PRs in these other peritoneal lesions are scarce, and the effect of ovarian steroids on them is not well understood.

We re-evaluated ER and PR expression in endometrium and typical endometriosis and extended our study to include other peritoneal lesions. The highest score of ERs and PRs was observed in the epithelial and stromal cells of the normal uterine endometrium at the early proliferative phase of the menstrual cycle. The ER and PR scores declined throughout the secretory phase. In typical endometriotic lesions, The ER and PR scores were constantly high independent of the menstrual cycle. The expression pattern of ER mRNA was mostly in parallel with that of ERs. In typical endometriosis, ERs and PRs were found in both glandular epithelial cells and their surrounding stromal cells. Expression of ER mRNA was found in typical endometriotic peritonea and in pelvic peritoneum with columnar epithelial cells, but not in normal pelvic peritoneum (mesothelium). Estrogen receptor and PRs were negative in mesothelium, but were positive in the nuclei of fibroblasts in the connective tissue.

Our findings indicate that the columnar type cells in mesothelium are more similar to the epithelial cells in endometrium and endometriosis than to flat mesothelium. The relation of the columnar cells with their adjacent mesothelium and endometriotic lesions appears to be a key point that will decide whether endometriosis is an implant of

the endometrium or is a metaplasia of mesothelium. In our previous study, clusters of columnar cells were found in mesothelium in cases in which endometriosis was absent.[126] The study suggested that the columnar cells did not originate from endometriosis and that those found in the periphery of endometriotic lesions may manifest eventually as endometriosis. Although there is no unequivocal evidence for this path, there are some supporting data: Murphy et al.[127] found a glandular orifice adjacent to normal mesothelium, and Nakamura et al.[128] documented columnar and ciliated cells in many peritoneal infoldings that dip into sub-peritoneal stroma. We strongly presume that pelvic peritoneum may have the potentiality for metaplastic changes under the influence of steroid hormones and/or a stimulating substance such as growth factors. In fact, we already described that HGF, as a pleiotropic growth factor, has the parallel potentiality involving in cellular changes of peritoneal mesothelium to a columnar phenotype and consequent gland-like invagination mimicking endometriosis.[3]

7.4. Expression of ERs and PRs in macrophages

Since mesenchymal cells retain estrogen receptor, production of different cytokines by endometrial stromal cells and its modulation by estrogen has been

demonstrated.[16] Considering that infiltrated M ϕ is one of the cell components of endometriotic lesion in pelvic environment, reports describing expression of steroid receptors by M ϕ and the secretion of different macromolecules in response to steroid hormones are scanty.

Several reports demonstrated that these inflammatory cells retain the mRNA encoding both ER and PR.[71,129] Majority of CD68 immunoreactive Mϕ as isolated from women with or without endometriosis showed stronger nuclear staining for ER but were less reactive to PR. In contrast, tissue localization of ER and PR was equally demonstrated in the same position of CD68 immunoreactive Mϕ in the serial section of intact tissues derived from the eutopic endometrium of woman with endometriosis.[71] This indicates that besides glandular epithelium and stroma, ER and PR are also being synthesized and expressed by the infiltrated Mϕ in intact tissue. This was further confirmed at gene level and revealed that basal Mϕ isolated from the PF of women with or without endometriosis contained the mRNA encoding for ER and PR. No phase of the menstrual cycle dependent variation in the expression of these receptor mRNAs was evident for either group.[71]

7.5. Production of macromolecules by E2-, P-, and LPS-stimulated Mϕ

Several reports have already demonstrated the potential role of ovarian steroid hormones in the regeneration of endometrium after menstruation and the growth of endometriosis.[6,7] However, information regarding inflammatory cell mediated growth or persistence of endometriosis by ovarian steroids is limited.

We and others found that direct stimulation of Mϕ in culture with E2 and P resulted in a variable increase in the secretion of HGF, VEGF, IL-6 and TNFα by peritoneal fluid Mϕ.[71,129] The production of HGF was significantly increased by E2 in women with endometriosis than that of control women or non-treated macrophages. A marked increase in the secretion of VEGF was observed by treatment with both E2 and P in women with endometriosis and also in women without endometriosis when compared with non-treated PF macrophages. No phase of cycle differences were seen. When we performed a blocking experiment on E2 by using ER antagonist, tamoxifen, we found that tamoxifen significantly reversed the secretion of and tended to reverse the secretion of HGF by the estrogen-treated PF macrophages towards the non-treated macrophages.[71] This indicates that it is the direct effect of estrogen on the PF macrophages that was able

to produce significant amount of HGF and VEGF and is being mediated by ER as located on these inflammatory cells. We also found a substantial amount of increase in the levels of IL-6 and TNFα in response to ovarian steroids without displaying any significant difference with non-treated cells.[71]

It has been reported that activation with LPS significantly increased the amount of a number of macromolecules secreted by inflammatory cells.[71,73] We observed that activation of basal macrophages further enhanced the response of these cells to ovarian steroids. In fact, exogenous treatment with E2 was able to further increase the amount of both HGF and VEGF secretion by PF macrophages when these cells were activated with LPS.[71] Although progesterone increased the secretion of VEGF by non-activated Mϕ, it was unable to further enhance the secretion of either HGF or VEGF by activated PF macrophages.[71] These results confirmed that irrespective of activation status, PF macrophages were independently stimulated to produce HGF and VEGF by E2. This also indicates that in addition to primary and secondary inflammatory mediators, ovarian steroids equally produce a pelvic inflammatory reaction mediated by macrophages. An inflammatory response and ovarian steroid hormones may function either alone or in

combination to regulate the production of a variety of macromolecules by PF macrophages in pelvic microenvironment.

It is generally believed that ovarian steroid hormones are essential for the growth or persistence of ectopic endometrium and corresponding eutopic endometrium in women with endometriosis.[130] Since the major cellular constituents of PF are macrophages, comprising between 82 and 99% of the total cell population,[131] it is quite reasonable to speculate that these cells may be responsive to ovarian steroids.

7.6. *Synergistic effect between HGF and E2 in the growth of endometriosis*

The presence of c-Met receptor and estrogen receptor in endometrial and endometriotic cells and Mϕ might be expected to enable these cells to respond to endogenous or exogenous HGF and E2.[71,73,132] When we examined the effect of HGF and E2 on the proliferation of endometrial gland cells, stromal cells and Mϕ, we found that these cells significantly proliferated in response to HGF and E2 either alone or in combination when compared with non-treated cells.[71] A synergistic effect between HGF and E2 on cell proliferation was observed. A similar pattern of cell proliferation was also found in gland cells and stromal cells derived from ectopic endometrium. These results

indicate that an innate immune system in pelvic environment and ovarian steroid hormones function in an orchestrated fashion and are involved in the growth of endometriosis. Our results are summarized in Figure **9**.

8. Conclusions

We now know that besides steroid hormones, innate immunity plays a pivotal role in the initiation of an array of inflammatory reactions against regurgitated endometrial cells and subsequent development of peritoneal endometriosis. Currently prevalent concepts on the genesis of pelvic endometriosis are retrograde dissemination of eutopic endometrial tissues during menstruation,[4] coelomic metaplasia of the peritoneum as a secondary Mullerian system,[133,134] and compromised immuno-surveillance.[18-20] However, none of these theories can explain the pathogenesis of endometriosis uniformly. Based on our serial studies on the etiological role of bacterial endotoxin (LPS), we would propose a novel concept for the genesis of pelvic endometriosis via LPS/TLR4-mediated engagement of innate immune response.

According to this concept, it would appear possible to integrate two conflicting thoughts of transplantation and metaplasia as reflecting the different phases of initiation

and progression of pelvic endometriosis. Transplantation and consequent implantation of regurgitated endometrial cells during menstruation may trigger strong inflammatory reaction in early endometriosis. In addition, a variety of pro-inflammatory factors are also secreted from the infiltrated mononuclear cells of innate immune system. During progression of the affected lesion, cellular changes of juxtaposed mesothelium into endometrioid cells and gland-like structures subsequently ensues and was described as metaplasia of peritoneal mesothelium.[3] As a pleiotropic growth factor, HGF being produced by macrophages and stromal cells has been shown to serve this unique role. The multi-functional role of HGF can be performed with the aid of systemic or focal hormonal environment characterized by consistent estrogen synthesis. Our presenting findings demonstrate for the first time that besides other pro-inflammatory mediators, ovarian steroids also participate in the generation of a pelvic inflammatory response by producing different macromolecules including HGF by peritoneal Mϕ. These pro-inflammatory mediators including HGF may be involved in the growth of endometriosis either alone or in combination with estrogen (Figure **9**).

A complete understanding of the mechanisms of endocrine-immune cross talk

in the mammalian species and the function of innate immunity via toll-like receptor system will be helpful for the future development of innovative therapies for manipulation of endometriosis.

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