Among them, mutations of angiotensinogen (*AGT*), methlenetetrahydrofolate reductase (*MTHFR*), *Factor V Leiden*, and prothrombin genes seem to be the most likely candidate in Japanese although no mutation in single gene can correctly predict the disease. The detection rate of *AGT* and *MTHFR* gene polymorphisms are high in Japanese, and relationship between preeclampsia and DNA polymorphism in these two genes were also reported. And, in European countries, strong relationship between preeclampsia and *Factor V Leiden*, prothrombin genes polymorphisms were reported.
In the present study, we determined the composite genetic risk for preeclampsia by simultaneous genotyping of these loci.

Material And Methods

Patients participating in this study were recruited from the obstetric population followed at Nagasaki University Hospital between December 1997 and March 2000. Informed consent was obtained from all patients, and the ethics committee of our university approved the study protocol. Fifty-two pregnant women with preeclampsia in their first pregnancy and 113 normotensive gravid women were recruited for this retrospective study. All subjects were Japanese women with singleton gestations. According to the working group of the National High Blood Pressure Education Program (NHBPEP 2000), preeclampsia is defined by the de novo appearance of hypertension after 20weeks of gestation, systolic blood pressure $\geqq$140 mmHg or diastolic blood pressure $\geqq$90 mmHg, accompanied by new-onset proteinuria, defined as $\geqq$300 mg per 24 h. Genomic DNA was extracted from peripheral blood leukocytes by standard procedures (Quiagen DNA extract kit), and the genotype was determined by a PCR restriction fragment length polymorphism method (RFLP) as described below.

Angiotensinogen M235T polymorphism

A 165-base-pair DNA fragment of the angiotensinogen gene was amplified by polymerase chain reaction (PCR) using the sense primer 5' CAG GGT GCT CAC ACT GGA CCC C-3' and antisense primer 5' CCG TTT GTG CAG GGC CTG GCT CTC T-3', according to the method described by Kamitani et al.[4] After endonuculease digestion with *Tth*111-I, PCR fragments were electrophoresed in 10% polyacrylamide gels and stained with ethidium bromide.

MTHFR C677T polymorphism

A 189-base-pair DNA fragment of the *MTHFR* gene was amplified by the PCR using the sense primer 5' TGA AGG AGA AGG TGT CTG CGG GA-3' and antisense primer 5' AGG ACG GTG CGG TGA GAG TG-3', according to the method described by Morita et al.[5] After endonuclease digestion with *Hin*fI, PCR fragments were electrophorased in 10% polyacrylamide gels and stained with ethidium bromide.

Factor V gene analysis

A 287-base-pair DNA fragment of Factor V gene was amplified by the PCR using the sense primer 5' GGA ACA ACA CCA TGA TCA CAG CA-3' and antisense primer 5' TAG CCA GGA GAC CTA ACA TGT TC-3', according to the method described by Zoller et al.[6] After endonuclease digestion with *Mnl*I, PCR fragments were electrophorased in 8% polyacrylamide gels and stained with ethidium bromide.

Prothrombin G20210A polymorphism

A 640-base-pair DNA fragment of the prothrombin gene was amplified by PCR using the sense primer 5' CGG TGT GTG TGT AGG AAC TCC-3' and antisense primer 5' CAA TGT CAG ATG CTG GGG ACT-3', according to the method described by Poort et al.[7] After endonuclease digestion with *Alw 44I*, PCR fragments were electrophoresed in 1% agarose gels and stained with ethidium bromide.

Statistical analysis

Differences between groups were examined for statistical significance using the Fisher's exact test or chi-square test. Odds ratio and 95 percent confidence intervals (95%CI) were calculated. A p value less than 0.05 denoted the presence of a statistically significant difference.

RESULTS

We studied 52 pregnant women with preeclampsia in their first pregnancy and 113 normotensive gravid women (Table 1).

Angiotensinogen

Table 2 shows the M235T genotyping results. M235T homozygosity (genotype TT) was detected in 33 of 52 (63.5%) patients with preeclampsia and 51 of 113 (45.1%) control subjects. The frequency of each genotype in the control group was consistent with those reported previously by other investigators.[4,8,9] Compared with the other genotypes (TM and MM), the frequency of aa was significantly higher in preeclampsia than in controls ($p<0.05$). The observed genotype frequencies were not different from those predicted from Hardy-Weinberg equilibrium.

MTHFR

Table 3 shows the results of C677T genotyping.
C677T homozygosity (TT) was identified 18 of 52 (34.6%) in control subjects. The frequency of each genotype in the control was consistent with those reported previously by other investigators.[5,10] Compared with the other genotypes (CT and CC), the frequency of

Table 1. Clinical Characteristcs

Variable	Preeclampsia (n=52)	Control (n=113)	
	Mean (95%CI)	Mean (95%CI)	p
Age	30.1 (28.3, 32.0)	30.4 (29.5, 31.3)	NS
BMI (kg/m^2)	21.1 (18.4, 23.8)	20.7 (21.0, 22.4)	NS
Systolic BP (mmHg)	164 (153, 172)	116 (113, 119)	< .001
Diastolic BP (mmHg)	102 (95, 109)	71 (69, 73)	< .001
Birth Weight (g)	2,244 (1,857, 2,598)	2,994 (2,687, 3,298)	< .001
Weeks of gestation	35.0 (32.6, 37.2)	39.1 (38.2, 40.0)	< .001

BMI: Body mass index
CI : confidence interval; BP : blood pressure, NS : not significant

Table 2. AGT genotype distribution in patients with pre-eclampsia and control subjects

	MM (%)	MT (%)	TT (%)
PE (n=52)	4 (7.7)	15 (28.9)	33 (63.5)
Controls (n=113)	11 (9.7)	51 (45.1)	51 (45.1)

PE : preeclampsia

Table 3. MTHFR genotype distribution in patients with pre-eclampsia and control subjects.

	CC (%)	CT (%)	TT (%)
PE (n=52)	17 (32.7)	17 (32.7)	18 (34.6)
Controls (n=113)	48 (42.4)	54 (47.8)	11 (9.7)

PE : Preeclampsia

Table 4. Odds ratios of preeclampsia in the presence of *AGT, MTHFR* and combined *AGT + MTHFR* gene polymorphisms.

		Odds ratio	95%CI
AGT homozygous mutation (TT)	(n=33)	2.11	1.08-4.15
MTHFR homozygous mutation (TT)	(n=18)	4.91	2.11-11.4
Both homozygous mutaiton (TT/TT)	(n=8)	4.79	1.66-13.8

TT was significantly higher in preeclampsia than in control subjects ($p<0.01$). The observed genotype frequencies were not different from those predicted from Hardy-Weinberg equilibrium.

Analysis of the effect of modification was performed to evaluate the estimates of risk associated with *AGT* gene mutation (genotype aa) and *MTHFR* gene mutation (genotype aa), and their combination (Table 4). The frequency of double homozygous (TT/TT) in the Preeclampsia is 13.4% (8/52), and 3.5% (4/113) in control. The calculated risk associated with the presence of both mutations (odds ratio 4.79, 95%CI: 1.66-13.8) did not exceed the risk of *MTHFR* single gene mutation (odds ratio 4.91, 95%CI: 2.11-11.4).

Factor V and prothrombin gene

Previous studies have shown that *factor V Leiden* mutation and prothrombin G20210A mutation are very rare in Asia.[11] In our population sample, no Leiden or prothrombin G20210A mutations were detected in all 165 women.

Discussion

Preeclampsia is one of the most serious complications during pregnancy and a syndrome that affects virtually all-maternal organ systems. The condition is still the leading cause of maternal and infant morbidity and mortality.[12] Several studies have examined the etiology and pathophisiology of preeclampsia, but the exact mechanism remains unknown. Furthermore, several studies reported the possible association between genetic factors and preeclampsia.[13,14]

In the present study, we investigated the genotypes of four candidate genes, *AGT, MTHFR, factor V Leiden*, and prothrombin gene in the same patients with history of preeclampsia. Our results demonstrated that pregnagnt woman with both homozygous for *AGT* gene mutations and *MTHFR* gene mutations have a higher risk for preeclampsia comparing to the controls with other genotypes.

Jeunemaitre et al.[15] were the first to report the genetic association between the *AGT* gene and hypertension. Ward et al.[8] proposed that preeclampsia could be viewed as a pregnancy-induced proteinuric hypertension, and thus the condition could be associated with *AGT* gene mutation. They were able to demonstrate a significant association between preeclampsia and a molecular variant of *AGT*, M235T. Based on this finding they suggested that increased concentration of angiotensinogen in individuals carrying variants of *AGT*, such as M235T, might be associated with increased production of angiotensinogen II. They also argued that chronic stimulation in M235T carriers could increase vascular tone and promote vascular hypertrophy, consequently causing preeclampsia during pregnancy. In Japanese, the frequency of M235T is higher than in Caucasians, but as in Caucasians, several reports confirmed the association between M235T mutation and preeclampsia,[8,15] but

others could not confirm these findings.[16, 17]

Hyperhomocysteinemia has been identified as an important risk factor for occlusive vascular disease,[18] and some reports suggested a relationship between repeated fetal loss or abruptio placentae and hyperhomocysteinemia.[19] *MTHFR* is one of the key enzymes essential for normal homocystein metabolism, and abnormalities in this enzyme can lead to hyperhomocysteinemia. Frosst et al.[20] were the first group to identify a common mutation in *MTHFR* gene (C677T) in hyperhomocysteinemia, and significantly higher concentrations of plasma homocystein in individuals homozygous for this mutation. Based on these findings, they suggested that this mutation is an important genetic factor in vascular disease. Other groups later supported these results.[4] Vascular damage consisting of thrombosis of small uterine arteries may be involved in the pathophisiology of preeclampsia.[21, 22] Souda et al.[9] reported the association between preeclampsia and *MTHFR* gene mutation, C677T. However, other investigators could not confirm such relationship.[23-27]

Our results showed a significant correlation between mutations of these two genes and preeclampsia, and that the presence of *MTHFR* gene mutation has a strong impact on the incidence of preeclampsia. Our analysis also demonstrated that *AGT* and *MTHFR* gene mutations play a role in the pathogenesis of preeclampsia. Our results indicate that combination analysis of genetic mutations is potentially clinically useful for genetic screening of patients at risk for the development of preeclampsia. However, in our combination study we could not find additive effects both mutations; i.e., the calculated risk associated with the presence of both mutations was not higher than that of *MTHFR* mutations only. The reason for the lack of the additive effects may be simply due to the small sample size. To examine this issue, we are currently attempting to analyze more patients for mutations of these genes. It is probable that a group of genes could accurately predict the risk of preeclampsia, while individual genes may have only a limited predictive power. Therefore, combination analysis of only two genes may not establish significant predictive power. However, available data suggest that a very common allele is necessary to explain the observed inheritance of preeclampsia. Thus, we believe in the existence of a few "major loci", and many "minor loci", and postulate that *MTHFR* gene may be at the front and *AGT* gene at the back in such a scheme.

Our results showed the lack of mutations of *factor V Leiden* and prothrombin genes in our 165 patients with preeclampsia. These results are different from those of previous studies.[28] In Caucasian, several reports suggested the presence of a significant correlation between mutation of *factor V Leiden* gene and preeclampsia.[29, 30] However, the majority of these studies were from European countries and to our knowledge, no such correlation has been previously reported in Asian-based studies. In this regard, Rees et al. reported the world distribution of *factor V Leiden* mutation, and demonstrated confinement of *factor V Leiden* mutation to European countries with the exception of two cases.[11] These genodemographic findings are in agreement with our results.

In conclusion, based on the results of the present study, we speculate that *AGT* and *MTHFR* are likely to be implicated in preeclampsia in Japanese women. We suggest that preeclampsia is a heterogeneous disease. To identify the underlying genetic factors associated with preeclampsia, one has to consider the variability in the incidence of preeclampsia among different countries and races, and that such variability probably reflects different prevalence of mutations of candidate genes.

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

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Endometriosis, a chronic disease characterized by endometrial tissue located outside the uterine cavity 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 (lipopolysaccharide) and is mediated by toll-like receptor 4 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 report 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 are 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.

Introduction

Endometriosis, the presence of functional endometrium outside 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 studies 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 among 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] compared with 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]

As mesenchymal cells retain estrogen receptor (ER), production of different cytokines by endometrial stromal cells and their 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 discuss the orchestrated role of HGF, innate immune system and ovarian steroid hormones in inducing pelvic inflammation and consequent development of pelvic endometriosis.

Pathogenesis and Natural Course of Pelvic Endometriosis

Common Concepts in the Pathogenesis of Endometriosis

In addition to transplantation and implantation theory of Sampson[4] and celomic metaplasia theory of Meyer,[17] immuno-surveillance of the refluxed endometrial cells is an attractive theory for the development of pelvic endometriosis (Fig. 1). The immune tolerance or immune deficiency 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, PF, peritoneum, and endometriosis.[30,31]

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 and 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 pre-treatment 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 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 en-

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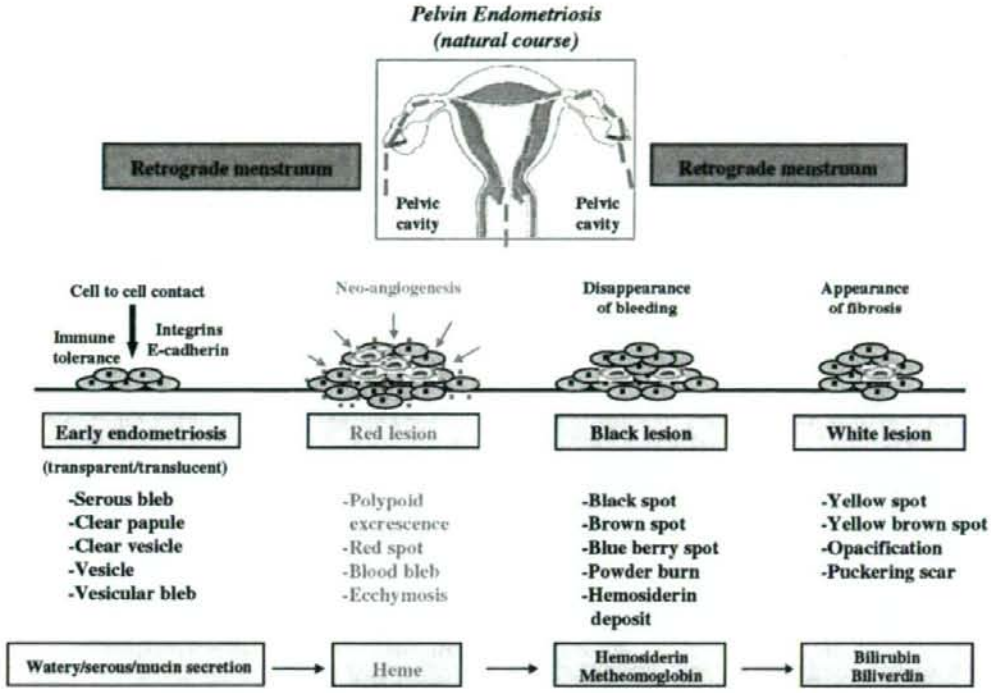

Fig. 1 Shows the diagrammatic representation of the natural course of pelvic endometriosis in pelvic cavity. After initial attachment of refluxed endometrial cells with peritoneal cells producing early endometriotic lesions, the consequent events of mitosis, angiogenesis, metabolic degradation of heme and appearances of fibrosis result in the generation of different morphologic appearances of pelvic endometriosis as shown in this figure.

dometriotic biopsies, but not from human endometrial biopsies, are invasive in an *in vitro* collagen invasion assay.[36]

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 may 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 PF of women with endometriosis. There was no concomitant increase in bilirubin concentrations in the PF 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, may be insufficient to detoxify heme in women with endometriosis.

The lesions of early endometriosis are either transparent or translucent because they lack formation of vasculatures around them. We named these early lesions as non-opaque lesions[38] because these lesions contain either watery, serous or mucinous secretion and there is no collection of blood in the stroma by histology (Fig. 2a,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 laparoscopy (Fig. 2c,d).[38] With the progression of time, there is deoxygenetion process from hemoglobin to methemoglobin 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 (Fig. 2e,f). Black lesion again changes to white lesion because of collection of bilirubin or biliverdin and collection of fibrous tissue. In this stage, gland gradually becomes smaller and stroma sometimes disappears because of deposition of fibrous tissue. Finally old lesions disappear and there is new focus of endometriosis because of 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 do not 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 Fig. 1.

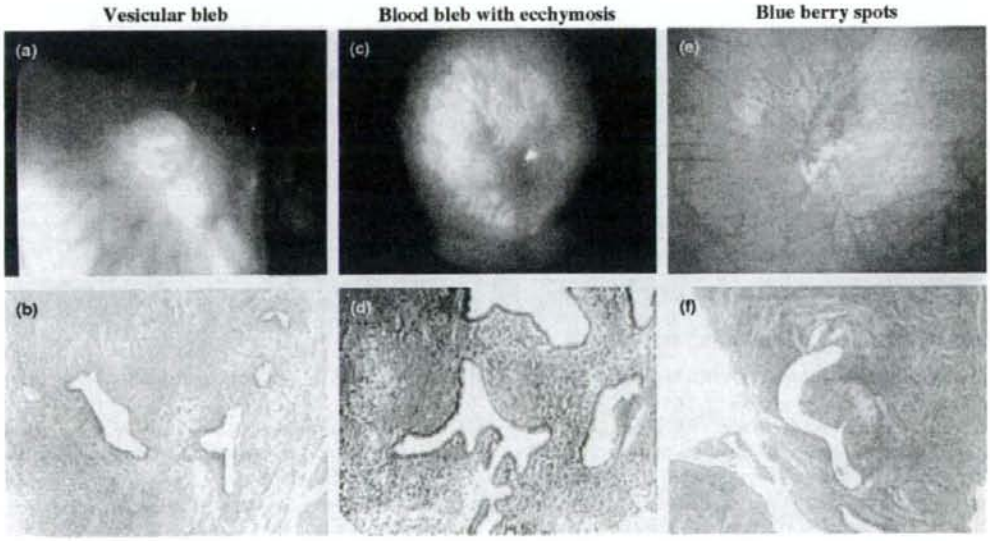

Fig. 2 Shows the laparoscopic and histologic appearance of blood-filled opaque red lesions and non-opaque red lesions (a–d) and blue berry spots (e, f) in pelvic cavity. It can be noted here that non-opaque endometriotic lesions such as vesicular bleb (a) lack oozing of blood in stroma (b) and opaque red lesions such as blood bleb with ecchymosis (c) are accompanied by oozing of blood in the stroma of these lesions (d). In contrast, black lesions such as blue berry spots (e) are manifested by color change and disappearance of blood from the stroma of these lesions (f).

Role of Hepatocyte Growth Factor in Endometriosis

Biology of HGF and its Receptor, c-Met

Hepatocyte growth factor 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-kDaα-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. As 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.

HGF Concentrations in Different Body Fluids

Elevations in the 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 advanced stage 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 out the contentious issue 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]

As 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 com-

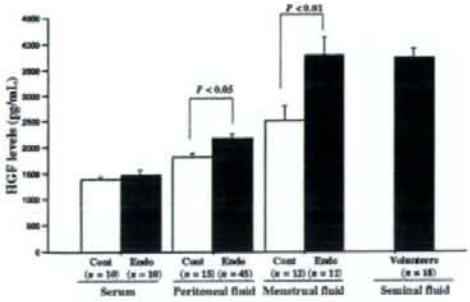

Fig. 3 Shows the levels of hepatocyte growth factor (HGF) in the different body fluids of women with and without endometriosis. The level of HGF in seminal fluid was used as a positive control. Although not significant in serum, HGF levels were found to be significantly higher in the menstrual fluid ($P < 0.01$) and peritoneal fluid ($P < 0.05$) of women with endometriosis than that of control women (without endometriosis).

pared with that in women without endometriosis (Fig. 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 PF and lowest in the serum of women with endometriosis (Fig. 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.

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. In a very similar manner to 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.

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 (50–100 ng/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 (Fig. 4a,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.

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 Fig. 4c,d, addition of 50 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 50 and 100 ng/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.

HGF as Morphogenic Factor in Endometriosis

Though celomic 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.

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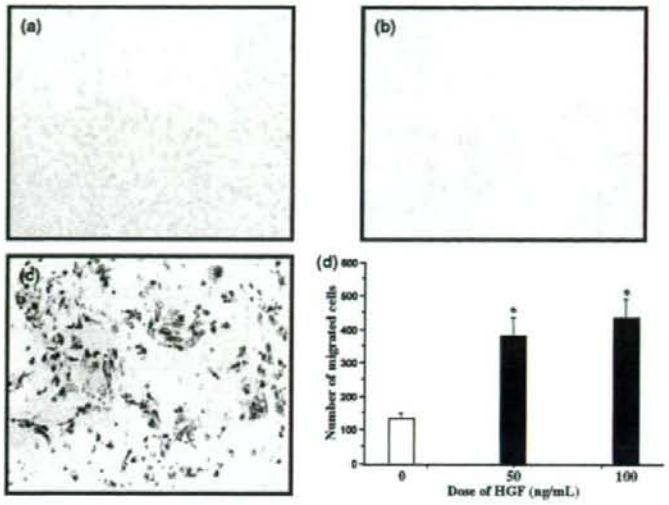

Fig. 4 Shows the effect of hepatocyte growth factor (HGF) on the scattering (a, b) and migration (c, d) of endometrial cells derived from women with endometriosis. After application of 100 ng/mL, HGF was found to scatter endometrial stromal cells (b) in a collagen non-coating culture plate when compared with non-treated cells (a). Boyden's chamber assay showed that addition of 50 and 100 ng/mL of HGF were able to migrate epithelial cells (c) and migrated cells were significantly higher ($P < 0.001$) when compared with HGF non-treated cells (d).

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 within 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 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 Fig. 5.

Role of Mφ in Pelvic Inflammation and Growth of Endometriosis

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 PF. 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

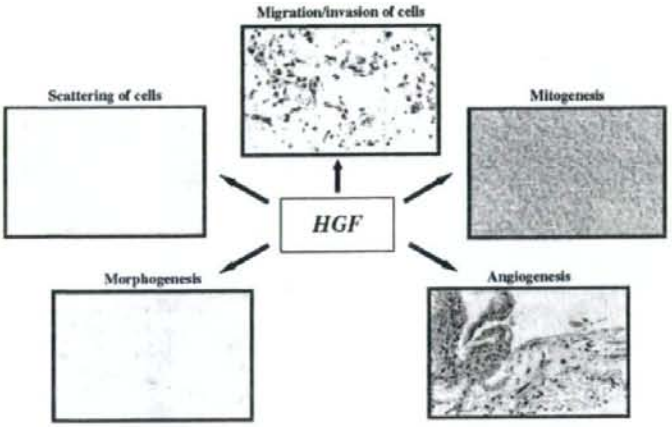

Fig. 5 Shows the multifunctional role of hepatocyte growth factor (HGF) in the development of endometriosis such as migration/invasion of cells, scattering of cells, cell proliferation effect (mitogenesis) on cells of eutopic and ectopic endometrium, immunoreaction of HGF to transforming cells of pelvic peritoneum (morphogenesis) and strong immunoreaction of HGF to vascular endothelial cells (angiogenesis).

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 expressions 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]

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 PF Mφ.[71] The mRNA expression of HGF was found to be significantly higher in women with endometriosis than those without endometriosis. No difference in either the expression of c-Met mRNA or phases of menstrual cycle was seen between these two groups of women (Fig. 6a). 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

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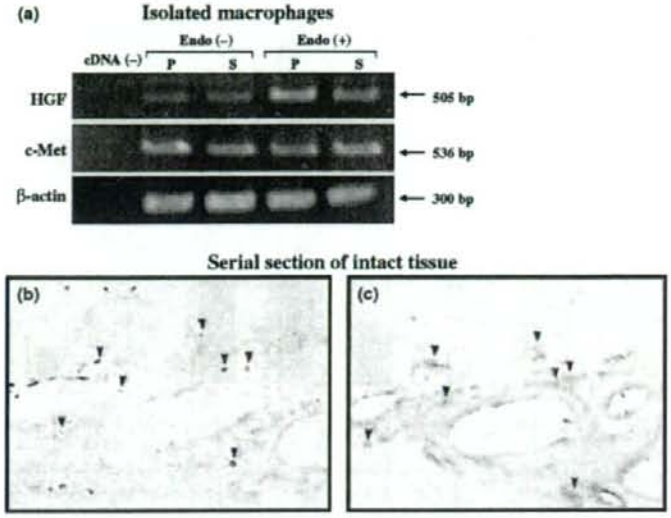

Fig. 6 Shows production of hepatocyte growth factor (HGF) by macrophages. Upper panel (a) shows expression of mRNA encoding for HGF and its receptor, c-Met in isolated macrophages derived from the peritoneal fluid of women with endometriosis (endo+) and without endometriosis (endo–). The mRNA expression of HGF was found be to be higher in women with endometriosis than those without endometriosis. P, proliferative phase; S, secretory phase of menstrual cycle. Lower column (b and c) shows immunoreaction of HGF in CD68-positive macrophages in a serial section of tissue derived from the eutopic endometrium of women with endometriosis. Arrowheads show the localization of HGF (c) in the same position as CD68-stained macrophages (b) in the stroma of eutopic endometrium.

eutopic endometria of women with endometriosis (Fig. 6b,c). This indicates that HGF is being synthesized and secreted by the infiltrated Mφ of intact tissue and PF.

Role of Endotoxin or Lipopolysaccharide 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. As 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, tumor necrosis factor-α (TNF-α) and other growth factors in response to LPS.[9,12,72]

We found that the production of HGF, VEGF, IL-6, and TNF-α 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 (Fig. 7). In fact, a threefold and a twofold 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 three-, five- and fourfold 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.

TLR4-Mediated Cytokine Production and Growth of Endometrial Cells

The major component of the outer membrane of Gram-negative bacteria, endotoxin or 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

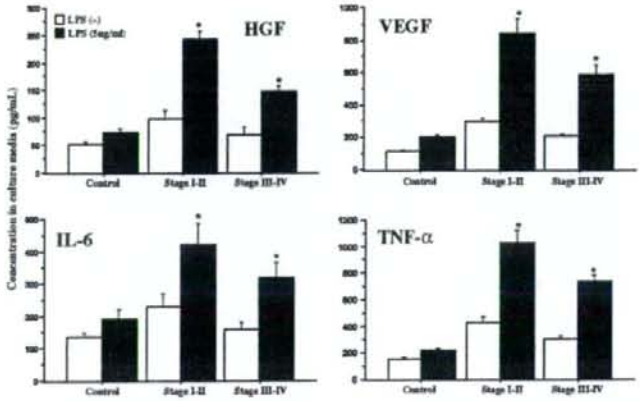

Fig. 7 Shows concentrations of hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), interleukin (IL)-6 and tumor necrosis factor-α (TNF-α) in the culture media of basal macrophages (non- lipopolysaccharide (LPS)-treated) and LPS-treated (5 ng/mL) macrophages and according to revised-ASRM staging of endometriosis. The asterisks denote the significant difference between stage I–II and stage III–IV endometriosis. The levels of HGF, VEGF, IL-6 and TNF-α in the LPS-treated culture media were significantly higher ($P < 0.05$ for all) in women with early endometriosis (stage I–II) than that of women with advanced endometriosis (stage III–IV) or control women. Although a substantial increase was observed, basal macrophages did not show any significant difference in all macromolecules levels between these two groups of women.

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 pre-treatment 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]

Bacterial Endotoxin (LPS) Concentrations in Different Body Fluids

As LPS or bacterial endotoxin acts as a primary inflammatory mediator and consequently stimulates immune cells for the production of secondary inflammatory mediators such as cytokines, chemokines, growth factors[9] but we do not know exactly about the presence of endotoxin in the pelvic environment of women with or without endometriosis. We measured endotoxin levels in the menstrual blood and corresponding PF of women with and without endometriosis. We found that the concentration of bacterial endotoxin is two- to fourfold higher in the menstrual fluid when compared with that in PF 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 PF 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 PF. 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*. 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 PF. 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]

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 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 aromatze 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 studies have already demonstrated the potential role of ovarian steroid hormones in the regeneration of endometrium after menstruation and the growth of endometriosis.[7,97] 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.[97,98] In fact, the association between ovarian steroid hormones and innate immune system is not well described.

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* are the most commonly isolated pathogenic bacteria from clinical uterine diseases in cattle and also in human vaginal cavity.[99,100] Possibly the ascending migration of *E. coli* towards endometrial cavity is possible might be causing 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.[82,100] For example, in humans, rodents and cattle, progesterone

suppresses uterine immune function by decreasing the proliferative capacity of lymphocytes as demonstrated earlier *in vitro*,[101] thereby increasing the susceptibility to bacterial infection.[98] 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.[100] 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.[98]

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.[102–104] 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[105,106] as well as immunoregulatory cytokine production.[107,108] 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[109] and to be associated with viral infection or enhanced tumor growth.[110,111] NK cell activity in women taking oral contraceptives is significantly lower compared to non-users.[112,113] Considering the effect of HRT on NK cell-mediated cytotoxicity, there exist only few and inconsistent reports with variable effects on NK cells.[106,114] 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.[115] 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,[116,117] which is most probably attributable 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.[118] 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.[119]

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.[120,121]

Estrogen (E2) and Progesterone (P) Levels in Different Body Fluids

We already reported increased immunoexpressions of estrogen and PRs in eutopic endometrium and ectopic endometrial lesions from our laboratory.[6] However, information regarding concentrations of estrogen (E2) and progesterone (P) in the 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.[122] Estrogen and P concentrations in the seminal fluid

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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 (Fig. 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 increased trend 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.

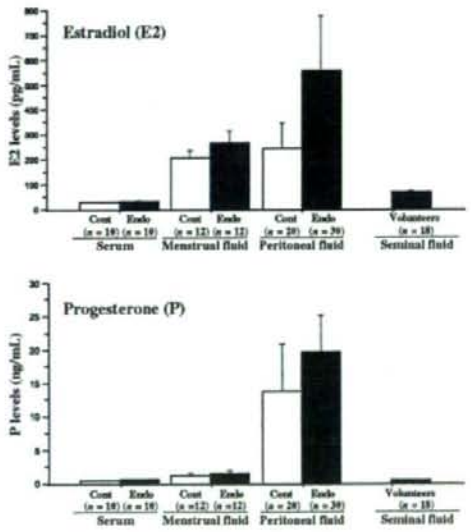

Fig. 8 Shows levels of estradiol (E2) and progesterone (P) in different body fluids collected from women with and without endometriosis. The level of ovarian steroids in seminal fluid was used as a positive control. A higher increase of E2 and P in the peritoneal fluid and menstrual fluid derived from women with endometriosis was observed when compared with corresponding levels in control women. cont, control women; endo, women with endometriosis.

Expression of E2 Receptor (ER) and P Receptor (PR) in Endometriosis

The expressions of ER and PR 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,[123,124] 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). ER 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.[125] 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.[126] found a glandular orifice adjacent to normal mesothelium, and Nakamura et al.[127] 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]

Expression of ERs and PRs in Macrophages

As mesenchymal cells retain ER, 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.

Several reports demonstrated that these inflammatory cells retain the mRNA encoding both ER and PR.[71,128] 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]

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 TNF-α by PF Mφ.[71,128] 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 TNF-α 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

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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.[129] As the major cellular constituents of PF are macrophages, comprising between 82% and 99% of the total cell population,[130] it is quite reasonable to speculate that these cells may be responsive to ovarian steroids.

Synergistic Effect Between HGF and E2 in the Growth of Endometriosis

The presence of c-Met receptor and ER in endometrial and endometriotic cells and Mφ might be expected to enable these cells to respond to endogenous or exogenous HGF and E2.[71,73,131] 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.

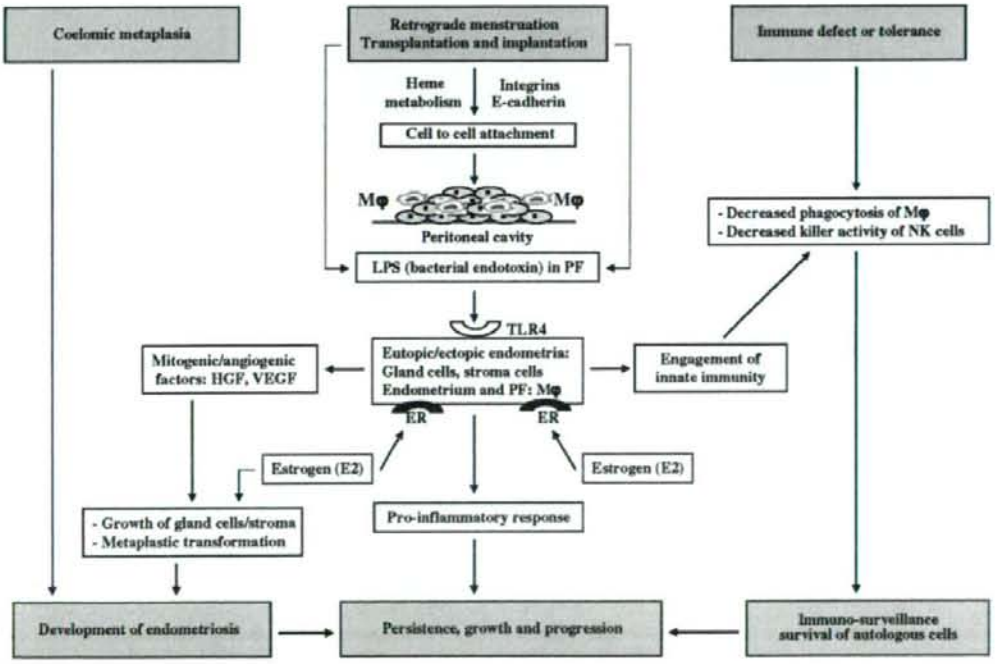

Fig. 9 Shows our proposed concepts on the immunopathogenesis of pelvic endometriosis by hepatocyte growth factor, bacterial endotoxin (LPS) via toll-like receptor 4, macrophages and ovarian steroid hormone (estrogen) and their cross-talk in the development, persistence and progression of endometriosis. The details of the development of early endometriosis by initial cell to cell contact and tissue invasion, the immuno-endocrine relationship and engagement of innate immune system in endometriosis are described in the text.

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 Fig. 9.

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] celomic metaplasia of the peritoneum as a secondary Mullerian system,[132,133] and compromised immuno-surveillance.[18–20] However, none of these theories can explain the pathogenesis of endometriosis consistently. 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 ensue 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 (Fig. 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 handling endometriosis.

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