Comprehensive Endometrial Immunoglobulin Subclass Analysis in Infertile Women Suffering from Repeated Implantation Failure with or without Chronic Endometritis

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Problem
Chronic endometritis (CE) is a local inflammatory condition with unusual plasmacyte infiltration in the endometrial stromal area. CE is frequently found in infertile women with repeated implantation failure (RIF). In this study, we comprehensively investigated the endometrial immunoglobulin (Ig) subclass expression in infertile women suffering from RIF with versus without CE.

Method of study
Endometrial biopsy specimens obtained from 28 infertile women with RIF and CE (the RIF-CE group), 23 infertile women with RIF but without CE (the RIF-non-CE group), and 22 proven fertile women undergoing hysterectomy for benign endometrial pathology (the control group) were immunostained for Ig subclass expression.

Results
The density of IgM+, IgA_1+, IgA_2+, IgG_1+, and IgG_2+ stromal cells were significantly higher in the RIF-CE group than that in the RIF-non-CE and control group. The density of IgG_2+ stromal cells was significantly higher than that of any other Ig subclass-positive cells ($P < 0.045$) in the RIF-CE group. In serial section staining, the immunoreactivity for CD138 and Ig subclasses in the endometrial stroma was detectable in adjacent cells of some specimens in the RIF-CE group.

Conclusions
The endometrium of infertile women with RIF-CE was characterized by increase in IgM, IgA, and IgG expression and predominance of IgG_2 over other Ig subclasses.

Introduction

Chronic endometritis (CE) is a histopathologic diagnosis recognized as unusual plasmacyte infiltrates within the endometrial stromal compartment.[1,2] The major cause of CE is microbial infection by common bacteria (including Escherichia coli, Streptococcus species, and Enterococcus faecalis) and Mycoplasma/Ureaplasma species.[3] This view is supported by the fact that the antibiotics with the spectrum activity against these microorganisms are effective in eradication of endometrial stromal plasmacytes in CE.[4,5] CE is usually asymptomatic or oligosymptomatic with nondescript gynecologic manifestations and is

prone to be unnoticed in clinical practice. Recent studies, however, demonstrated the association of CE with repeated implantation failure (RIF) following *in vitro* fertilization–embryo transfer, unexplained infertility, and unexplained recurrent pregnancy losses.[4–8]

Plasmacytes are antibody-producing immunocompetent cells of B cell lineage that appear in various chronic inflammatory lesions. Immature B cells can bear only antibody of immunoglobulin (Ig) M subclass, but encounter with specific antigens induces class-switch DNA recombination in these cells, elicits their differentiation into plasmacytes, and stimulates production of a variety of Ig subclasses.[9] Ig subclass expression by plasmacyte infiltrates depends on the types of inflammatory responses. For example, ulcerative colitis is recognized by accumulation of overwhelming number of IgG_1-bearing plasmacytes within the intestinal mucosa, whereas mucosal invasion by IgG_2-producing plasmacytes is one of the specific features seen in Crohn's colitis.[10] Meanwhile, higher level of IgA and IgM is detectable in mucosal plasmacytes with refractory interstitial cystitis.[11]

Despite that there is a growing body of knowledge on etiology and pathogenesis of CE, Ig subclass production by endometrial plasmacytes and its expression in the endometrium in this pathology remains poorly understood. Given that plasmacytes are absent in the non-pathologic human endometrium, these local unusual lymphocyte infiltrates and surrounding immunologic milieu in CE potentially pose negative impacts on endometrial receptivity. In this study, we aimed to characterize the Ig subclass expression comprehensively in the endometrium of infertile women suffering from RIF and CE.

Materials and methods

Definition of Repeated Implantation Failure

Morphologically good embryos were defined as day 3, grade 1 or 2, seven-to-eight-cell embryos according to Veeck's classification,[12] whereas morphologically good blastocysts were defined as day 5 blastocysts with score 3BB or above according to Gardner and Schoolcraft scoring system.[13] Serum pregnancy test (Evanet HCG; Nissui Pharmaceutical Inc., Tokyo, Japan) was performed on the eleventh day after transfer of day 3 embryos or on the ninth day after transfer of day 5 blastocysts. According to the manufacturer's guidance, the value <2 IU/L was regarded as a negative pregnancy test. RIF was defined as consecutive negative pregnancy tests following transfer of three or more morphologically good embryos and/or blastocysts.

Subjects

From January 2011 to December 2012, 179 infertile women met the inclusion criteria of RIF in our IVF center. Of them, 172 patients (96.1%) opted for the RIF workup program (hysteroscopy and local endometrial injury),[5] as these procedures have been shown to improve the pregnancy outcome in the following embryo/blastocyst transfer cycle.[14] Fluid hysteroscopy was performed on the days 6–12 of the menstrual cycle using a 3.1-mm-diameter flexible endoscope with the continuous flow system (Olympus, Tokyo, Japan). Local endometrial injury was performed using a 3-mm-wide curette (Atom Medical, Tokyo, Japan), and endometrial samples were retrieved from the curette tip. Under informed consent, the samples obtained from 28 infertile women with RIF complicated with CE (the RIF-CE group) and 23 infertile women with RIF but without CE (the RIF-non-CE group) were subjected to immunostaining for Ig subclass expression. The archival endometrial samples from 22 age- and body mass index-matched proven fertile women undergoing hysterectomy with benign endometrial pathology were used as control (Table S1). This study was approved by the Ethical Committee of the Oak Clinic Institutional Review Board (Approval Number 1250819081).

Immunohistochemistry

After being washed thoroughly in cold phosphate-buffered saline (pH 7.4), the endometrial samples were fixed overnight in 4% paraformaldehyde (in phosphate buffer, pH 7.3) and embedded in paraffin. They were cut into 4-μm sections and mounted onto 3-aminopropyltriethoxysilane-coated slides. After being dewaxed in limonen (Falma Inc., Tokyo, Japan) and rehydrated in a graded series of ethanol, the sections were subjected to antigen retrieval (Table S2) and then immersed in 3% hydrogen peroxide for 5 min to block endogenous peroxidase. After being washed, the sections were covered with 10% fetal calf serum (JRH Biosciences, Lenexa, KS, USA) for 10 min to suppress non-specific antibody binding. The sections were then incubated with either of primary antibodies (Table S2) or isotype-

matched control antibodies for 2 hr at room temperature. After being washed, the sections were incubated with horseradish peroxidase-conjugated secondary antibody (LSAB kit; Dako, Kyoto, Japan) for 30 min at room temperature, washed, and developed with diaminobenzidine (Dako). The sections were stained with hematoxylin for counterstaining. In some samples, the immunoreactivity of the individual antigens was determined by staining serial sections for CD138 and either of Ig subclasses. Immunoreactivity for each antigen was evaluated by two independent observers who were unconnected to the study. The density of immunoreactive cells was counted in 20 non-overlapping stromal areas under a light microscope (×400 magnification). CE was diagnosed according to the presence of plasmacytes based upon punctate immunostaining for CD138 in the stromal compartment and nucleic heterochromatin counterstaining.[2]

Statistics

Each dataset was analyzed for normal distribution using goodness-of-chi-squared fit test and compared with two-tailed Student's *t*-test, nonparametric Mann–Whitney *U*-test, Steel-Dwass test, or contingency tables in combination with Pearson's chi-squared test or Fisher's exact test. *P* values with <0.05 were considered as significantly different.

Results

Expression of Ig Subclasses in Endometrial Epithelium with or without CE

According to immunostaining for CD138 and nucleic heterochromatin pattern, CE was diagnosed in 59 (34.3%) of 172 patients undergoing RIF workup program. Due to the tissue volume, 28 samples from the RIF-CE group and 23 samples from the RIF-non-CE group were available for comprehensive Ig subclass analysis. Immunostaining for IgM, IgA_1, and IgA_2 was detected mainly on the apical side of the glandular epithelium in all endometrial samples examined (Fig. 1a). All glands were immunostained for IgA_1 and IgA_2 in each endometrial sample examined, regardless of the presence or absence of CE. By contrast, the proportion of the glands immunostained for IgM varied among the individual samples (36.3–100% in the RIF-CE group, 40.0–100% in the RIF-non-CE group, and 37.5–100% in the control group). There were no significant differences in the total percentage of the glands immunostained for IgM among the RIF-CE, RIF-non-CE, and control group ($P = 0.38$).

Meanwhile, immunostaining for IgG_1 or IgG_2 in endometrial epithelial cells showed marked intrasample variances. Immunostaining in epithelial cells for IgG_1 was detected mainly on the apical side of the endometrial glandular epithelium in 16 of 28 (57.1%) RIF-CE samples, 12 of 23 (52.2%) RIF-non-CE samples, and 14 of 22 (63.6%) control samples, whereas immunostaining in epithelial cells for IgG_2 was detected in 12 of 28 (42.9%) RIF-CE samples, 15 of 23 (65.2%) RIF-non-CE samples, and 11 of 22 (50.0%) control samples. The proportion of the glands immunostained for IgG_1 (~45.5% in the RIF-CE samples, ~37.5% in the RIF-non-CE samples, and ~40.0% in the control samples) and those immunostained for IgG_2 (~23.1% in the RIF-CE samples, ~27.3% in the RIF-non-CE samples, and ~28.6% in the control samples) also varied among the samples. There were no significant differences in the total percentage of the glands immunostained for IgG_1 or IgG_2 among the RIF-CE, RIF-non-CE, and control group ($P > 0.23$). On the contrary, immunostaining for IgG_3, IgG_4, IgE or IgD was not detectable in the endometrial epithelium in any of the samples examined.

Expression of Ig Subclasses in Endometrial Stromal Compartment with or without CE

Punctate immunostaining for IgM, IgA_1, IgA_2, IgG_1, and IgG_2 was scattered in the endometrial stromal compartment in all RIF-CE samples examined (Fig. 1a). Meanwhile, immunostaining for IgM, IgA_1, IgA_2, IgG_1, and IgG_2 was detected more sparsely in the endometrial stromal compartment in 13 of 23 (56.5%), 23 of 23 (100%), 23 of 23 (100%), nine of 23 (39.1%), and 11 of 23 (47.8%) RIF-non-CE samples, respectively. In serial section staining, the immunostaining for IgM, IgA_1, IgA_2, IgG_1, or IgG_2 was, at least in part, adjacent to that for CD138 in some endometrial stromal cells in the RIF-CE samples (Fig. 1b). By contrast, immunostaining for IgG_3 was detectable sparsely in the stromal compartment in one of 28 (3.6%) RIF-CE samples examined, one of 23 (4.3%) RIF-nonCE samples examined, and one of 22 (4.5%) control samples examined. Immunostaining for IgE was detectable only in one of 28 (3.6%) RIF-CE samples examined (data not shown). By contrast,

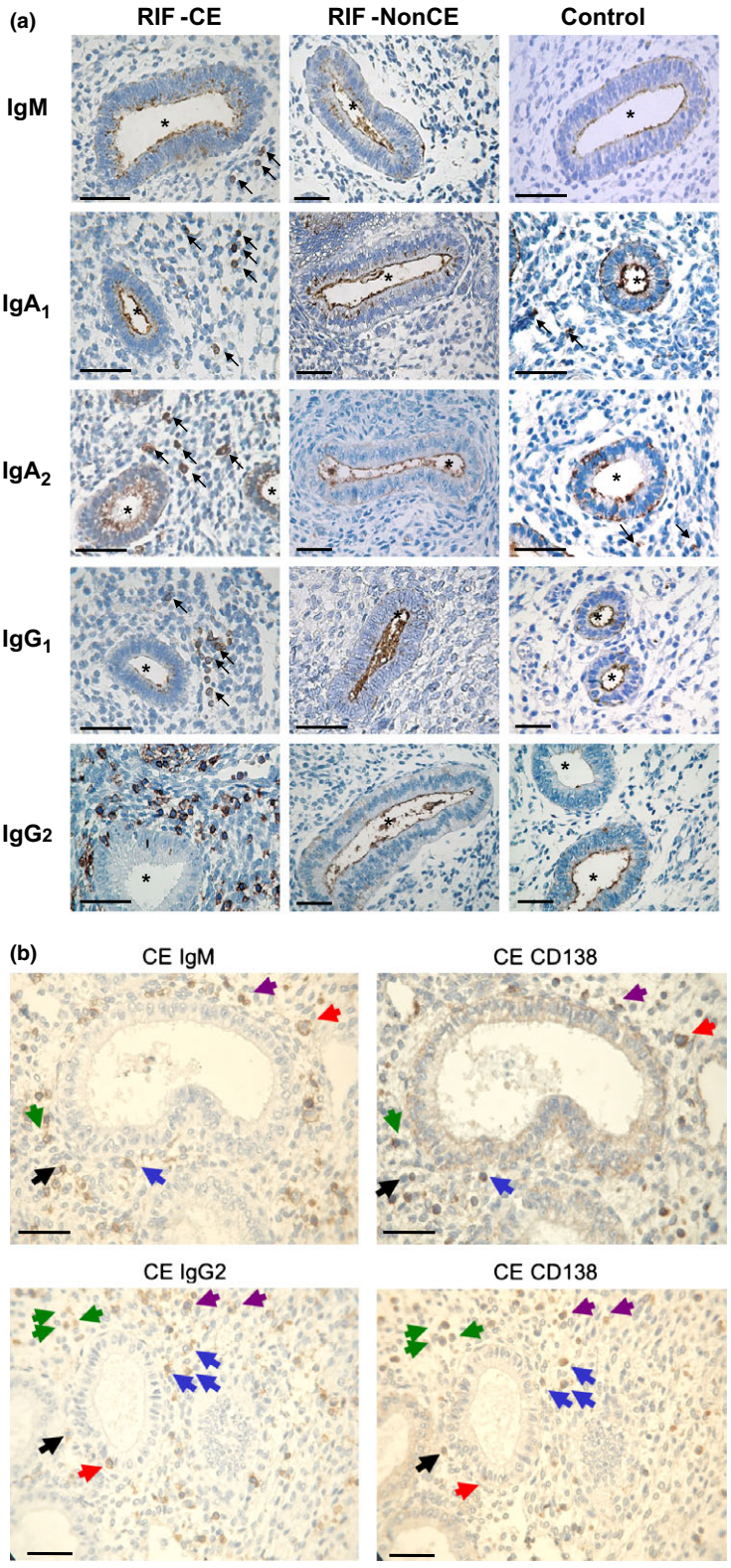

Fig. 1 Representative photographs of immunohistochemistry for Ig subclasses in the human endometrium. The scale bars indicate 50 μm. (a) Immunostaining for IgM, IgA_1, IgA_2, IgG_1, and IgG_2 in a representative RIF-CE, RIF-non-CE, and control sample. Asterisks indicate endometrial gland cavities. Arrows indicate immunoreactive stromal cells. (b) Serial section immunostaining for IgM and CD138 as well as IgG_2 and CD138 in the RIF-CE samples. The photos were overexposed to focus on the punctuate immunoreactivity in the endometrial stromal compartments. Arrows indicate immunoreactive stromal cells.

immunostaining for IgG_4 or IgD in the endometrial stromal compartment was not detectable in any of the endometrial samples examined.

The density of IgM+, IgA_1+, IgA_2+, IgG_1+, and IgG_2+ stromal cells was significantly higher in the RIF-CE samples than in the RIF-non-CE samples and control samples ($P < 0.0001$, Fig. 2). When the Ig subclass expression was compared within the RIF-CE samples, the density of IgG_2+ stromal cells alone was significantly higher than that of any other Ig subclass+ stromal cells ($P < 0.045$).

Discussion

Early reports showed the presence of Ig-bearing cells in the non-pathologic human endometrium. Endometrial stromal compartment contains IgG predominantly over IgM and IgA, whereas endometrial epithelial cells mainly bear IgA and IgM.[14] The endometrial Ig subclass profiling in CE, however, remains largely unexplored. In this study, we first demonstrate that the endometrium with CE was characterized by the predominance of IgG_2+ stromal cells over other Ig subclass+ stromal cells.

Local endometrial injury emerges as a promising medical intervention to improve the pregnancy outcome in infertile patients with RIF.[15] Taking advantages of the benefits of this procedure, we obtained the endometrium to search for stromal plasmacytes and Ig subclass expression in these patients during the proliferative phase, as this phase is superior in diagnosing CE to the secretory phase.[2] Using immunostaining for the plasmacyte marker CD138 and hematoxylin counterstaining, we identified CE in 34% of these biopsy samples obtained from women with RIF. Regardless of the presence or absence of CE, IgA_1 and IgA_2 were expressed constitutively in the epithelial component, implicating that IgA subclasses play a role in front line defense against foreign body invasion into this mucosal tissue.[14] In addition, all endometrial samples had some IgM+ epithelial cells. Meanwhile, we found that the endometrium with CE expresses IgM, IgA, IgG_1, and IgG_2 in the stromal compartment with intersample variance. The density of Ig-bearing endometrial stromal cells was significantly higher in CE than in non-CE. The results of serial section immunostaining support the idea that plasmacytes in CE lesions, at least in part, bear Ig subclasses.

Studies suggest that CE and endometriosis share some common inflammatory backgrounds in terms of local antibody-producing cell infiltration. For instance, the eutopic endometrium in both endometriosis and CE contains CD20+ B cells and CD138+ plasmacytes, which are rarely seen in this mucosal tissue under the non-pathologic conditions.[7,16] In addition, the eutopic endometrium in endometriosis was shown to express IgG at a high rate, but not IgM or IgA subclass.[17] However, the expression of IgM/IgA and CD138 in the serial sections of the CE samples suggests the potential differences in endogenous Ig subclass repertoire between CE and endometriosis. IgG_4-related disease is a systemic pathologic entity characterized by serum IgG_4 elevation, tissue fibrosis,

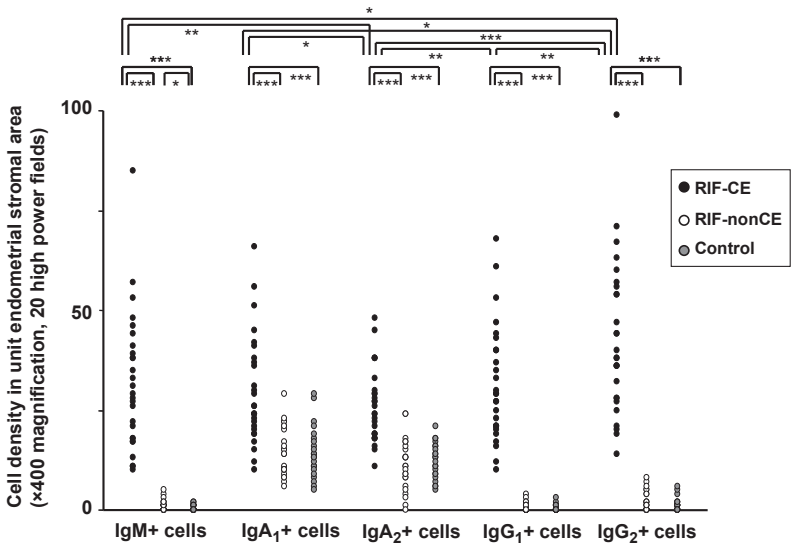

Fig. 2 Dotgram of IgM+, IgA_1+, IgA_2+, IgG_1+, and IgG_2+ cell density in endometrial stromal compartment of the RIF-CE, RIF-non-CE, and control samples. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ with Steel-Dwass test.

and infiltration of IgG_4-bearing plasmacytes into a wide range of organs.[18] We failed to detect IgG_4+ endometrial plasmacytes in any of our cohort, indicating that CE is unlikely to be associated with IgG_4-related diseases.

In conclusion, we demonstrate that the endometrium of the infertile women with RIF and CE expresses higher level of IgM, IgA, and IgG than that with RIF but without CE. This unique Ig subclass expression in CE, at least in part, is likely to result from *in situ* production of endometrial plasmacyte infiltrates. Formation of ectopic germinal center structures is a common finding seen in several chronic inflamed organs.[19] Given the massive B cell invasion into the endometrium in severe CE cases,[7] the presence of various Ig subclasses in the CE lesions implicates that local Ig class-switch recombination may possibly occur in this mucosa.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Demographics of infertile patients with RIF with or without CE and control fertile women.

Table S2. List of primary antibodies used for immunohistochemistry.