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Evidence for estradiol-induced apoptosis and dysregulated T cell maturation in the thymus

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Abstract

In an attempt to delineate the immunological alterations that may occur following treatment with estrogen, groups of C57BL/6 mice were treated with 75 mg/kg body weight of β-estradiol-17-valerate (E2) or the vehicle. The thymus from these mice were harvested on days 1, 4 and 7 following treatment. The thymocytes from E2-treated mice when cultured in vitro for 24 h, showed increased levels of apoptosis when compared to controls. The apoptosis was demonstrable by both TUNEL assay and AnnexinV/propidium iodide (PI) staining. Also, thymic atrophy and increased apoptosis of thymocytes when cultured in vitro were seen when lower doses of E2 (5 mg/kg) were administered. The thymus from E2-treated mice on days 4 and 7 also showed a decrease in the percentage of $CD4^+CD8^+$ (DP) T cells and an increase in the percentage of $CD4^-CD8^-$ (DN), $CD4^+$ and $CD8^+$ T cells. However, the total cellularity of all T cell subsets in the thymus was decreased following E2 treatment. Earlier studies from our laboratory and elsewhere have demonstrated that in thymocytes undergoing apoptosis, there is increased expression of surface markers including CD3, αβTCR and CD44 with a simultaneous decrease in the expression of J11d. Similar changes were observed in thymocytes from mice on days 4 and 7 following E2 treatment. These data therefore confirmed that the thymocytes were indeed undergoing apoptosis following E2 treatment. Together, our studies suggest for the first time that estrogen may induce thymic atrophy by triggering apoptosis.

Keywords: TCDD; Immunotoxicity; Apoptosis; Estrogen; Thymus

Abbreviations: E2, β-estradiol; ER, estrogen receptors; CD, cluster of differentiation; FITC, fluorescein isothiocyanate; Ig, immunoglobulin; TCR, T cell receptor; DN, double-negative ($CD4^-CD8^-$); DP, double-positive ($CD4^+CD8^+$); MFI, mean intensity of fluorescence; PBS, phosphate-buffered saline; PE, phycoerythrin; PI, propidium iodide; SEM, standard error of mean; TUNEL, TdT-mediated FITC-dUTP nick end-labeling; TdT, terminal deoxynucleotidyl transferase.

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1. Introduction

Estrogens play an important role in the growth, differentiation and functioning of the male and female reproductive systems. Estrogens also affect the non-reproductive tissues such as the bone, central nervous system, immune system, skin and cardiovascular systems. Estrogens have a profound effect on cells expressing estrogen receptors (ER). The ER belong to the steroid receptor family and are found in an inactive state, associated with heat-shock proteins. In the presence of estrogen, these receptors disassociate from the heat-shock proteins and estrogen–estrogen receptor complexes form. These receptor–ligand complexes homodimerize and then bind with high affinity to the estrogen-responsive elements and act as transcription factors regulating the expression of estrogen-responsive genes (Yamamoto, 1985; Ing and O'Malley, 1993). ER may be found as intranuclear receptors as well as on the cell surface (Razandi et al., 1999). ER are found on the lymphoid and non-lymphoid cells in the thymus (Couse et al., 1997).

Estrogens are steroids that are endogenously produced by the ovaries or may be provided in an exogenous form (Roy et al., 1997). Several microbes and plants also produce estrogens, such as enterolactone and coumestrol respectively. In addition, many synthetic chemicals such as diethylstilbesterol have been produced for pharmacological purposes. Moreover, environmental pollutants such as polychlorinated hydrocarbons may also bind to the estrogen receptors and behave as environmental estrogens. In recent years, concern has been raised about the possibility of such environmental contaminants with potent estrogenic activity, especially acting synergistically, to produce severe reproductive, developmental and other adverse effects (Arnold et al., 1996; Cooper and Kavlock, 1997). The widespread use of estrogens as contraceptives or in the treatment of postmenopausal women in preventing osteoporosis, Alzheimer's disease and cardiovascular diseases has raised the question of whether estrogens can induce immunotoxicity as well as lead to the development of breast and uterine cancer.

β-estradiol (E2) is a naturally occurring estrogen that has been shown to have significant immunomodulatory properties characterized by induction of thymic atrophy (Silverstone et al., 1994; Hirahara et al., 1994). The exact mechanism by which E2 induces thymic atrophy is not clear. Some studies have suggested that apoptosis may not be the pathway of E2-induced thymic atrophy (Staples et al., 1998). Furthermore, others have demonstrated that E2 affects prothymocytes (Holladay et al., 1993) or that E2 activates extrathymic T cell differentiation while inactivating intrathymic pathway (Okuyama et al., 1992).

Recent studies have also suggested that estrogen can induce apoptosis in non-lymphoid cells (Hughes et al., 1996; Robertson et al., 1996; Shevde and Pike, 1996). Because the apoptotic cells are rapidly cleared in vivo by phagocytic cells, it was possible that E2 may induce apoptosis of thymocyes in vivo that remains difficult to detect. Earlier studies from our laboratory have shown that thymocytes exposed in vivo to an apoptotic agent, upon in vitro culture, exhibit increased levels of apoptosis (Kamath et al., 1997). Furthermore, we and others (Kishimoto et al., 1995; Kamath et al., 1999) have demonstrated that apoptosis induction correlated with the dysregulated expression of several cell adhesion molecules on the surface of thymocytes. Thus, in the current study we used these two strategies to investigate whether E2 can induce apoptosis in thymocytes in vivo. The data suggested that the thymic atrophy induced by E2 may result from its ability to induce apoptosis in thymocytes.

2. Materials and methods

2.1. Mice

Adult female C57BL/6 mice were purchased from Charles River Co. The mice were housed in polyethylene cages containing wood shavings in laminar flow units (Animal Storage Isolators, Nu Aire Inc., Plymouth, MN) and given rodent chow and tap water ad libitum. Mice were housed in rooms maintaining a temperature of 74 ± 2°F and on a 12 h light/dark cycle.

2.2. Estradiol exposure

β-estradiol-17-valerate (1,3,5 [10]-Estratrine-3,17 β-diol 17-valerate) (Sigma Chemical Co., St. Louis, MO) was added to olive oil for preparation of a dosing solution. Mice received a single subcutaneous injection of E2 at a dose of 5 or 75 mg/kg or the vehicle.

2.3. Cell preparation

Mice were euthanized after estradiol or vehicle treatment and the thymus was surgically removed. The thymus was placed in 10 ml of RPM1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (Summit Biotechnology, Ft. Collins, CO), 10 mM HEPES, 1 mM glutamine, 40 μg/ml gentamicin sulfate, and 50 μM 2-mercaptoethanol, referred to as complete tissue culture medium, inside a 4 × 6 in sterile plastic bag. Single-cell suspension was made with a laboratory homogenizer (Stomacher, Tekmar Co., Cincinnati, OH) at high setting for 1 min and kept on ice. Cells were pelleted by centrifugation and resuspended in 0.83% ammonium chloride to lyse the erythrocytes. Cells were further washed twice in medium.

2.4. Total cellularity

Thymocytes were prepared as described above and resuspended in medium. Twent-five microlitre of the single cell suspension was added to 25 μl of trypan blue dye and viable cells were enumerated by exclusion of trypan blue under a phase contrast microscope.

2.5. Detection of apoptosis using the TUNEL method

The FITC-dUTP nick end-labeling kit was used to detect and quantitate apoptosis at the single cell level, which is based on labeling of DNA strand breaks using terminal deoxynucleotidyl transferase (TdT) and FITC-dUTP, commonly called the TUNEL technique (Boehringer Mannheim, Indianapolis, IN) (Kamath et al., 1997). Briefly, the cells (2×10^6) were washed twice with phosphate-buffered saline (PBS) and fixed with 4% p-formaldehyde for 30 min at room temperature. The cells were next washed with PBS, permeabilized on ice for 2 min and incubated with FITC-dUTP and TdT for 1 h in the incubator. Fluorescence of the cells was measured by flow cytometry, as described (Kamath et al., 1997). The analysis was performed by a Coulter Epics V flow cytometer. Five thousand cells were analyzed per sample.

The thymocytes from vehicle or E2-treated mice were assayed for apoptosis either immediately after collection or following in vitro culture for 24 h. For in vitro culture, the thymocytes were cultured in 96-well tissue culture plates in 0.2 ml tissue culture medium at a concentration of 1×10^6 cell/well for 24 h. Next, the cells were harvested, cell viability measured by trypan blue dye exclusion and stained with FITC-dUTP to detect apoptosis.

2.6. Detection of apoptosis using AnnexinV and propidium iodide

In some experiments, the thymocytes were studied for apoptosis using AnnexinV/PI staining, as described (Vermes et al., 1995). Briefly, thymocytes were washed with PBS and stained with AnnexinV and PI for 20 min at room temperature. The cells were washed twice with PBS and fluorescence of cells was measured by flow cytometry.

2.7. Detection of phenotypic markers on thymocytes

Two million thymocytes from E2 or vehicle-treated mice were stained with fluorescein-conjugated monoclonal antibodies to CD3 (hamster IgG), CD4 (rat IgG2a), CD8 (rat IgG2a), CD44 (rat IgG2b), αβTCR (hamster IgG), IL-2R (rat IgG2b) and J11d (rat IgM) (Pharmingen, Torregana, CA). The cells were incubated with the antibodies for 30 min on ice and then washed twice with PBS. Negative controls consisted of cells that were stained with appropriate fluorescein-conjugated normal antibody isotypes (Pharmingen, San Diego, CA). Cells were then

fixed with 1% p-formaldehyde. Fluorescence was measured by flow cytometry as described (Kamath et al., 1997). The data were analyzed and expressed as percent positive cells expressing the surface marker. In addition, the mean channel number, which represents the density of expression of the surface marker, was determined for the control and E2-treated cells, and the percent change in the mean intensity of fluorescence (MFI) was calculated as follows:

$$\frac{\text{MFI for E2 histogram} - \text{MFI for control histogram}}{\text{MFI for control histogram}} \times 100$$

The data from multiple experiments were pooled and depicted as mean percent change in MFI ± SEM.

2.8. Two-color fluorescent staining for CD4 and CD8 markers

Thymocytes were also stained for the expression of CD4 and CD8 by using a double-staining technique. Cells were incubated with Cy-5-conjugated anti-CD4 mAb and phycoerythrin (PE)-conjugated anti-CD8 mAb for 30 min at 4°C. The cells were washed twice, resuspended in PBS and analyzed using a flow cytometer.

2.9. Statistical analysis

Each experiment was repeated at least three times and each vehicle or E2-treated group consisted of four to six mice. The E2-treated groups were compared with the vehicle controls using Student's t-test, and $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Thymic cellularity in mice treated with E2

C57BL/6 mice were injected with 75 mg/kg body weight of E2 or vehicle and 1, 4 and 7 days later, the mice were sacrificed and thymic cellularity was calculated. The total number of cells per

Table 1
Effect of estradiol (E2) on cellularity and percentages of T cell subsets in the thymus

	Cellularity of the thymus × 10[6a]		T cell subsets[c]							
			$CD4^+$		$CD8^+$		$CD4^+CD8^+$		$CD4^-CD8^-$	
Days	Vehicle	E2	Vehicle	E2	Vehicle	E2	Vehicle	E2	Vehicle	E2
1	96.0 ± 5.3	54.2 ± 8.6*[,b]	14.9 ± 4.1 (14.3)	21.7 ± 4.6 (11.7)	4.3 ± 0.4 (4.1)	4.1 ± 1.3 (2.2)	76.0 ± 5.0 (72.9)	69.0 ± 5.6 (37.4)	4.9 ± 0.6 (4.7)	5.3 ± 1.1 (2.8)
4	95.33 ± 5.8	19.8 ± 2.2*	20.5 ± 6.0 (19.5)	44.1 ± 7.7 (8.7)	4.8 ± 2.1 (4.6)	7.8 ± 3.4 (1.5)	71.7 ± 7.4 (68.3)	40.5 ± 6.0* (8.0)	2.8 ± 1.1 (2.6)	7.6 ± 1.3* (1.5)
7	95.67 ± 6.2	8.0 ± 1.5*	20.5 ± 3.7 (19.6)	46.3 ± 2.9* (3.7)	5.5 ± 1.6 (5.3)	15.5 ± 2.2* (1.2)	69.7 ± 4.3 (66.7)	22.8 ± 5.5* (1.8)	4.5 ± 1.1 (4.3)	15.4* ± 4.8 (1.2)

[a] C57BL/6 mice were treated with E2 (75 mg/kg) or the vehicle. On days 1, 4 and 7 following treatment with E2, the thymocytes were harvested and total cellularity was determined.

[b] Data represent mean ± SEM obtained from four to six mice.

[c] T cell subsets were determined using flow cytometry. The data represent mean ± SEM of percentage of T cell subsets in the thymus obtained from four to six mice. The numbers in parentheses represent the mean total cellularity of each T cell subset found in the thymus.

* $p < 0.05$ when compared to vehicle controls.

thymus is depicted in Table 1. E2 caused a significant decrease in thymic cellularity on all days tested when compared to thymocytes from vehicle-treated mice. The decreased thymic cellularity in E2-treated mice (23.5 ± 5.1 million cells in E2-treated vs. 98 ± 7.1 million in vehicle-treated group; $P < 0.05$ was observed even 1 month following treatment, but returned to levels comparable to those found in controls by 2 months of treatment (86.3 ± 5.1 million in E2-treated and 95.5 ± 7.4 million in vehicle-treated group; $P < 0.05$. Thus, these studies clearly demonstrated that E2 induced early and sustained thymic atrophy.

3.2. Effect of estradiol on T cell subsets in the thymus

We next examined if E2 treatment (75 mg/kg) led to alterations in T cell differentiation in the thymus. For this purpose, a double-staining procedure was used to enumerate the four subpopulations of T cells. The thymocytes were stained with Cy-5 anti-CD4 and PE anti-CD8 mAbs. The dual parameter histograms were gated based on negative controls consisting of normal isotype antibodies. Fig. 1 shows representative flow cytometric data depicting the percentages of $CD4^+$, $CD8^+$, $CD4^+CD8^+$ (DP) and $CD4^-CD8^-$ (DN) T cell subsets in the thymus of a control and E2-treated mouse and data from multiple experiments is summarized in Table 1. The data indicated that E2 induced a significant decrease in the percentage of DP T cell subpopulation in the thymus and an increase in the DN, $CD4^+$ and $CD8^+$ subpopulations on days 4 and 7 following treatment. It should be noted that despite a decrease in the thymic cellularity observed on day 1 following E2 treatment, there was no significant change in the percentages of the thymocyte subpopulations. Table 1 also shows the total number of T cell subsets found in the thymus following E2 treatment. These data demonstrated that E2 treatment caused a significant decrease in the cellularity of all T cell subsets, particularly on days 4 and 7.

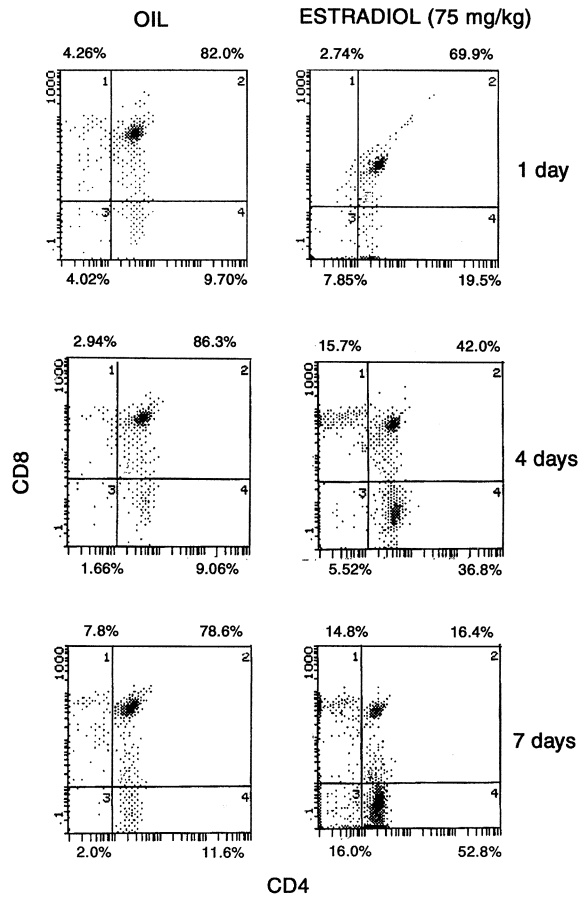

Fig. 1. Flow cytometric analysis of T cell subsets in the thymus following treatment with estradiol. Mice were injected with 75 mg/kg of estradiol or the vehicle. At different days after treatment, the thymocytes were harvested and stained with Cy5-anti CD4 and PE-anti CD8 mAbs. The cells were analyzed using a flow cytometer. The dual parameter histograms were gated based on negative controls consisting of cells stained with Cy5 or PE-conjugated normal antibody isotypes. The upper left quadrant represents $CD8^+$ T cells; the upper right, $CD4^+CD8^+$; the lower left, $CD4^-CD8^-$; and the lower right $CD4^+$ T cells. The percentages of cells in various quadrants have been depicted.

3.3. Detection of apoptosis

Whether the thymic atrophy observed following E2 treatment (75 mg/kg) was due to induction of apoptosis in the thymocytes was investigated next. We were not able to detect apoptosis in freshly isolated thymocytes from vehicle and E2-treated

mice (data not shown). However, increased apoptosis in thymocytes was demonstrated following 24 h in vitro culture of the in vivo E2-exposed thymocytes when compared to similar cells from vehicle-treated mice. The data shown in Fig. 2 indicated that when thymocytes harvested on days 1, 4 and 7 from mice treated with E2 were cultured for an additional 24 h in vitro with medium alone, they exhibited increased levels of apoptotic cells when compared to similarly cultured thymocytes from vehicle-treated controls. The percent of apoptotic cells increased over time following E2 treatment. Also, when cell viability was measured, E2-exposed thymocytes upon in vitro culture showed more non-viable cells when compared to the controls (data not shown). On days 1 and 7, in addition to increased percentage of apoptotic cells, there was also increased intensity of fluorescence in apoptotic thymocytes from E2 treated mice when compared to the controls. Because increased intensity of fluorescence correlates with late apoptotic cells (Kamath et al., 1997), these data suggested that E2-treated mice exhibited thymocytes that underwent apoptosis earlier than thymocytes from control mice.

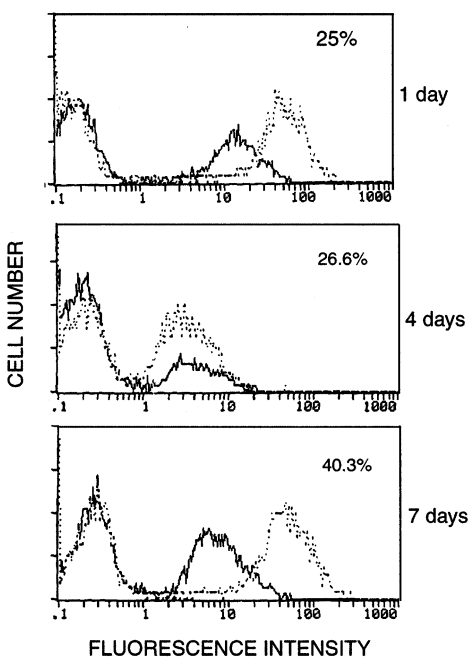

Fig. 2. Effect of estradiol on apoptosis induction in the thymus. Thymocytes from mice treated with 75 mg/kg of estradiol or vehicle were harvested at different days after treatment and cultured for 24 h in medium. The thymocytes were then stained with FITC-dUTP to detect apoptosis. Bold histograms depict thymocytes from vehicle-treated mice and broken histograms represent thymocytes from estradiol-treated mice. The bold histogram was substracted from the broken histogram and the difference in percentage of cells was expressed in each panel.

To further confirm that E2 was inducing apoptosis in thymocytes, we used Annexin/PI staining to detect apoptotic cells. To this end, C57BL/6 mice were injected with E2 (75 mg/kg) or the vehicle and on day 4, the thymocytes were harvested and cultured for an additional 24 h in medium. Next, the cells were stained with AnnexinV and PI. The data shown in Fig. 3 indicated that E2-exposed thymocytes showed increased percentage of AnnexinV$^+$/PI$^-$ and AnnexinV$^+$/PI$^+$ cells when compared to the controls. Because AnnexinV$^+$/PI$^-$ cells represent early apoptotic cells and AnnexinV$^+$/PI$^+$ cells are suggestive of late apoptotic/necrotic cells (Vermes et al., 1995), these data together suggested that E2 treatment led to significant increase in apoptotic cells when compared to the vehicle controls.

3.4. Effect of estradiol on the surface phenotype of thymocytes

Earlier studies from our laboratory and elsewhere have demonstrated that in thymocytes undergoing apoptosis, phenotypic changes occur (Kamath et al., 1998, 1999; Kishimoto et al., 1995). In order to study the effect of E2 on the expression of various surface markers on thymocytes, mice were injected with E2 (75 mg/kg) or vehicle and 1, 4 and 7 days later, the cells were harvested and analyzed. The cells were stained for expression of CD3, CD44, αβTCR and J11d using mAbs and analyzed using a flow cytometer. In these experiments, groups of four to five mice were used, and each mouse thymus was analyzed separately. The data from representative experiments on days 1, 4 and 7 following E2 treatment are depicted in Figs. 4–6, respectively. The data indicated that E2 administration induced no significant changes in the percentages of cells expressing the surface markers on all days tested,

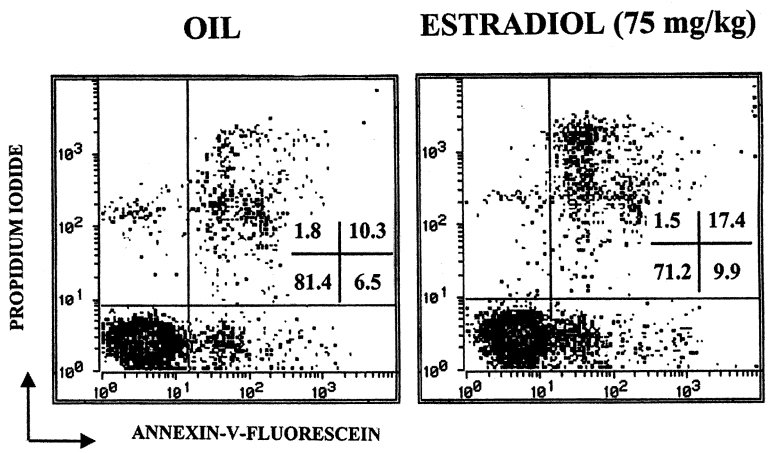

Fig. 3. Detection of estradiol-induced apoptosis using AnnexinV/PI staining. Thymocytes from mice treated with 75 mg/kg of estradiol or vehicle were harvested 4 days after treatment. The cells were cultured for 24 h in medium and stained with AnnexinV and PI. The cells were analyzed for dual staining using a flow cytometer. The percentage of cells in various quadrants has been depicted.

except for an increase on day 4 and 7 in the percentages of cells bearing αβTCR and CD3, when compared to the controls.

In addition to the percentage of cells expressing the surface markers, comparisons were also made in the MFI that indicated the density of expression of the surface marker on a per cell basis. The MFI obtained with each marker in the E2-treated mice was compared to the MFI for that marker in the controls, and the data were expressed as mean percentage increase in MFI as discussed in Section 2. Based on this, a 100% increase in MFI represents a two-fold increase in the density of cell surface markers. As seen from the MFI values depicted in Figs. 4–6 for individual histograms and as summarized in Fig. 7, on day 1 after E2 injection, there were no significant changes in the density of expression of all the markers studied. On days 4 and 7 following E2 administration, there was a marked increase in the density of expression of CD3, CD44 and αβTCR. In contrast, the expression of J11d showed a significant decrease, especially on days 4 and 7. These results suggested that E2 treatment led to an increase in the density of expression of CD3, CD44 and αβTCR and a decrease in the expression of J11d. These changes correlated with earlier studies demonstrating that there is an upregulation of cell surface molecules such as CD3, αβTCR and CD44 and a downregulation of other surface molecules such as J11d in thymocytes undergoing spontaneous or steroid-induced apoptosis (Kishimoto et al., 1995). These studies therefore provided additional evidence to support our findings that E2 can induce apoptosis in thymocytes which may be responsible for the thymic atrophy.

3.5. Lower doses of E2 can also induce apoptosis in thymocytes

To address whether E2 would induce apoptosis at lower concentrations, we injected C57BL/6 mice with 5 mg/kg of E2 and 1, 4 and 7 days later thymic cellularity was determined. The data shown in Fig. 8 indicated that E2 caused a significant decrease in thymic cellularity on days 4 and 7 but not on day 1. Furthermore, when thymocytes exposed to E2 for 1, 4 and 7 days in vivo were cultured in vitro for 24 h, they exhibited significant increase in AnnexinV$^+$/PI$^-$ as well as AnnexinV$^+$/PI$^+$ cells when compared to the vehicle controls (Fig. 9). This increase in early apoptotic and late apoptotic/necrotic cells was more significant on days 4 and 7, correlating with significant thymic atrophy seen on days 4 and 7 (Fig. 8). It should be noted that when normal thymo-

cytes are cultured in vitro, they undergo spontaneous apoptosis and appear initially as AnnexinV$^+$/PI$^-$. However, with increasing culture duration, the same cells appear AnnexinV$^+$/PI$^+$ cells. Thus, the AnnexinV$^+$/PI$^+$ cells seen in the current study represented cells that were mainly late apoptotic rather than necrotic cells.

4. Discussion

Estrogens have previously been shown to cause thymic atrophy (Holladay et al., 1993; Silverstone et al., 1994; Staples et al., 1999). However the mechanism of thymic atrophy remains unclear. Earlier studies have suggested that estrogens induce thymic atrophy by affecting prethymic stem cells in bone marrow or fetal liver (Holladay et al., 1993; Silverstone et al., 1994). In contrast, other investigators have demonstrated that E2 alters the intrathymic T cell development (Screpanti et al., 1989; Brunelli et al., 1992; Rijhsinghani et al., 1996). Luster et al. (1984) suggested that thymic atrophy may not be due to a direct effect of E2 on lymphocytes, but may be mediated through the estrogenic effects on thymic epithelial cells which have higher expression of ER.

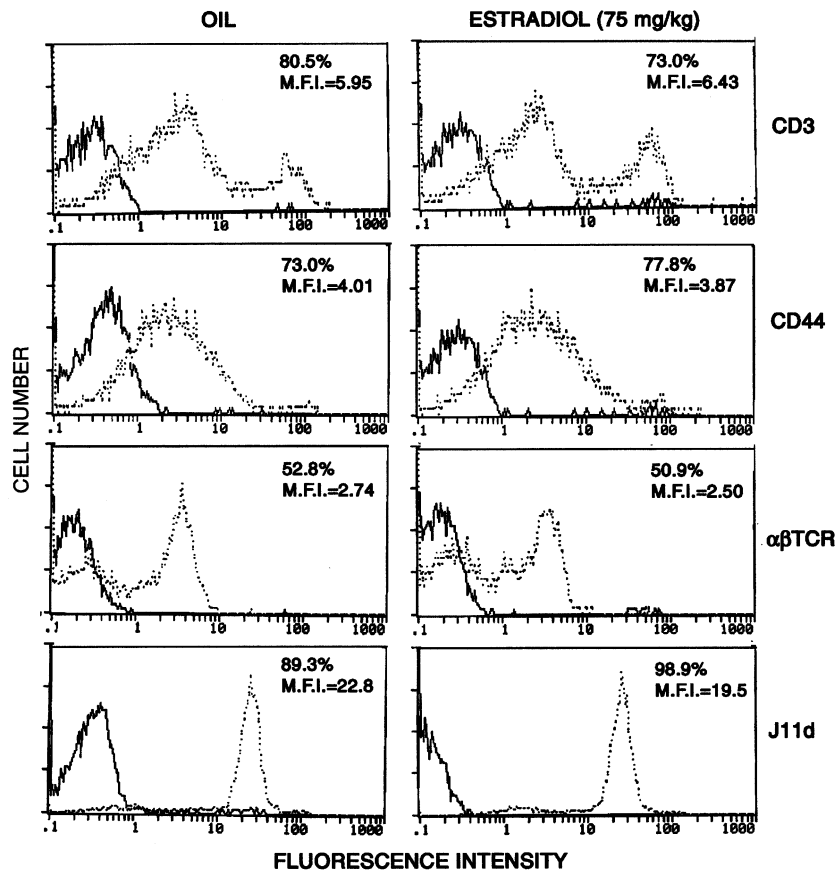

Fig. 4. Effect of estradiol administration on the surface phenotype of thymocytes from C57BL/6 +/+ mice. Estradiol (75 mg/kg) or the vehicle (olive oil) was injected into mice and 1 day later, the thymocytes were harvested and stained with FITC or PE-conjugated mAbs against CD3, CD44, αβTCR and J11d. Cells were analyzed using a flow cytometer. The bold histogram represents antibody isotype controls and the broken histogram represents thymocytes stained for various surface markers. The percentage of positive cells and MFI for stained cells have been depicted for each histogram. The figure represents data from a representative experiment.

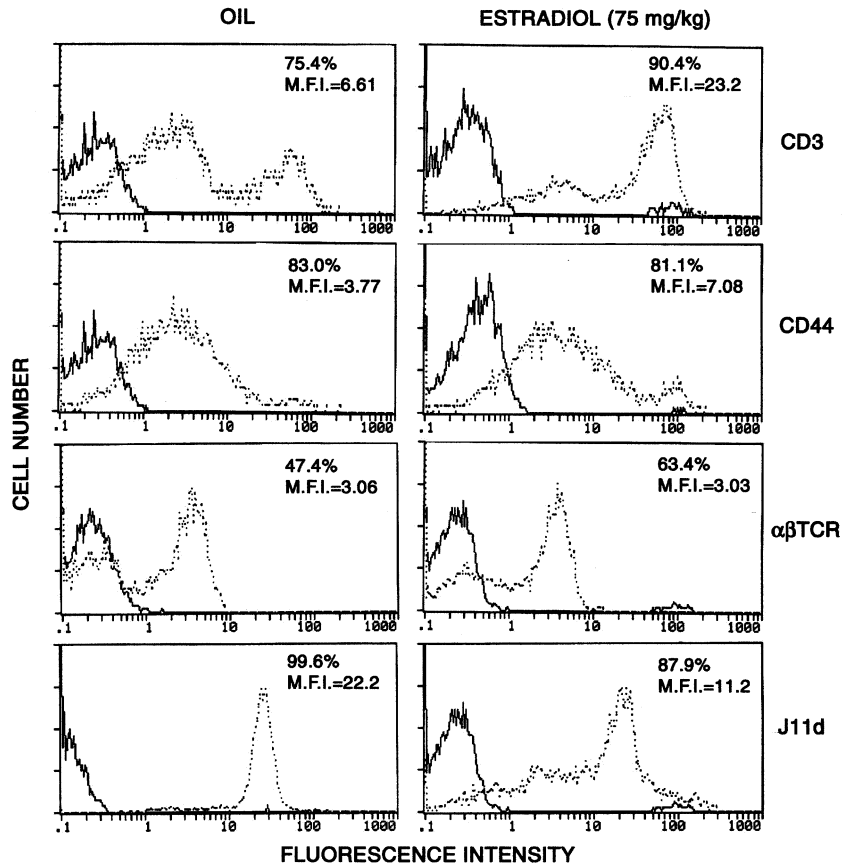

Fig. 5. Effect of estradiol administration on the surface phenotype of thymocytes from C57BL/6 +/+ mice. Estradiol (75 mg/kg) or the vehicle (olive oil) was injected subcutaneously into mice and 4 days later, the thymocytes were harvested and stained with FITC or PE-conjugated mAbs against CD3, CD44, αβTCR and J11d. Cells were analyzed using a flow cytometer as depicted in Fig. 3.

Estrogen has recently been shown to induce apoptosis in non-lymphoid tissues (Hughes et al., 1996; Robertson et al., 1996; Shevde and Pike, 1996). However, whether estrogen induces thymic involution by triggering apoptosis is not clear. Staples et al. (1998) failed to observe apoptosis in freshly isolated thymocytes following E2 treatment. This is consistent with the data obtained in the current study wherein we observed that thymocytes from E2-treated mice failed to exhibit apoptosis when tested directly and immediately after isolation. However, we have reported previously that apoptosis induced by chemicals in vivo is difficult to detect because of the rapid clearance of apoptotic cells by phagocytic cells (Kamath et al., 1997, 1998, 1999; Pryputniewicz et al., 1998). To overcome this problem, we have used a novel strategy in which the thymocytes or T cells exposed in vivo to chemicals or vehicle are cultured in vitro with medium for an additional 12–24 h. We have shown that this procedure permits the detection of increased apoptosis induced by chemicals such as dioxin and dexamethasone when compared to the vehicle controls (Kamath et al., 1997, 1998, 1999). In the current study, we used a similar approach to investigate the mechanism by which E2 triggers thymic involution. Our results demonstrated that E2 does indeed trigger apoptosis in thymocytes. It should be noted that Staples et al. (1998) did not test whether apoptosis could

be detected in E2 exposed thymocytes upon in vitro culture. Moreover, while some studies failed to detect apoptosis when diethylstilbesterol, a synthetic estrogen, was added to in vitro cell cultures (Donner et al., 1999), others have been able to demonstrate apoptosis (Robertson et al., 1996). Such discrepancies may result from the fact that thymocytes were used in the former study while prostate cancer cells were tested in the latter.

Kishimoto et al. (1995) demonstrated that in thymocytes undergoing spontaneous or steroid-induced apoptosis, there is downregulation of some surface markers due to degradation of cellular components. In contrast, some molecules on apoptotic cells may be upregulated due to the breakdown of intracellular trafficking. Our earlier studies on dioxin-induced thymocyte apoptosis have shown that apoptosis correlated with an upregulation of CD3, αβTCR, CD44 and IL-2R and a downregulation of J11d, CD4 and CD8 molecules (Kamath et al., 1998, 1999). In the current study, we observed similar alterations in E2-treated thymocytes, thereby corroborating that E2 treatment leads to apoptosis. From the current study, it is not clear whether the increased density of expression of cell surface markers resulted from increased expression within a single population or whether it included multiple cell types. Further studies on cell surface markers in purified cell populations should resolve this.

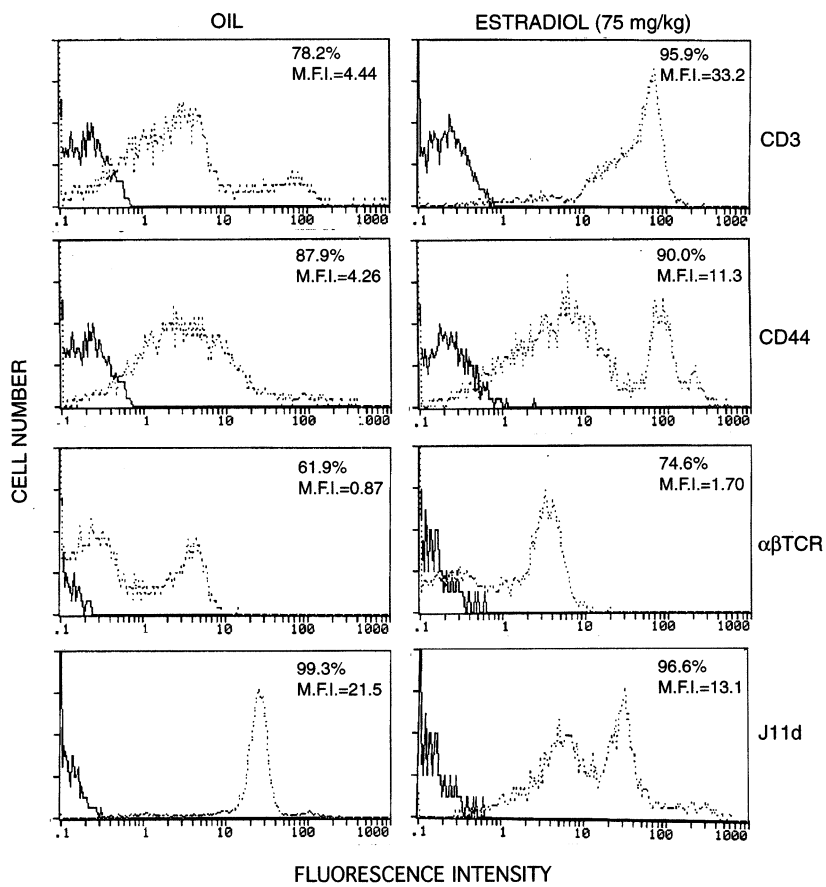

Fig. 6. Surface phenotype of thymocytes from C57BL/6 +/+ mice treated with estradiol. Estradiol (75 mg/kg) or the vehicle (olive oil) was injected subcutaneously into mice and 7 days later, the thymocytes were harvested and stained with FITC or PE-conjugated mAbs against CD3, CD44, αβTCR and J11d. Cells were analyzed using a flow cytometer as shown in Fig. 3.

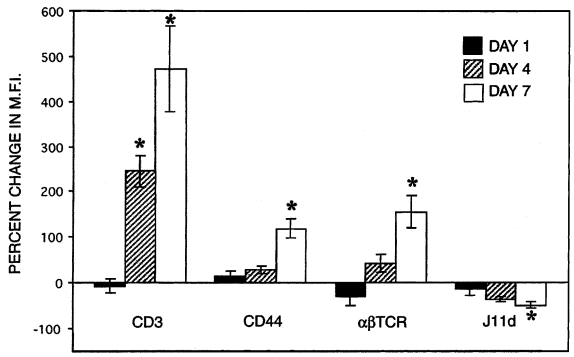

Fig. 7. Changes in the density of surface marker expression upon estradiol treatment. The thymocytes were stained for various markers as described in Figs. 4–6. The MFI obtained with each marker in estradiol-treated mice were compared to the MFI of the controls, and the data were expressed as mean percentage change in MFI as described in Section 2. The percentage changes in MFI from four to five individual experiments were pooled to obtain the mean and plotted. An asterisk denotes statistically significant difference ($p < 0.05$) when compared to the controls.

Estrogen mediates its effects by binding to the ER, which may be of two types: ERα and ERβ. ERα is expressed in the thymocytes and stromal elements. However, the expression of ERβ in the thymic tissue depends on the species. It is not found in mouse thymic tissue (Tremblay et al., 1997; Couse et al., 1997), whereas it is expressed in the rat thymus at low levels and in human thymus at high levels (Mosselman et al., 1996;

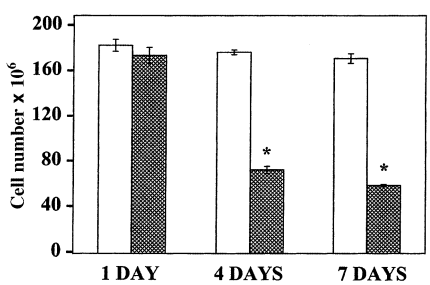

Fig. 8. Detection of thymic cellularity in mice treated with lower doses of estradiol (5 mg/kg). Groups of five mice were treated with estradiol (hatched bars)or the vehicle (empty bars) and 1, 4 and 7 days later, the thymocytes were harvested. Vertical bars represent mean thymic cellularity ± SEM. An asterisk denotes statistically signficant difference ($p < 0.05$) when compared to the controls.

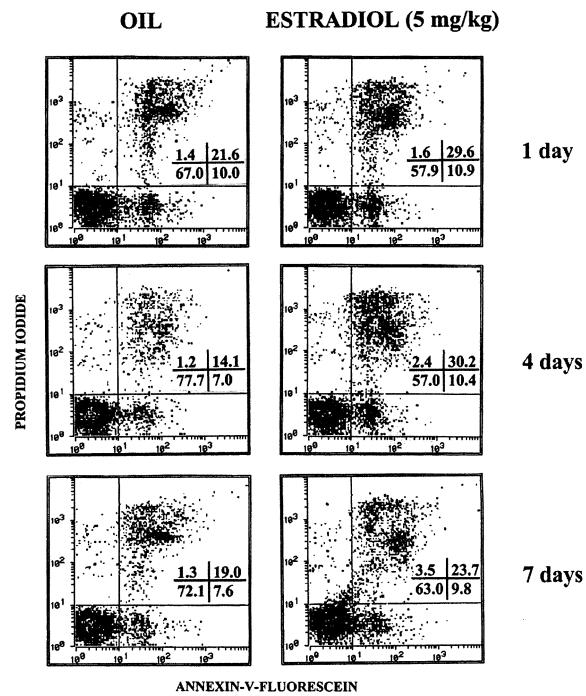

Fig. 9. Detection of apoptosis in mice treated with lower doses of estradiol (5 mg/kg). Groups of five mice were treated with estradiol or vehicle as described in Fig. 8. On days 1, 4 and 7, the thymocytes were harvested, cultured for 24 h in vitro and stained with AnnexinV and PI and analyzed for dual fluorescence using a flow cytometer as described in Fig. 3.

Brandenberger et al., 1997; Kuiper et al., 1997). Staples et al. (1999) have demonstrated that E2-induced thymic atrophy may involve ERα-dependent and independent mechanisms.

Studies have demonstrated that there is a reduction in lymphocytes in thymus and spleen but not in bone marrow or lymph node following administration of estrogen for 2 weeks in female C57BL/6 mice (Ahmed et al., 1985). There was a reciprocal correlation between the levels of circulating leukocytes and estrogen, with low levels of leukocytes seen during the rise in the preovulatory estrogen levels (Al-Afaleq and Homeida, 1998). Most studies have demonstrated that treatment with E2 leads to a depletion of the DP thymocytes and an increase in the SP and DN thymocytes (Lai et al., 1998; Screpanti et al., 1989; Staples et al., 1999; Rijhsinghani et al., 1996). In the current

study, we made a similar observation. However, in addition, we noted that the density of expression of certain cell surface markers was altered.

Thymus is a site of T cell differentiation whereby T cells that recognize self-peptides presented by self-MHC encoded molecules or T cells with little or no avidity for self-MHC undergo apoptosis (Ashwell et al., 2000). Thus, although the majority of the thymocytes undergo programmed cell death, demonstration of apoptosis in thymocytes directly is difficult. The current study as well as our previous studies (Kamath et al., 1997, 1998) demonstrated that analysis of the density of expression of cell surface markers on thymocytes may constitute a sensitive indicator for apoptosis in thymocytes induced by chemicals, in the absence of direct demonstration of apoptosis.

Several pathways operate to induce apoptosis. Staples et al. (1998) demonstrated that E2-induced thymic atrophy was independent of overexpression of bcl-2, an inhibitor of apoptosis. We have shown that dioxin-induced apoptosis in thymocytes is dependent on Fas–FasL interactions (Rhile et al., 1996; Kamath et al., 1997, 1998). Thus, it is possible that other pathways involving the TNF receptor family, including Fas–FasL interactions, may be involved in apoptosis of thymocytes induced by E2. Moreover, Hughes et al. (1996) demonstrated that estrogen promoted apoptosis of murine osteoclasts by facilitating the TGF-β-induced apoptosis. Further studies to delineate these pathways are necessary to investigate the mechanism by which E2 triggers apoptosis in thymocytes.

In the current study, we used two doses of estrodiol (5 and 75 mg/kg) to study thymic atrophy and apoptosis. While a higher dose of estradiol induced thymic atrophy as early as 1 day after treatment, the atrophy induced by a lower dose was not seen on day 1 but was observed on day 4 and 7. The apoptosis, however, was detected in thymocytes exposed to both 5 and 75 mg/kg of estradiol. These dose ranges are similar to those used in earlier studies which demonstrated thymic atrophy and T cell subset alterations (Rijhsinghani et al., 1996; Screpanti et al., 1989; Staples et al., 1999). Furthermore, estrogen at 0.1–1.0 mg/mouse has been considered to be a pharmacologic dose (Okuyama et al., 1992) and compares well with the doses used in the current study (0.125–0.375 mg/mouse). Yahata et al. (1996) demonstrated that estrogen, when injected into mice at a dose of 0.01 mg/mouse every 4 days for 30 days, led to serum estrogen levels of 300 pg/ml, while the serum estradiol levels in normal female mice ranged from 47 to 66 pg/ml. In humans, estradiol levels have been shown to be present in the range of 118 ± 80 pg/ml in women of age ranging between 20 and 39 years (Giglio et al., 1994).

Together, these studies suggest that the doses of estrogen used in the current study are clinically relevant. Synthetic estrogens such as diethylstilbestrol (DES) were used extensively in millions of pregnant women in the US and Europe from 1947 to the 1980s at massive doses as high as 125 mg/day (~ 2.5 mg/kg body weight) during the last month of pregnancy. Such an intake of high doses has been responsible for increased risk of adenocarcinoma of the vagina and cervix in the daughters of the women treated with DES (Melnick et al., 1987). Thus further studies on estrogens and their ability to induce apoptosis should shed new light on a wide range of physiological effects exerted by this hormone.

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