Apoptosis Induction of Mouse Splenic Cells by Exposure to High-Level 17β-Estradiol and Endocrine Disrupting Chemicals

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The immunotoxicological effect of 17β-estradiol and other estrogenic compounds was determined with respect to lymphocyte apoptosis. High concentrations (10^{-6}–10^{-5} M) of 17β-estradiol inhibited lymphocyte proliferation. DNA fragmentation was observed by agarose gel electrophoresis when lymphocytes were exposed to 17β-estradiol, confirming the occurrence of apoptosis. After exposure to 17β-estradiol, the lymphocytes underwent apoptosis within 12 hr of culture through three sequential alterations of membrane structure, as judged by the results of staining with three kinds of dyes, namely annexin V, which binds on the surface of the cell membrane, and propidium iodide and trypan blue, which cross the cell membrane. Diethylstilbestrol killed more cells whereas bisphenol A, a weak estrogen, induced apoptosis in fewer cells. These results suggest that 17β-estradiol and other estrogenic compounds induce lymphocyte apoptosis.

Key words —— 17β-estradiol, lymphocyte, apoptosis, endocrine disrupting chemicals

INTRODUCTION

It is commonly known that the sex hormones play important roles not only in the sexual organs, but also in the nervous system[1–3] and immune systems.[4,5] 17β-Estradiol affects the differentiation and the maturation of T cells in the thymus[6,7] and B cells in the bone marrow,[8] which are concerned with the homeostasis of variable immune actions[9–11] such as the weight of the spleen and thymus, the secretion of thymic hormones,[12] the secretion of cytokines and the production of antibodies.[13]

A number of endocrine disrupting chemicals (EDCs) exist in the environment and have hormonal, mainly estrogenic, effects. Because endogenous 17β-estradiol modulates the immune functions as mentioned above, EDCs may be also assumed to affect the immune system as well. However, little is known about their modification of immune responses because EDCs have usually been studied in respect to developmental toxicity, teratogenicity or influence on sexual organs.

The lymphocyte mitogenesis test is one of the *in vitro* immunotoxicity tests used to evaluate the safety of drugs.[14,15] In this test, lymphocytes are stimulated by a polyclonal mitogen specific for either B cells or T cells as an indication of humoral immunity or cell-mediated immunity, respectively. We preliminarily evaluated the effect of 17β-estradiol on the immune function using this lymphocyte mitogenesis test and found that this chemical suppressed the lymphocyte proliferation. We further investigated the immunosuppressive activity of estrogenic compounds focused on cell death. Apoptosis, which refers to programmed death, is supposed to be triggered by cellular functional mechanisms, and necrosis is supposed to result from extrinsic damage.

Apoptosis is achieved through several steps.[16,17] A Fas ligand[18] binds to the Fas protein on the surface of the cell, then several kinases and proteases including caspase[19,20] are activated, and finally endonucleases[21] cut the DNA between nucleosomes to make fragments in lengths of some integer times 180 base pairs.[22] Then, the electrophoresed genomic DNA will show a ladder-like pattern. At apoptosis, cell membrane structure also changes. Phosphatidyl serine, which usually exists on the inner side of the

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lipid bilayer, is able to flip-flop into outer side.[23] Other events occur when a cell undergoes apoptosis. The alteration of membrane structure may enhance membrane permeability, and pigments such as propidium iodide (PI) or trypan blue are able to enter the cell.

Lymphocytes undergo apoptosis when they are exposed to oxidative stress, heavy metals or ultraviolet irradiation. Lymphocyte apoptosis also occurs in the process of lymphocyte maturation. If this mechanism is disordered, it may cause autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) which occur more frequently in women.[5,24–26] If estrogenic compounds modulate apoptosis of lymphocytes, they may cause autoimmune diseases.

We determined whether the cell death induced by 17β-estradiol is apoptosis or necrosis. To determine the effects of estrogenic compounds on apoptosis of splenic cells, cells were exposed to the chemicals, and DNA fragmentation and alterations of lipid composition of the cell membrane were detected as typical indications of apoptosis, and the permeability of PI and trypan blue across cell membranes was detected as an indication of cell death.

MATERIALS AND METHODS

Male BALB/c mice, 5 weeks old, were purchased from Japan SLC Co. (Shizuoka, Japan). The mice were kept in a room maintained at 23 ± 1°C with 47–67% relative humidity for 1 week. 17β-estradiol, lipopolysaccharide (LPS), concanavalin A (Con A) and phenol-red-free RPMI-1640 medium were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other reagents were of the highest grade commercially available.

Lymphocyte Mitogenesis Test —— The lymphocyte mitogenesis test was conducted as previously reported.[14,15] The spleen was aseptically isolated, and the splenic cells were flushed out by injecting 10 ml of RPMI-1640 medium with a syringe into the spleen. The RPMI-1640 medium was supplemented by 5 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES), 50 μM 2-mercaptoethanol, 50 mg/l potassium benzylpenicillin, 50 mg/l streptomycin sulfate, 0.18% $NaHCO_3$ and 10% fetal calf serum. The suspended cells were left for 2–3 min on ice to precipitate and remove fragments of connective tissue, and then cells were washed twice with the medium and counted with a hematocytometer. The cells were dispensed into each well of a 96-well flat bottom polystyrene multiwell plate (Thermo Labsystems Oy, Helsinki, Finland) at 10^5 cells/well in 200 μl of the medium. As the mitogen, 100 μg/ml LPS was added for B cell mitogenesis or 2 μg/ml Con A for T cell mitogenesis. The 17β-estradiol and EDCs were dissolved in dimethylsulfoxide (DMSO) at the concentration of 10–200 mmol/l. All samples were sterilized by filtration and stored at –20°C. The EDCs were serially diluted with DMSO and applied to the cells. The final concentration of DMSO was limited to 0.1%, at which DMSO only weakly suppresses lymphocyte mitogenesis. The plates were incubated at 37°C in 5% CO_2. After 4 days of cultivation, the total amount of DNA in grown cells was determined by the ethidium bromide fluorescence method.[14,27] The plate was centrifuged at 4°C, 500 × g for 10 min, and the supernatants were removed. After the cells were washed once with 200 μl of PBS, 200 μl of 0.1% sodium dodecyl sulfate (SDS) was added and left for 30 min to lyse the cells. Then, 100 μl of 50 μg/ml ethidium bromide was added to each well, left for 15 min and the complex formed by DNA and ethidium bromide was determined fluorometrically at Em 620 nm and Ex 515 nm as an indication of cell number. The stimulation index (SI) was calculated as below.

$$SI = (E - E_0)/(E' - E_0)$$

E : fluorescence intensity of DNA of cells exposed to the mitogen.

E': fluorescence intensity of DNA of cells not exposed to the mitogen.

E_0: background fluorescence intensity.

Genomic DNA Electrophoresis —— Splenic cells (1×10^6 cells/dish) were incubated at 37°C, being exposed to estradiol. Cells were harvested, transferred to tubes and centrifuged; the medium was removed. To the harvested cells, 200 μl of PBS, 20 μl of 10% SDS and 1 μl of 10 mg/ml RNase A were added, and left at 37°C for 30 min to lyse cells and degrade RNA. To dissolve the proteins, 10 μl of 10 mg/ml of proteinase K was added and left at 37°C for 30 min. After 300 μl of NaI solution [6 M NaI, 0.5% sodium-*N*-lauroylsarcosinate, 10 mg/ml glycogen, 26 mM Tris–HCl (pH 8.0) and 13 mM EDTA] were added, the samples were incubated at 60°C for 15 min. After precipitation and washing with 2-propanol, DNA was dissolved in 15 μl of Tris–EDTA buffer, and electrophoresed using 2% agarose gel at

100 V for 30 min.

Flow Cytometric Analysis with Annexin V and PI Staining —— Splenic cells (5×10^6 cells/dish) were cultured for 0–12 hr and harvested to tubes. Erythrocytes were removed by hemolysis. Cells were suspended in 1 ml of NH_4Cl buffer, left for 10 min at room temperature, and 10 ml of PBS added. The tubes were centrifuged at $500 \times g$ for 10 min, and the supernatant was removed. The cells were washed with 10 ml of PBS and resuspended in PBS, adjusting the cell concentration to 1×10^7 cells/ml. Annexin V staining was done with fluorescein isothiocyanate (FITC)-conjugated annexin V and PI from an ApoAlert Annexin V kit (Clontech Laboratories Inc., Palo Alto, CA, U.S.A.) following the instructions provided by the manufacturer. Cells were washed with the binding buffer of the kit, and resuspended in 200 μl of the binding buffer. Annexin V solution and PI solution were added, and cells were left for 10 min at room temperature. Finally, cells were analyzed by a flow cytometer (Jasco Co., Tokyo, Japan) following the manufacturer's operating guide. Each examination was exerted 3–5 times and the representative data were shown.

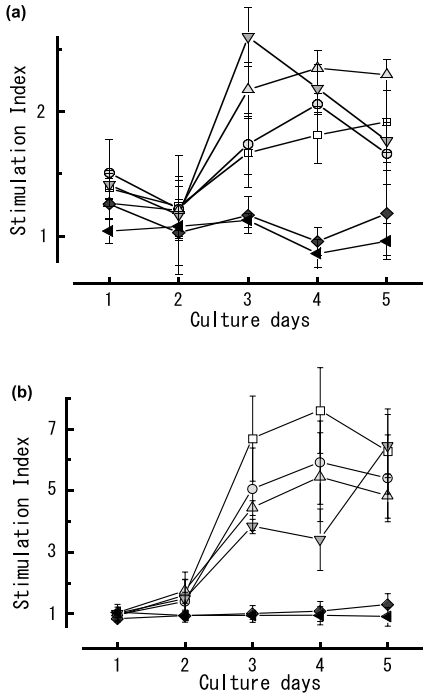

Fig. 1. Lymphocyte Mitogenesis was Suppressed by 17β-Estradiol during 5 days Cultivation

The concentrations of 17β-estradiol were: □ no addition, ● 1×10^{-9} M, ▲ 1×10^{-8} M, ▽ 1×10^{-7} M, ◆ 1×10^{-6} M, ◀ 1×10^{-5} M. (a) Lymphocytes were stimulated by LPS. (b) Lymphocytes were stimulated by Con A.

RESULTS AND DISCUSSION

Lymphocytes of mouse spleen were exposed to 17β-estradiol under growth stimulation by LPS or Con A as a polyclonal mitogen specific for either B cells or T cells, respectively. After cultivation for 5 days, the extent of cell growth was calculated. Figure 1 is the time course of suppression of mitogenesis by each concentration of 17β-estradiol. The mitogenesis occurred in 2–3 days when cells were exposed to no 17β-estradiol. Low concentrations (10^{-9}–10^{-8} M) of 17β-estradiol did not modulate the lymphocyte mitogenesis. However, high concentrations (10^{-6}–10^{-5} M) of 17β-estradiol inhibited the lymphocyte proliferation within 2 days of cultivation. High concentrations of 17β-estradiol are seen when 17β-estradiol is administrated for therapeutic use. Besides therapeutic administration, lymphocytes can be exposed to high levels of 17β-estradiol. 17β-Estradiol from river water[28,29] might be biologically concentrated through the food chain and consumed by humans. Therefore, it is possible that human lymphocytes are exposed to high levels of estrogenic compounds and lymphocyte mitogenesis is suppressed.

We hypothesized that the inhibition of mitogenesis is due to lymphocyte death, and further investigated the immunosuppressive activity of estrogenic compounds by focusing on it. Lymphocytes die by apoptosis in several situations, such as negative selection in the thymus and the end of immune response. We then determined whether the cell death induced by 17β-estradiol is apoptosis or necrosis because the death of lymphocytes exposed to high levels of 17β-estradiol is probably due to its toxicity. As shown in Fig. 2, the characteristic ladder-like pattern that is one of the typical characters of apoptosis was observed in electrophoresed DNA fragments. These facts confirmed the occurrence of lymphocyte apoptosis.

Because it was difficult to estimate the frequency of apoptosis by genomic DNA electrophoresis method, frequency of lymphocyte apoptosis was assessed by more quantitative experiment, utilizing flow cytometry. After cultivation, cells were retrieved and placed in a tube, and erythrocytes were removed by hemolysis. We did not use density-gradient centrifugation because living lymphocytes are separated from dead ones in this method, but we wanted to calculate percentages of both living cells

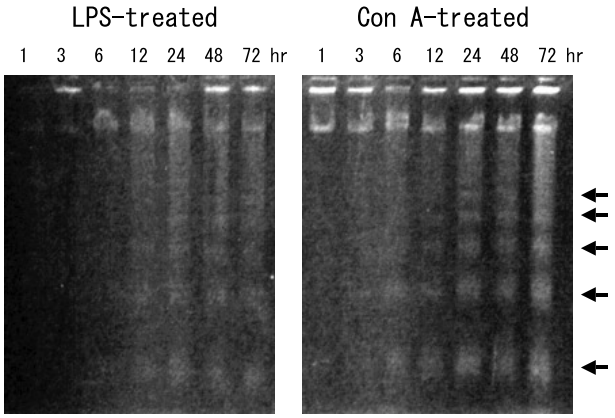

Fig. 2. The Splenic Cells were Exposed to LPS or Con A and 10^{-5} M of 17β-Estradiol and Incubated for the Indicated Period

The genomic DNA was extracted and electrophoresed. DNA of cells was cut regularly and ladders were observed. The representative results are shown.

and dead cells in the total cells. Figure 3a shows the forward-scattered light plotted along the X-axis and the side-scattered light along the Y-axis; approximately three regions were observed. It had been preliminarily confirmed that lymphocytes were plotted in region A. Because the intensity of forward-scattered light is generally accepted to indicate cell size, the cells plotted in region B were smaller cells than region A, and much smaller cells were plotted in region C. The cells plotted in regions A, B, and C were suspected to be living lymphocytes, dead lymphocytes and debris, respectively. We isolated the mononuclear cell fraction from the precipitate fraction by density-gradient centrifugation using Lympholyte-M (Cedarlane Laboratories Ltd., Hornby, ON, Canada) according to the manufacturer's protocol; then erythrocytes were removed from both fractions by hemolysis and similarly analyzed by flow cytometry. From the mononuclear cell fraction we obtained living lymphocytes (plotted in region A) and dying cells (plotted in region B), and neither of these cells was stained by trypan blue (data not shown). However, the dying cells were stained by PI, whereas the living cells were not (Fig. 3b). Only debris was obtained from the precipitate fraction of density-gradient centrifugation (data not shown), and this debris was stained by trypan blue (data not shown). Moreover, PI-stained cells plotted in region C at lower intensity than the region B cells (Fig. 3b). Therefore, the cells plotted in region C in Fig. 3 were supposed to the remnants of dead cells that had already leaked DNA fragments. We stained the cells with FITC-conjugated annexin V and measured the fluorescence of each cell by flow cytometry, gating both the living and the dying lymphocytes (in Fig. 3c). Annexin V is a specific phosphatidyl serine-binding protein that is not able to bind to normal living cells but binds to apoptotic cells because an apoptotic cell has the annexin V-binding target on its surface. If annexin V has been conjugated with a fluorescent dye such as FITC, apoptotic cells will be detected by a fluorometric technique, namely flow cytometry.[30–32] Data are also shown in Fig. 3d, plotting the green fluorescence intensity of FITC along the X-axis and the red fluorescence intensity of PI along the Y-axis. The dying lymphocytes were stained by FITC-annexin V (Figs. 3c and 3d). Most of the living lymphocytes were not stained by FITC, but some of them were (Figs. 3c and 3d). The unstained cells were named region A1 cells and the stained cells region A2 cells (Fig. 3d). Cells in the early phase of apoptosis must distribute to region A2. Annexin V perhaps binds to the constitutive phosphatidyl serine of normal living cells, or background binding. Table 1 summarizes the dye staining of the cells. The cells went from A1 through A2 and B, and finally to C.

To confirm sequential changes of flow cytometric phenotype in Table 1, we estimated the time course of cell death when splenic cells were exposed to 17β-estradiol under growth stimulation with LPS and Con A, respectively. In Fig. 4, the percentages of groups A1, A2, B and C to all cells are shown. The cells, which were exposed to mitogen and 17β-estradiol, transited from A1 (living), through A2 (early apoptotic phase) and B (late apoptotic phase), to C (debris). Same results were seen about cells that were exposed to only 17β-estradiol (data not shown).

In the annexin V and PI staining, few cells showed a typical apoptotic pattern (region A2). In the early apoptotic cells, annexin V bound to phosphatidyl serine induced on the outside of the cell membrane lipid bilayer, and PI did not penetrate the membrane. We detected apoptosis by the DNA ladder method but detected only a few cells by flow cytometry. It was supposed that very rapid alteration of the membrane lets PI penetrate it. Phosphatidyl serine flip-flopped to the outside of the membrane, membrane permeability rapidly changed, and PI (Molecular weight = 668.40) entered the cells. Later, the next change in the membrane constitution occurred, and trypan blue (Molecular weight = 976.84) also entered the cells. While these events occurred,

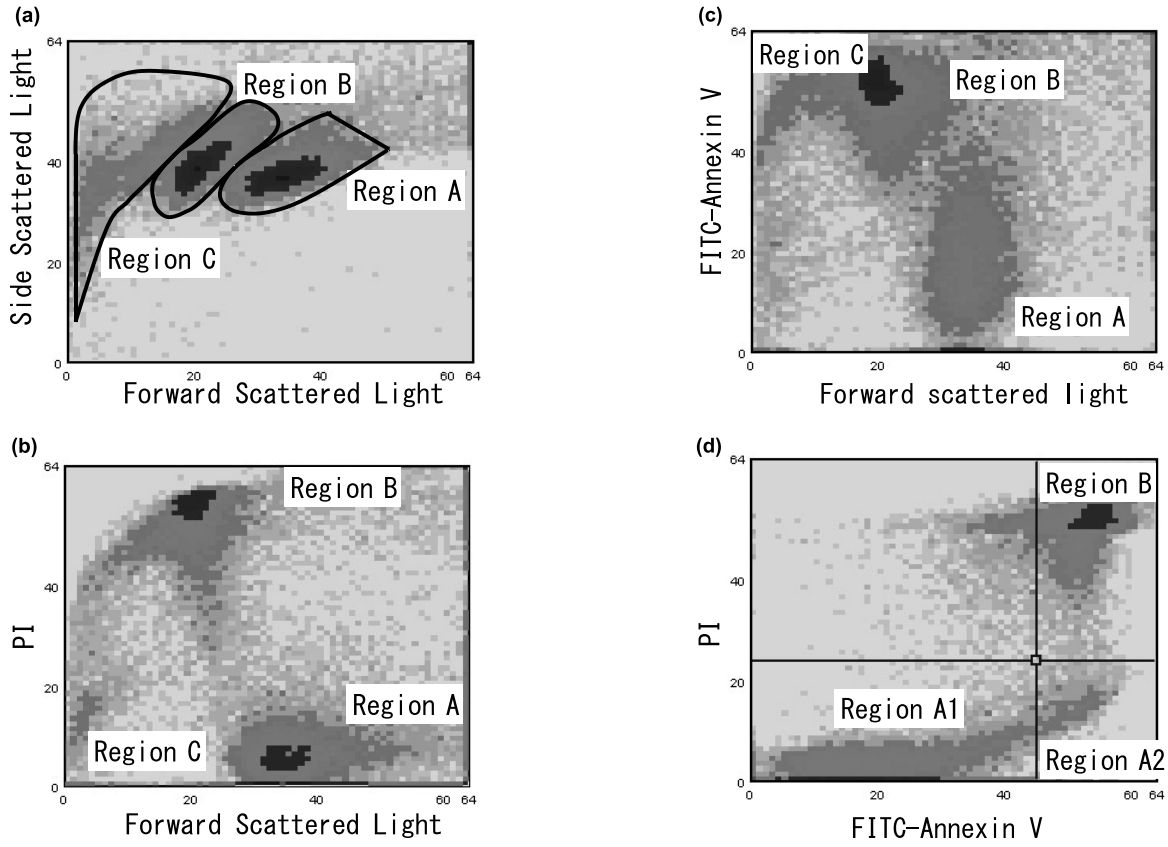

Fig. 3. Flow Cytometric Analysis of Cell Death

Representative results are shown. (a) Approximately 3 regions were observed: A, B and C. (b) The cells plotted in region A were not stained by PI, but cells in region B were. (c) Most of the cells in region A were not stained by annexin V, but cells in region B were. (d) The cells in region A1 were not stained by either annexin V or PI. The cells in region A2 were stained by annexin V but not by PI. These cells were suspected to be apoptotic. The cells in region B were stained by both annexin V and PI.

Table 1. Summary of the Relationship between the Flow Cytometric Phenotype (Figs. 3a and 3d) and the Results of Dye Staining (Figs. 3b, 3c, and 4), and the Proposed Sequence of Steps in Cell Death

Flow cytometric phenotype	Density gradient centrifugation	Trypan blue staining	PI staining	Annexin V staining	The sequential steps of cell death
A1	Mononuclear cell fraction	−	−	−	Alive
A2	Mononuclear cell fraction	−	−	+	In early apoptosis
B	Mononuclear cell fraction	−	+	+	In late apoptosis
C	Precipitate	+	±	+	Debris

the genomic DNA was cut by each nucleosome and the fragments existed stably (Fig. 2). The membranes of lymphocytes exposed to 17β-estradiol rapidly shifted from the early apoptotic stage to the late stage of cell death.

In the flow cytometric examination, the intensity of forward-scattered light is usually accepted to indicate cell size, but the construction of the cell membrane also modifies it. In Table 1, the shift of regions in flow cytometry plotting exactly correlated with the pattern of pigment staining, or the translocation of phosphatidyl serine and the membrane permeability for PI and exclusion of trypan blue. The cells in region C were confirmed to be not yet divided into apoptotic bodies by microscopic examination (data not shown). The shifting from region A via B to C (Fig. 3) might be attributed to alteration of the membrane structure in some stages of apoptotic cell death.

The dose–response relationship between the 17β-estradiol concentration and lymphocyte death was estimated. Figure 5 shows the percentages of cells

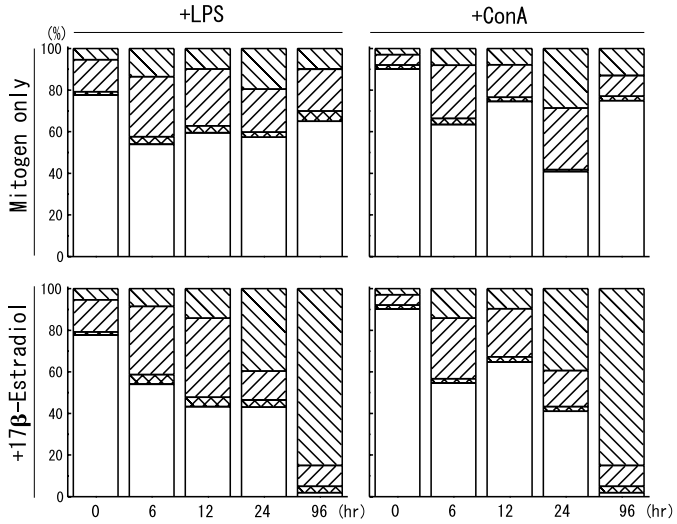

Fig. 4. The Time Course of Cell Death due to 1×10^{-5} M of 17β-Estradiol
□: Living cells. ▨: Early apoptotic cells. ▨: Late apoptotic cells. ▨: Debris.

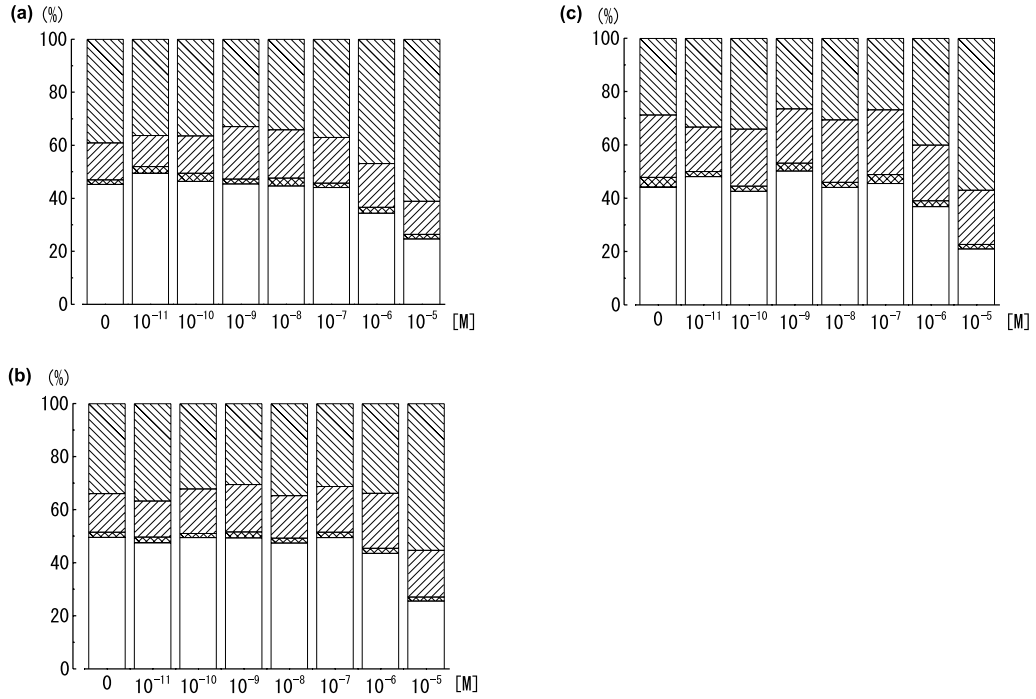

Fig. 5. Dose–Response Relationship of the Concentration of 17β-Estradiol and Cell Distributions
Cells were exposed to 17β-estradiol for 12 hr. (a) Cells were not stimulated any mitogen. (b) Cells were stimulated by LPS. (c) Cells were stimulated by Con A. □: Living cells. ▨: Early apoptotic cells. ▨: Late apoptotic cells. ▨: Debris.

distributed to regions A1, A2, B and C to all cells. Lower concentrations (10^{-9}–10^{-8} M) of 17β-estradiol might slightly increase the cells not stimulated by mitogen in the late phase of apoptosis. Some previous reports[33,34] said that the low-level 17β-estradiol suppressed lymphocyte apoptosis. It might be suggested that 17β-estradiol prevents the change of membrane conformation between the late apoptotic phase and the final debris phase. High-level (10^{-6}–10^{-5} M) 17β-estradiol induced the debris, dead lymphocytes. It was reported that 2-methoxyestradiol, one of the metabolites of 17β-estradiol, induced apoptosis of the cells.[35] It is necessary to investigate that the compound, which induces lymphocyte

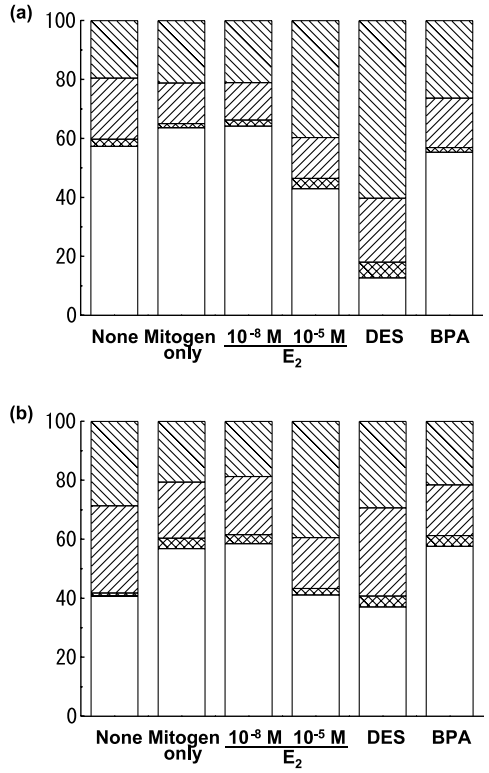

Fig. 6. The Cell Distributions to Regions in Fig. 3 after 12 hr Cultivation when Cells were Exposed to Mitogen, 10^{-8} M 17β-Estradiol, 10^{-6} M 17β-Estradiol, 10^{-8} M DES and 10^{-6} M BPA

(a) The cells were stimulated by LPS. (b) The cells were stimulated by Con A. ☐: Living cells. ▨: Early apoptotic cells. ▨: Late apoptotic cells. ▨: Debris.

apoptosis, is 17β-estradiol or its metabolites.

The cell distributions (Fig. 3, regions A1–C) when cells were exposed to each estrogenic compound for 12 hr were shown in Fig. 6. Diethylstilbestrol (DES) is a slightly stronger estrogen than 17β-estradiol, and DES killed a few more cells than the same concentration of 17β-estradiol. Bisphenol A (BPA) is a weak estrogen and killed few cells compared to the mitogen only. The extent of induction of apoptosis depended on each compounds. It perhaps seems to be correlated with its binding affinity to estrogen receptor α.

Apoptosis is achieved through several intercellular and intracellular events.[16,17] A Fas ligand from another cell binds to the Fas protein on the surface of the cell,[18] then several kinases and proteases including caspase are activated,[19,20] and finally endonucleases cut the DNA at each nucleosome into ordered fragments.[21] It is not elucidated which step in the mechanism described above 17β-estradiol enhances. However, we suppose that a lymphocyte exposed to a high concentration of estrogens might die via apoptosis. It suggests a relationship between autoimmune diseases and concentrated environmental 17β-estradiol or other estrogenic compounds. Further investigations of the mechanism by which 17β-estradiol affects lymphocyte apoptosis are necessary.

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