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Short Communication

High Level Estradiol Induces EBV Reactivation and EBV gp350/220(+)CD138(+) Double-positive B Cell Population in Graves' Disease Patients and Healthy Controls

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ABSTRACT

Graves' disease occurs predominantly in women. Epstein-Barr virus (EBV) mainly persists in human B lymphocytes, and its reactivation stimulates antibody production. We previously suggested that the EBV reactivation-induced production of TRAb and IgM at 100 nM estradiol (pregnant level) was lower than that at 0 nM estradiol and that class switch recombination may be increased by estradiol. In this study, we examined the effect of estradiol on EBV reactivation. We identified the expression of EBV-glycoprotein 350/220 (gp350/220) in the late phase of reactivation and plasma cell differentiation of EBV-infected cells using 72A1 antibody and CD138 antibody, respectively. We found the mean ratio of gp 350/220(+) CD138(+) cells at 100 nM estradiol was higher than that at 0 nM estradiol. These results suggested that EBV-infected cells could survive with keeping the ability of antibody production in 100 nM estradiol, which is consistent with the improvement of Graves' disease during maternity and exacerbation postpartum.

Key words anti-gp350/220 antibody (72A1); Epstein-Barr virus; estradiol; Graves' disease; reactivation

Most autoimmune diseases predominantly occur in women and are influenced by various factors.[1–5] Graves' disease, an autoimmune hyperthyroidism, often goes into remission during pregnancy and becomes aggravated after delivery.[6, 7] Estrogen regulates menstrual cycle and maternity and can stimulate antibody production from B cells.[5, 8] Estradiol is the most potent estrogen.

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Abbreviations: CSR, class switch recombination; gp350/220, EBV glycoprotein 350/220; EBV, Epstein-Barr virus; FCM, Flow-cytometry; Ig, immunoglobulin; LMP, latent membrane protein; PBMCs, peripheral blood mononuclear cells; TRAb, thyrotropin receptor antibody; TSHR, thyroid stimulating hormone receptor

Graves' disease is caused by thyrotropin receptor antibody (TRAb), which overstimulates the thyroid-stimulating hormone receptor. Nagata et al. reported that Graves' disease patients and healthy controls have Epstein-Barr virus (EBV)-infected lymphocytes with TRAb on their surface in human peripheral blood mononuclear cells (PBMCs); these PBMCs produce TRAb and the plasma cell population increases in response to reactivation of EBV in vitro.[9–11] In this study, we confirmed the presence of cells that were EBV-reactivated and plasma cell differentiated in healthy controls and Graves' disease patients, and examined the effect of estradiol on the ratio of these cells. We discussed the effects of pregnancy level of estradiol on EBV reactivation.

MATERIALS AND METHODS

Subjects

PBMC samples were obtained from 3 healthy controls and 4 Graves' disease patients diagnosed according to the guidelines of the Japan Thyroid Association (Table 1). All subjects provided written informed consent, and the study protocol was approved by the Medical Ethics Committee for Human Subject Research (No. 707, 707-1-13) at Faculty of Medicine, Tottori University, Yonago, Japan. The mean ages (± SD) of healthy controls and Graves' disease patients were 35.3 (± 19.85) years and 36.0 (± 4.24) years, respectively. One of the controls and all patients were female. Thyroid functions of the patients in Table 1 are clinical data. The levels of thyroid stimulating hormone (TSH), free thyroxine (FT4), and TRAb were measured by electro chemiluminescence immunoassay (ECLIA) (0.54–4.54 μIU/mL), ECLIA (0.97–1.72 ng/dL), and radio receptor assay (DYNOtest TRAb Human; Yamasa Corporation, Choshi, Japan) (1.0 IU/L >), respectively (normal range). Three patients were receiving treatment with antithyroid drugs (propylthiouracil). Control subjects were enrolled voluntarily, their thyroid functions were normal, and they had no family history of thyroid disease.

Table 1. Subject profiles

	No.	Sex	Age (years)	Treatment (for 1 day)	TSH (μIU/L)	FT4 (ng/dL)	TRAb (IU/L)	Allergy
Controls	1	F	27	–	nt	nt	nt	–
	2	M	58	–	nt	nt	nt	–
	3	M	21	–	nt	nt	nt	–
	Mean		35.3					
Patients	4	F	30	PTU 100 mg	2.03	1.32	3.3	–
(Graves' disease)	5	F	39	–	1.75	1.19	6.9	–
	6	F	36	PTU 50 mg	1.11	1.18	0.3 >	–
	7	F	39	PTU 150 mg	4.65	0.96	15.0	bronchial asthma, atopic dermatitis
	Mean		36.0		2.39	1.16	6.30	

F, female; FT4, free thyroxine; M, male; nt, not tested; PTU, propylthiouracil; TRAb, thyrotropin receptor antibody; TSH, thyroid stimulating hormone.

PBMC preparation

Peripheral blood samples were obtained from Graves' disease patients and healthy controls. PBMCs were separated using a Ficoll-Conray density gradient and stored at –80 °C until use.

Estradiol

17β-estradiol (Sigma-Aldrich, Saint Louis, MO) was dissolved in ethanol to prepare a 1 mg/mL stock solution and stored at 4 °C until use. Estradiol was added to the medium and maintained at 0 nM, 1 nM, or 100 nM, which corresponds to control, mid-luteal, or pregnancy levels, respectively.

Sampling protocol

PBMCs were cultured at 37 °C for 2 days in RPMI 1640/10% FBS with cyclosporine A (0.1 μg/mL) to inhibit T cell effects. PBMCs were transferred to an incubator at 33 °C to induce EBV reactivation and were regarded as day 0 samples. On days 0, 5, 10, and 12, half of the culture supernatant was sampled and replaced by fresh medium with estradiol, and the estradiol concentration was maintained at 0 nM, 1 nM, or 100 nM.

Flowcytometry (FCM) and confocal laser scanning microscopy (CLSM)

On day 12 after EBV reactivation induction, the collected PBMCs of 3 healthy controls (No. 1–3) and 4 Graves' disease patients (No. 4–7) were fixed by 2% paraformaldehyde and stored at 4 °C until use. We confirmed expression of EBV glycoprotein 350/220 (gp350/220) with anti-gp350/220 antibodies (72A1) and plasma cells with CD138 antibodies by FCM (No. 1–3 controls and No. 4–6 patients) and CLSM (No. 7 patient). gp350/220 binds to CD21/CR2 on the B cell surface. gp350/220 is a late phase protein of EBV reactivation and is also detected on the host cell membrane. We used the primary mouse monoclonal antibody 72A1 (Millipore, Billenca, MA) to detect gp350/220 and goat anti-mouse IgG H&L (Alexa Fluor 488) (Abcam, Cambridge, Britain) as a secondary antibody. Differentiation to plasma cells was detected by adding anti-human CD138 mouse IgG-Alexa Fluor 647 conjugated (AbD Serotec, Raleigh, NC). FCM analysis was performed using a Gallios analyzer (Beckman Coulter, Fullerton, CA). Gp350/220(+) CD138(+) cells were sorted with the cell sorter MoFlo XDP (Beckman Coulter) and examined by CLSM with TCS SP-2 (Leica, Wetzlar, Germany).

Statistical analysis

We adopted one-way ANOVA (SPSS statistics, IBM, Armonk, NY) to analyze the variation of the mean ratio of gp350/220(+) CD138(+) cell percentages.

RESULTS

Culture cells on day 12 after EBV reactivation contained gp350/220(+) CD138(+) double-positive cells

We detected gp350/220(+) CD138(+) double-positive cells in PBMCs of healthy controls (No. 1–3) and patients (No. 4–6) by FCM (Fig. 1). No. 2 and No. 3 are additional male subjects for FCM. The flowcytometer displays the cell percentages as the relative number against the lymphocyte population. To compare the effects of different estradiol concentrations, we examined the ratios of cell percentages at each estradiol concentration to that at 0 nM estradiol. In healthy controls and patients, the ratio of gp35/220(+) CD138(+) double-positive cells in culture with 100 nM estradiol was larger than

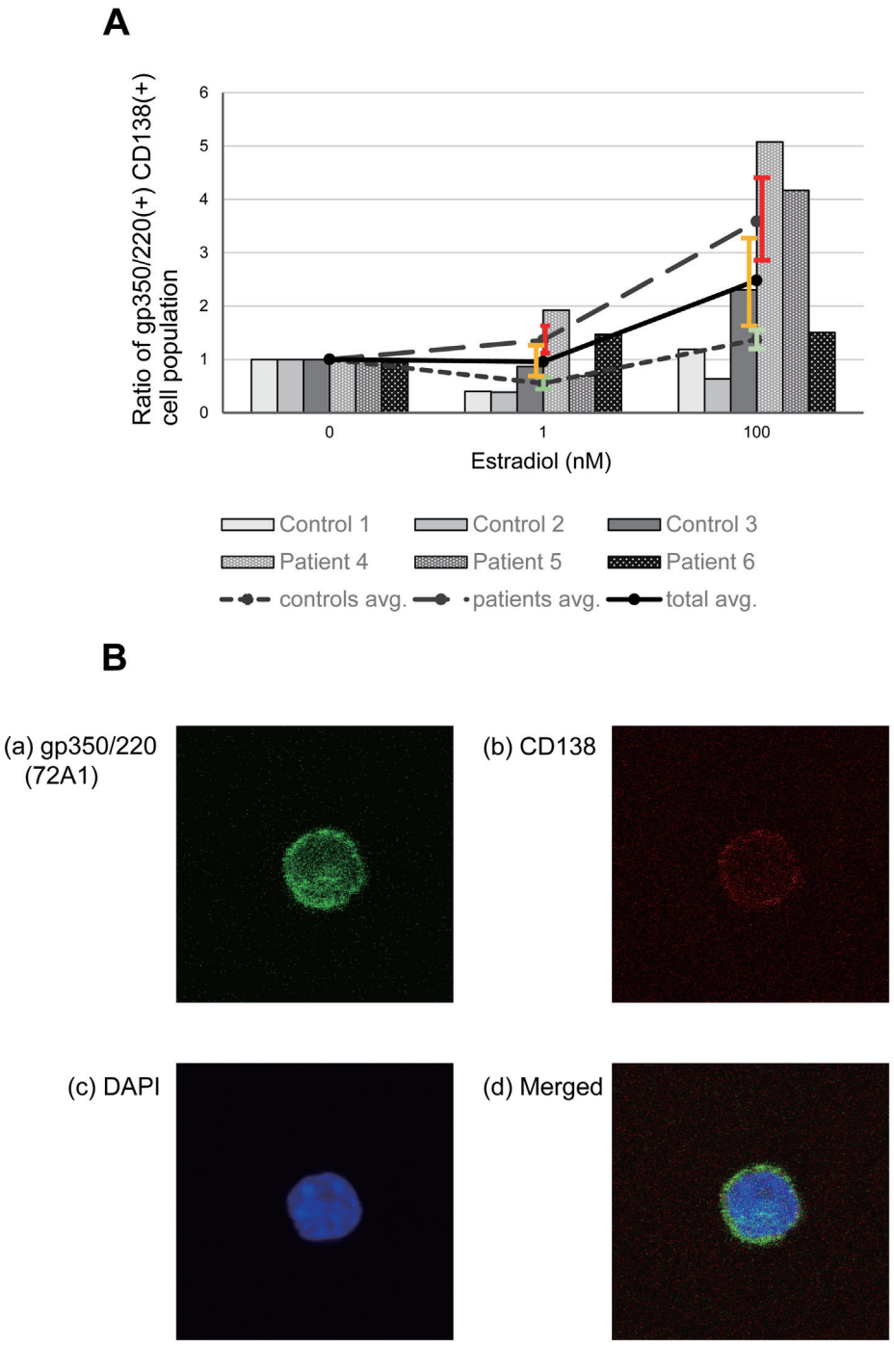

Fig. 1. Detection of gp350/220(+) CD138(+) double-positive cells in culture cells on day 12.
(**A**) Analysis of the population of gp350/220(+) CD138(+) double-positive cells in culture cells on day 12 by FCM (ratios of cell percentage at each estradiol concentration to that at 0 nM estradiol). No. 1–3 are controls, and No. 4–6 are patients. Two controls (No. 2 and 3) were male subjects for FCM. The bar chart shows the ratio of gp350/220(+) CD138(+) double-positive cells. The line graph showed the averages +/− standard error of the ratio of gp350/220(+) CD138(+) double-positive cells of 3 controls and 3 patients. Gp350/220(+) CD138(+) double-positive cell population was increased by addition of 100 nM estradiol ($P = 0.055$ in one-way ANOVA).
(**B**) Detection of gp350/220(+) CD138(+) double-positive cells in culture cells on day 12 using CLSM. Culture cells of patient No. 7 expressing gp350/220 in the cytoplasm and cell surface and CD138 on the surface. (a) Alexa Fluor488 (green) representing EBV gp350/220 was detected overall of the cells. 72A1 is the antibody against gp350/220. (b) Alexa648 (red spots) representing CD138 was observed on the surface of the cells. (c) DAPI (blue) represents the cell nucleus. (d) Merge of (a), (b), and (c).
Avg., average; CLSM, confocal laser scanning microscopy; DAPI, 4', 6-Diamidino-2-phenylindole; FCM, Flowcytometry; Gp350/220, glycoprotein 350/220.

that of cultures with 0 nM or 1 nM estradiol ($P = 0.055$) (Fig. 1A).

We observed gp350/220(+) CD138(+) double-positive cells by confocal laser microscope, and noted red spots of CD138 on cell surfaces and fine green dots of 72A1 in the cytoplasm and cell surfaces of patient No. 7 (Fig. 1B).

DISCUSSION

We found that the mean ratio of gp350/220(+) CD138(+) cells at 100 nM estradiol was higher than that at 0 nM estradiol. The result of CLSM indicated the presence of EBV-reactivated and plasma cell differentiated cells (Fig. 1). However, in our previous study, production of TRAb and IgM at 100 nM estradiol is lower than that at 0 nM estradiol,[12] which seems to contradict our current data, but we often observed abundant antibodies in culture supernatant in plate wells with only a few cells. In our previous study (0 nM estradiol), the percentage of gp350/220(+) cells or CD138(+) cells increased at day 12 compared with day 0 of reactivation.[10, 11] However, in the present study, the ratio of gp350/220(+) CD138(+) cells further increased on day 12 in culture with 100 nM estradiol, which suggests that there were less dead cells that had completed releasing antibodies than that at 0 nM estradiol. EBV-infected cells may survive with keeping the ability of antibody production in 100 nM estradiol culture.

EBV-infected cells could survive with keeping the ability of antibody production at high concentrations of estradiol, which suggests that EBV induces a survival signal. For instance, EBV-latent membrane protein 1 activates nuclear factor κB and induces the expression of anti-apoptotic B-cell lymphoma 2 (*Bcl-2*). Bcl-2 proteins are related to the survival of gp350/220(+) CD138(+) cells, and further experiments are needed to confirm this possibility.

We found that EBV-reactivated and plasma cell differentiated cell percentages increased under high estradiol concentrations, which could result in decreasing production of antibodies. If estradiol concentration decreased again, cells may produce antibodies. The present results and our previous results showing that high estradiol concentrations decrease TRAb and IgM production are consistent with the clinical observations of improvement of Graves' disease during maternity and exacerbation postpartum.[6, 12, 13]

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