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The mechanism of T cell dysfunction induced by Diethylstilbestrol.

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy at Virginia Commonwealth University.

by

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M.M., M.S., 1998, 1999

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List of Abbreviations

Abs	antibodies
AF	activation function
Ag	antigen
AICD	activation induced cell death
AIF	apoptosis inducing factor
Apaf	apoptotic protease activating factor
ATP	adenosine triphosphate
AICD	activation induces cell death
BCA	bicinchoninic acid
BH	Bcl-2 homology
bp	base pair
C57BL6	wild type mice
CD	cluster of differentiation
cDNA	complementary DNA
Con A	concanavalin
CPM	Count per minute
°C	degrees Celsius
dATP	deoxy ATP

DES	diethylstilbestrol
DiOC ₆ (3)	3,3'-dihexyloxacarbocyanine iodide
DISC	death induced signaling complex
DMSO	dimethylsulphoxide
DN	double negative
DNA	deoxynucleic acid
DP	double positive
dUTP	deoxyUTP
ECL	enhanced chemiluminescence
ER	estrogen receptor
ERE	estrogen responsive element
ERK	extracellular signal regulated kinase
FADD ^{-/-}	Fas associated death domain deficient
FasL	Fas ligand
FBS	fetal bovine serum
Fig	figure
FITC	fluorescein isothiocyanate
G	grams
Gd	gestational day
Gld	generalized lymphoproliferative disease
HPA	hypothalamic-pituitary-axis
Hr	hours

HY	male specific HY antigen
IFN	interferon
IL	interleukin
ip	intraperitoneally
ITAM	immunoreceptor tyrosine-based activation motif
JNK	Jun N-terminal kinase
kDa	kilodaltons
kg	kilogram
LN	lymph node
Lpr	lymphoproliferative
$\Delta\psi_m$	loss of mitochondrial membrane potential
mAb	monoclonal antibody
MAP	mitogen activated protein
μCi	microcurie
μg	microgram
μl	microliter
μM	micromolar
kDa	kilodalton
MHC	major histocompatibility complex
MIF	mean intensity of fluorescence
μg	microgram
μl	microliter

μM	micromolar
min	minute
ml	milliliter
mM	millimolar
mol	mole
mRNA	messenger RNA
ng	nanogram
NK	natural killer cell
OD	optical density
p	p-value
PAGE	polyacrylamide gel electrophoresis
PARP	poly ADP ribosyl-polymerase
PBS	phosphate buffered saline
PD	post delivery
PE	phycoerythrin
pT α	pre T cell receptor alpha chain
RNA	ribonucleic acid
rpm	revolutions per minute
RT-PCR	reverse transcription-polymerase chain reaction
SAPK	stress activated protein kinase
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis

SEM	standard error of mean
SMAC	second mitochondria derived activator of caspases
SP	single positive
TCR	T cell receptor
Tdt	terminal deoxynucleotide transferase
Th	T helper cell
TNF	tumor necrosis factor
TNFR	tnf receptor
TUNEL	Tdt-mediated dUTP nick end labeling
Vs	versus
WT	wild type
XIAP	X-chromosome linked inhibitor of apoptosis

Abstract

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Nicole C. Brown, M.M., M.S., Ph.D.

A dissertation submitted in partial fulfillment of the requirements for the degree of
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Estrogens have the ability to alter the immune system. Diethylstilbestrol (DES), a synthetic estrogen, is known to have estrogenic activity and induce thymic alterations. We investigated the mechanism by which DES is able to alter T cells and thus the immune system. First, we studied the effect of DES on mature T cells by using the T cell leukemia cell line, Jurkat. We found that DES treatment reduced cell viability and increased apoptosis. Additionally, apoptosis was found to involve both death receptor and mitochondrial pathways. Furthermore, estrogen receptor beta was found to be expressed in these cells and increased following DES treatment. Secondly, we studied the effect of DES on developing T cells using two different mouse models, timed

pregnant and HY-TCR transgenic. The pregnant mouse model showed that DES exposure in utero reduced thymic cell viability and induced apoptosis at gestational day (gd)-17. Apoptosis was found to involve the death receptor pathway. Additionally, alterations in T cell subsets was most pronounced at gd-17 as well. The HY-TCR tg mouse model showed that DES exposure altered both positive and negative selection of T cells. Furthermore, DES was found to alter the ability of T cells to proliferate during an immune response. Finally, we studied the intrathymic interaction between thymic stromal cells and thymic T cells. We found that cell:cell interaction was important for inducing T cell apoptosis in the thymus. Additionally, FasL expression was increased on thymic stromal cells following DES exposure. Furthermore, the presence of both FasL on stromal cells and Fas on T cells was important for inducing T cell apoptosis in the thymus.

Chapter I. Introduction

Immune system

The immune system is a network of heterogeneous cellular populations and chemical signals (cytokines, chemokines) (Arai et al., 1990). Immune cell types represented include: myeloid (macrophage) and lymphoid cells (T cells and B cells). These cells play roles in the generation of an immune response. There are two basic types of immune responses, innate and cell-mediated (Dempsey et al., 2003). In the innate response, preexisting immune components are directed against a foreign threat. These responses do not need to be induced (pre-formed) and are nonspecific defenses. These defensive mechanisms are numerous. Nonspecific defenses can be barriers (mechanical, chemical, immunological, or genetic), inflammatory responses, fevers, and interferon. Other mechanisms represented are complement and phagocytosis. Mechanical barriers are typically found at entry points. These barriers are anatomical or physiological such as skin, membranes (mucous), or cilia (Diamond et al., 2000; Jameson, 2004). Chemical barriers are secreted molecules such as lysozyme or defensin (Lehrer et al., 1993; Fellermann and Stange, 2001). Immunological barriers are normal flora that reside in the body. Genetic barriers are guided by hereditary characteristics that are specific for individuals (Kennedy, 1989; Wells et al., 2003).

Inflammatory responses serve to help mobilize the immune response by recruiting immune cells to the site (Laroux, 2004; Sherwood and Toliver-Kinsky, 2004).

Complement proteins are plasma proteins that facilitate removal by phagocytosis (Cooper and Nemerow, 1985; Tomlinson, 1993; Gasque, 2004). Phagocytosis is mediated through the recruitment of neutrophils and macrophage (Kobayashi and Deleo, 2004).

The purpose of this initial response is to limit the damage to the host and to provide time for the development of the adaptive immune response. The adaptive response is the specific clonal selective process in which antigen specific lymphocytes arise. An important characteristic of this response is memory, which allows for a more rapid response against further challenges (Vitetta et al., 1991; Gray, 1992; Mackay, 1993). B lymphocytes play a role in the humoral response. During this response, B cells produce antibodies that are antigen specific (Vitetta et al., 1989). Antibodies are plasma proteins that function to bind and neutralize/remove the antigen. The other side of adaptive immunity is the T cell (Jankovic et al., 2001). T cells are represented by several effector functions. T helper cells function to facilitate other cells such as B cells or macrophages to carry out their effector functions (Parker, 1993). Cytotoxic T cells function to kill cells that are virally infected. An additional class of T cells are the suppressor T cell. These cells suppress the activities of other cells (O'Garra and Vieira, 2004).

The thymus and T cells development

The thymus gland is a critical microenvironment that facilitates the development of thymocytes. The thymus can be segregated into regions or zones: cortex and medullary zones. Thymocyte precursors from the bone marrow migrate into the thymus, entering the subcapsular cortical area. These thymocyte precursors bear none of the T cell characteristic surface markers such as CD3, CD4, or CD8. Upon entry into the subcortex, these cells engage the cortical epithelial cells. These epithelial cells represent the thymic stroma. At this stage, the precursors go through a period of proliferation called lymphopoiesis. During this proliferation, the precursors known as double negative (DN) cells go through a process by which they lose their ability to become cells other than thymocytes. At the DN1 stage, cells could potentially become other cells of non T cell origin outside of the thymic compartment. The DN2 stage is marked by the inability to become B lymphocytes or natural killer cells. The potential to become T lymphocytes or dendritic cells remains intact (Shortman and Maraskovsky, 1998; Shortman et al., 1998). The DN3 stage represents that commitment to the T lymphocyte lineage. The T cell receptor beta gene rearrangement can be observed (Godfrey et al., 1993; Godfrey and Zlotnik, 1993; Petrie et al., 1995). The DN4 stage is represented by the a pre-double positive stage. Thus, DN3 and DN4 are associated with T cell receptor (TCR) beta gene rearrangement when the preTCR is exposed.

The DN stage can be represented by hallmark surface molecules, CD117 (cKit), CD25 (IL-2 receptor) and CD44. The DN1 stage express CD117+CD25-, while DNII have CD117+CD25+. At the DNIII, CD117-CD25+ is expressed, whereas DNIV has

CD117-CD25- (Shortman and Wu, 1996; Zuniga-Pflucker and Lenardo, 1996; Prockop and Petrie, 2000). Thus, down regulation of CD117 coincides with VJ rearrangement. Additionally, the expression of CD44 and CD25 can be hallmarks. DNI can be expressed by CD25-CD44+. DNII precursors express CD25+CD44+. The last two stages DNIII and DNIV express CD25+CD44- and CD25-CD44- respectively (Godfrey et al., 1993; Godfrey and Zlotnik, 1993).

Following the end of the DN stage, the beta chain undergoes rearrangement (beta selection). The rearrangement of the beta chain is allelically excluded, in that gene rearrangement is limited. The rearrangement is not infinite (Dudley et al., 1994; von Boehmer et al., 1999). Productive gene rearrangement of beta chain signals the pairing with pT α and CD3 in the membrane. Further signaling through the pre-TCR leads to no further beta rearrangement (Saint-Ruf et al., 1994; von Boehmer and Fehling, 1997). At this stage, cells are rescued from apoptosis and undergo proliferation. There is an upregulation of CD4 and CD8, while CD25 is downregulated. At this point, the alpha chain undergoes its rearrangement. Unlike beta rearrangement, alpha is not allelically excluded, meaning that the process is continuous (Levelt and Eichmann, 1995). In fact the process will continue until there is a productive rearrangement or the cell dies. Double positive (DP) T cells migrate further into the thymus to the corticomedullary junction for further development. DP T cells undergo two selective processes that may not be mutually exclusive. Positive selection drives the survival and differentiation of the thymocytes that are able to recognize peptide presented by self: major histocompatibility complex (MHC). During negative selection, thymocytes are further tested for their self

tolerance. Tolerance can be described a failure to respond (von Boehmer et al., 1989; Kisielow and Miazek, 1995; Kisielow and von Boehmer, 1995). Self tolerance is critical to proper immunity, because it prevents cellular responses to self antigens. Such interactions lead to autoimmunity.

Several models have been proposed for lineage commitment. The models primarily focus on strength and duration. The stochastic model suggests that the interaction leads to the down regulation of the unselected MHC. This model represents a randomness to the lineage process. The thymocytes are already randomly predetermined as to the lineage. Thymocytes require the appropriate signal to continue development. In this model, signals lead to enforced expression of the rescued MHC (Itano et al., 1994). A second model, the quantitative signal model, suggests that the co-receptor can be enhanced, which leads to increased signaling and thus selection (Wiest et al., 1993). The third model is focused on the duration of the interaction, in which the length of TCR:MHC interaction determines the lineage. Long interactions mediate positive selection, while short interactions lead to negative selection (Mariathasan et al., 2001). A fourth model suggests that differential ERK signaling leads to lineage commitment. The role of ERK primarily focuses on its activation state and TCR internalization. During positive selection, the interaction is not strong (low affinity), resulting in weak signaling because the ITAMs are not fully activated (McGargill et al., 2000). This interaction promotes no receptor internalization and thus leads to continuous signaling. In negative selection, the interaction is transient but strong. Negative selection has been found to involve ERK as well as p38 (Hogquist, 2001; Mariathasan et al., 2001). During negative

selection, ERK signaling is sustained resulting in TCR internalization and editing (McGargill et al., 2000).

The model that is most supported is the differential avidity/ affinity model of engagement, which indicates that positive selection is determined by a lower affinity engagement and negative selection is a high avidity interaction (Sprent et al., 1988; Kisielow and Miazek, 1995). Thus, cells that have low or intermediate responsiveness to MHC are positively selected and those that have strong interactions to MHC:self peptide are negatively selected. Interactions that do not meet these requirements lead to death by neglect during positive selection. Death by neglect refers to cell death due to lack of cell support. During negative selection, those thymocytes that are self reactive die by the induction of apoptosis.

The processes of positive selection are mediated through interaction with the thymic epithelial cells (cortical epithelial cells). This process takes approximately 3-5 days. Thymic epithelial cells line the inner surface of the thymic capsule, in addition to the vessels (Manley, 2000; Petrie, 2002). These cells are characterized by being keratin expressing, CD45- and having a flat, regular, polygonal morphology (Gray et al., 2002). Thymic epithelial cells express MHC molecules, which play important roles in thymocytes development. Other stromal components such as macrophage and dendritic cells are integral part of negative selection. The thymocytes spend approximately 12-16 days in the medulla of the thymus (Scollay and Godfrey, 1995). Macrophage and dendritic cells function as antigen presenting cells. During development the antigen presented in the thymus is self antigen.

Given that T cell development is under strict parameters, most thymocytes do not leave the thymus. Some 10-100 million precursors per day begin the process, only approximately 3-5% actually survive the selection process and leave the thymus (Shortman et al., 1990). The mature thymocytes leave the thymus for the periphery, where they take up residence in lymphoid tissues such as the spleen or lymph nodes. In these organs, the mature T cells encounter foreign and self antigens. Typically, the mature T cells respond to the foreign antigens but tolerate the self antigens. The response to the foreign antigens follows the presentation of antigens to T cells. In these organs, antigens are processed and presented in the context of MHC on the surface of antigen presenting cells. The interaction between the TCR and the MHC leads to T cell activation.

T cell receptor and antigen recognition

Mature T cells express a functional TCR on its surface. The TCR provides specificity for each individual T cell. T cell recognition of antigen is dependent on the TCR and MHC:peptide complex on the antigen presenting cell (APC). The MHC molecule has two class designations. MHC class I molecules are found on nucleated cells and present small peptides to T cells. These small peptides are in the form of endogenous antigens such as viral protein or tumor protein. Class II molecules are found on APCs such as macrophage, dendritic cells, and B cells. These molecules also present small peptides to T cells. Class II molecules present exogenous antigens such as bacterial fragments to T cells.

The TCR is an antigen specific receptor on T cells. The TCR recognizes presented antigen through the antigen recognition portion in its structure. This recognition structure is the variable domain which contain within it hypervariable regions. Hypervariable regions are variable sequences that determine antigen specificity. These regions are critical to antigen binding. Individual TCRs have different variable domains, which enable different TCRs to recognize specific antigens when presented by a MHC molecule. The TCR is thus specific for the peptide and MHC molecule.

The T cell surface, also, has accessory molecules such as CD2 or CD28 which have implications for antigen recognition. Additionally, coreceptors like CD4 or CD8 can be found on the T cell surface. CD4 and CD8 determine the effector function as well as MHC restriction. T cells are MHC restricted because they are limited in the ability to recognize a specific antigen. T cells will only recognize a antigen complexed with a specific MHC molecule. CD4 T cells recognize MHC class II, whereas CD8 T cells recognize MHC class I molecules. CD4 T cells are referred to as T helper (Th)cells. These cells can be further divided into Th1 or Th2 depending on the cytofine that they release. For instance, Th1 cells release interleukin (IL)-2, interferon gamma, and tumor necrosis factor beta. These cytokine produce a more cell mediated response. Th2 cells, on the other hand, release IL-4, -5, -6, and -10. These cytokine result in a humoral response.

T cell activation

Pairing between T cell and APC is dependent on the interaction of many molecules. For instance, TCR on CD4 T cells interact with MHC class II molecules on APC, CD2 interact with leukocyte function associated antigen (LFA-3), and LFA-1 with intracellular adhesion molecule (ICAM). The initial interaction between TCR and MHC:peptide complex is required along with a second signal for T cell activation. The second signal is provided by costimulatory molecules such as CD28 on T cells binding to B7 on APC (Santana and Rosenstein, 2003).

Upon ligation of the TCR and MHC, a series of biochemical reactions occur in the T cells. Protein tyrosine kinases, *lck* and *fyn*, phosphorylate the CD3 complex at immuno-tyrosine activation motifs. This is followed by recruitment and phosphorylation of *Zap70*. Activated *Zap70* then activates *SLP76* and linker for activation of T cells (LAT). Phospholipase C gamma 1 is recruited and activated, leading to the production of diacylglycerol (DAG) and inositol triphosphate (IP3). DAG activation leads to PKC activation and *RasGRP*, whereas, IP3 leads to calcium mobilization and NFAT activation. These reactions lead to proliferation and differentiation into effector cells. In their effector roles, T cells are able to activate other immune cells, thereby, driving the immune response and elimination of the immunological threat. Activation of T cells lead to its effector function (Del Prete, 1998; Lin and Weiss, 2001). Following the immune response, it is critical to remove the activated cells by initiating cell death (Hildeman et al., 2002; Strasser and Pellegrini, 2004).

Cell death mechanisms

Necrosis. Cells die for many reason and by different mechanisms. During necrosis, cells characteristically swell due to the loss of membrane integrity. This loss can be due to a chemical or physical injury. Many cells can undergo necrotic death . The internal and plasma membrane undergo lysis due to loss of ion homeostasis, which alters membrane integrity. This loss leads to organ swelling, including the nucleus which promote nuclear membrane disruption. Phosphatidylserine on the outer leaflet of the membrane becomes exposed. This process produces cellular debris that produces an inflammatory response, which recruits phagocytes to the site. Phagocytosis is required to remove the debris. Necrosis is marked , additionally by, mitochondria damage, calcium release and reactive oxygen species (Jaattela and Tschopp, 2003)

Apoptosis. The induction of apoptosis may be caused by general external or internal stimuli (development signals) or specific stimuli of death activators (growth factors, cytokines, FasL) or toxic stimuli (hormones, radiation, oxidants). External stimuli that initiate apoptosis include: mechanical damage, oxidative stress, corticosteroids, or toxic chemicals. Internal initiators may be represented by negative signals (DNA damage, increased intracellular oxidants) or withdrawal from positive signals (growth factors). Apoptosis is necessary during developmental processes and to remove cells that are harmful to host integrity. Death by apoptosis is a natural process that is run by an internal program. Characteristic morphological features of apoptosis include: cell shrinkage and fragmentation, membrane blebbing and ruffling, DNA fragmentation and

internal program. Characteristic morphological features of apoptosis include: cell shrinkage and fragmentation, membrane blebbing and ruffling, DNA fragmentation and laddering, and phagocytosis of apoptotic bodies. Other characteristics that are important are the release of cytochrome *c* and caspase activation (Krammer, 1999).

Apoptosis is a well regulated energy requiring process that involves activation and an enzymatic cascade. Two apoptotic pathways have been described, death receptor (extrinsic) mediated and mitochondrial (intrinsic) mediated. During death receptor mediated apoptosis, the cell receives an external signal in the form of a death ligand that binds a cognate cell surface receptor. The Tumor Necrosis Factor Receptor (TNFR) family represents a superfamily of proteins that facilitate apoptosis. This family has pleiotrophic activities in that depending on the signal, these receptors can initiate different programs from proliferation to death (Nagata, 1997; Ashkenazi and Dixit, 1998; Wallach et al., 1998). These molecules have several features in common including a death domain internally that functions in signaling and an extracellular 2 to 5 cysteine-rich repeat region in the ligand binding domain. These repeats serve as points of recognition for specific ligands. There are 6 known members currently (Schulze-Osthoff et al., 1998). The Fas (CD95/APO-1) molecule is the proteotypic member of the family. The Fas molecule is a 45 kDa type I transmembrane protein. Fas is ubiquitously expressed on many normal cells such as T cells (Suda et al., 1995). These molecules function as trimers to induce apoptosis.

The Tumor Necrosis Family (TNF) Ligand superfamily is a family of cognate ligands for the death receptors. These ligands are in the form of homotrimers. The FasL

molecule is a type II cell surface glycoprotein that is 40kDa. As a type II protein, FasL has a cytosolic N- terminus and an extracellular C-terminus. Unlike the ubiquitously expressed Fas molecule, the FasL molecule is sparsely expressed. Activated T cells have been shown to express FasL (Suda et al., 1995).

The importance of Fas/FasL expression on the T cell is seen during development and following activation. As discussed previously, developing thymocytes progress through different stages. The vast majority of developing thymocytes, approximately 95%, are deleted by apoptosis during negative selection (Jenkinson et al., 1989; Owen and Jenkinson, 1989; Nikolic-Zugic, 1991; Cairns et al., 1993; Castro et al., 1996). It is thought that the Fas/FasL interaction may be important during these processes in the thymus. The role in mature peripheral T cells is well characterized. Following the effector function performed by activated T cells in response to antigen, the response must be terminated. Termination of the response and cell removal is pivotal for the maintenance of homeostasis and tolerance. Activation induced cell death (AICD) is initiated by death receptors. Resting T cells are resistant to Fas mediated apoptosis (Salmon et al., 1994). Upon antigen engagement, resting T cells become activated, the cells upregulate Fas on the surface (Trauth et al., 1989). Activated T cells then cycle and proliferate. It is during this cycling period that cells become sensitive to apoptosis (Lissy et al., 1998). Upon a second activation, FasL is expressed and death is initiated. Activated T cells undergo apoptosis in an indirect manner due to antigen. Antigen engagement leads to the expression of death ligands such as FasL (Dhein et al., 1995). The mechanism is thought to be initiated by TCR ligation followed by the induction of

FasL expression on the surface. FasL, cleaved or membrane bound, bind Fas on the same or nearby cell. This interaction leads to apoptosis initiation (Alderson et al., 1995; Brunner et al., 1995; Dhein et al., 1995; Ju et al., 1995).

The mechanism of death receptor mediated apoptosis is well choreographed. Upon the ligation and trimerization of Fas by FasL, a death inducing signal complex (DISC) is formed via the death domain portion of the Fas receptor molecules. The DISC is a multi-protein complex required for Fas mediated apoptosis (Kischkel et al., 1995; Scaffidi et al., 1999). This complex is comprised of Fas, Fas-associated death domain (FADD), and pro-caspase 8. As an adapter molecule FADD binds the receptor through its death domain and pro-caspase 8 through its death effector domain. Once procaspase 8 is recruited and bound, the procaspase is cleaved. Caspases are made as proenzymes that require cleavage for activity. The procaspase has four domains including a prodomain, large fragment, a linker, and small fragment. Junctions between the segments are flanked by an aspartate residue. The proenzyme is cleaved at specific penta peptide (QACXG)sequences, that contains a cysteine active site (Cohen, 1997). Cleavage of the peptide occurs C-terminal to the aspartate residue. Procaspases can range from 32-56 kDa with four domains. The large fragment can be 17-21 kDa, whereas the small is from 10-13kDa (Earnshaw, 1999). Upon specific cleavage, a heterodimer comprised of the larger and small fragments is formed, representing a functionally active caspase (Liang and Fesik, 1997).

Upon the autocatalytic activation of procaspase 8, down stream caspases can be activated. The cleavage and activation of caspase-3, -6, and -7 promote the apoptotic

cellular changes. Cleavage of these effector caspases promotes cleavage of structural (laminins, actin), signaling (PKC delta) and regulatory proteins (repair proteins, replication) (Cross et al., 2000; Fadeel et al., 2000; Stennicke, 2000; Stennicke and Salvesen, 2000; Kaul et al., 2003). These cleavages are seen in the nuclear fragmentation and cellular shrinking characteristic of the apoptotic cell. The death receptor pathway has an additional pathway that is dependent on the cells type. The direct pathway of apoptosis from caspase-8 cleavage to caspase-3 cleavage is observed in type I cells. Thymocytes, peripheral T cells and some tumor cell lines such as SKW are considered type I cells. A second pathway can be activated in cases where the amount of caspase-8 is insufficient to promote DISC signaling. This reduction in caspase-8 leads to a reduction in DISC formation. In this scenerio, an additional molecule is recruited. Bid is a Bcl-2 family member that binds to caspase-8 and is cleaved. The Bcl-2 family is a diverse family of both proapoptotic and antiapoptotic members. The proteins can have Bcl-2 homology (BH) regions 1-4. All proapoptotic members have a characteristic BH3 domain. Recruited Bid is cleaved and activated. Upon activation the C-terminal region of truncated (tBid) Bid translocates from the cytosol to the mitochondria where it interacts, causing mitochondrial dysfunction. In this pathway, there is crosstalk between death receptor and mitochondrial pathways. Cells that utilize this pathway are subject to Bcl-2 regulation (Nagata, 1996b; Nagata, 1996a; Scaffidi et al., 1998).

A second pathway leading to apoptosis is the mitochondrial (stress) pathway. In this pathway, a stress such as cytotoxic drugs, DNA damage, or oxidative stress are stimulators. Several proteins, such as p53 or Bcl-2 proteins, pass along death signals to

the mitochondria (Marchenko et al., 2000). Upon their activation, these proteins translocate to the mitochondria and interact with Bcl-2 proapoptotic proteins promoting membrane dysfunction and the release of apoptogenic proteins. Mitochondrial dysfunction results from the interaction of proapoptotic molecules with the mitochondria causing loss of membrane potential. Loss of mitochondrial membrane potential ($\Delta\Psi_m$) has been suggested to be the result of formation of a permeability transition pore. The pore is known to be comprised of inner membrane proteins (adenine nucleotide translocator) and outer membrane proteins (porins, voltage dependent anion channel) (Qian et al., 1997). The pore allows the ions to flux. An asymmetric H^+ distribution develops across the inner membrane (Zamzami et al., 1995a; Zamzami et al., 1995b). The H^+ gradient is then dissipated across the membrane promoting uncoupling of oxidative phosphorylation processes. Additionally, hyperosmolarity of the mitochondrial matrix promotes swelling which disrupts the outer mitochondrial membrane. Disruption of the outer membrane leads to the release of intermembrane proteins such as SMAC, cytochrome *c*, procaspases-3, -9, and AIF. These intermembrane proteins help to activate caspases. Membrane rupture releases cytochrome *c* which binds apoptosis activating factor-1 (Apaf-1). This process requires dATP. Binding of Apaf-1 recruits procaspase-9 and cleaves it, producing activate caspase-9. The resulting complex that is formed is called the apoptosome (Li et al., 1997; Cain et al., 1999; Cain et al., 2000). Following caspase-9 activation, caspase-3 is activated and cleaves poly(ADP-ribose) polymerase (PARP). Following PARP cleavage, other downstream caspases such as caspase-6 and -7 are cleaved promoting cellular deconstruction.

The mitochondria is considered to be an early target during apoptosis and is thought to coordinate the death pathway (Kroemer et al., 1995; Kroemer et al., 1997; Green and Reed, 1998). Many signal and pathways intersect at the point of the mitochondria. In addition to these abilities, the mitochondria was originally described for its energy producing ability. This second role for the mitochondria in apoptosis is well established. These findings point to the mitochondria playing pivotal roles in both life and death of cells. Death mechanisms include: 1) electron transport disruption, oxidative phosphorylation, and ATP synthesis, 2) apoptogenic protein release, and 3) alteration in redox potential. The disruption of the electron transport pathway and energy metabolism are early events. Alterations in cellular redox lead to the increased production of reactive oxygen species which contribute to apoptosis development. The damage to the mitochondria becomes more substantial and cumulative as time progresses, until the effect is irreversible. At this point, death becomes eminent.

Neuroendocrine Influence of the Immune System

Recent evidence supporting crosstalk between the endocrine and immune systems has emerged. Both functional and structural roles have been suggested with regard to regulation. Immunologic mediators (biochemical) can gain entry to the brain (central nervous system) through organs that circumvent and stimulate the hypothalamus. The immune response is activated by the autonomic nervous system and neuroendocrine systems. Communication between endocrine and immune system is bidirectional

(Besedovsky and del Rey, 1996). As a result, the immune response can be altered by neurohormones. This is not surprising due to the presence of receptors for hormones (Gillette and Gillette, 1979), corticosteroids (Werb et al., 1978), and neurotransmitters (Strom et al., 1974; Richman and Arnason, 1979) that have been observed on immune cells. Neuronal and hormonal effects on the immune system can stimulate or inhibit (Berczi, 1989).

The immune system (lymphoid, myeloid) are susceptible due to nervous supply and hormonal input on nerves. The ability of lymphoid organs to produce both hormones and neuropeptides, in addition to, endocrine organs and nerve cells being able to produce different cytokines further points to an interdependence.

The thymus is not only regulated by cytokines, but thymic hormones as well. The thymus can produce several hormones including thymopoietin, TP-5, thymosin, thymic hormonal factor, and thymulin. The function and maintenance of the thymus is dependent on both endocrine and neural input (Savino et al., 1998; Savino and Dardenne, 2000). For instance, the pituitary plays a role in growth and function (Galy et al., 1990). As can be imagined, a proper balance of the endocrine input is critical to the functioning of the thymus (Berczi, 1989). A possible role has been suggested in thymocyte differentiation process for sex hormones.

Structurally, the thymus is supplied with sympathetic nerves (Felten et al., 1988). Thymocytes differentiate and proliferate under the influence of the autocrine (cell acts on self released molecules), paracrine (cell released molecules act on neighboring cells), and endocrine systems/hormones (molecules act on distal targets). The immature T cells

must be able to receive signals from the thymic environment (thymic epithelial cells) and act upon those signals (hormones). Signals either autocrine or paracrine influence development, maintenance, and growth of lymphocytes. Stromal contact provides signals for tolerance, survival, and apoptosis. Additionally, cytokine/chemokine signals cause lymphocyte activation, proliferation, differentiation, and effector function. Similarly, mature T cells upon antigen engagement receive hormonal or cytokine signals that direct functional behaviors.

Glucocorticoids have been found to promote thymocyte survival during development, by countering deletion signals. Not surprisingly, the glucocorticoid receptor (GR) has been found on lymphocytes (Miller et al., 1998) and additionally, a model has been proposed for the role in selection. It is thought that during selection, the balance between TCR and GR signals is important. Low to moderate TCR interaction promotes positive selection (Sprent et al., 1988; Schwartz et al., 1989; Ashton-Rickardt and Tonegawa, 1994; Hogquist et al., 1994). The thymus is equipped with steroidogenic enzymes in the thymic epithelial cells specifically (Vacchio et al., 1994). It is this ability to produce hormones that enables thymocytes to be subject to hormonal control during development.

Estrogen

Estrogen is a steroidal hormone that influences growth, differentiation, and function of many tissues or cells (Fillit et al., 1986; Turner, 1994; Farhat et al., 1996). Estrogens due to their lipophilic structure move freely in and out of cells. Lipid soluble hormones, like estrogen, interact with cytosolic or nuclear receptors. Steroidal estrogens have a characteristic phenolic A ring (Duax and Griffin, 1987). Estradiol is the proteotypical example. Upon estrogen receptor (ER) binding in target tissues, estrogen is mobilized. The ER is a nuclear receptor. As a nuclear receptor, the ER is located not on the cell surface, but is found intracellularly. The bound receptor translocates to the nucleus, where it interacts with DNA via binding an estrogen responsive element (ERE). Upon binding, transcription of estrogen responsive genes occurs (Beato et al., 1995; Jensen, 1995; Tsai-Morris et al., 1995).

Two isoforms, designated alpha and beta, of the ER have been discovered. Transcripts have been found in different organs or tissues. For instance, alpha has been found in many tissues including mammary glands, pituitary glands, and adrenals (Kuiper et al., 1996; Kuiper et al., 1997). The beta isoform has more recently been characterized (Mosselman et al., 1996), have transcripts in the prostate, ovaries, nervous system, and immune cells.

The structure of the isoforms are similar. Each contains an A/B domain, C domain, D domain, E domain, and F domain. The A/B domain functions in protein:protein interactions (ligand independent interactions) and has an activation

function (AF-1). The C domain functions in DNA binding. The D/E/F domains function in ligand binding and contains a second activation function (AF-2) (Evans, 1988; Mangelsdorf et al., 1995; Katzenellenbogen and Katzenellenbogen, 1996). The DNA binding domain has 96% homology and the ligand binding domain has 58% homology (Mosselman et al., 1996).

Communication between Estrogens, Immune system and Endocrine system (Endocrine disruption)

Environmental estrogens can have significant effects on the immune system. These endocrine disruptors can be natural, synthetic, or environmental estrogens. Because the immune system communicates to the endocrine, dysregulation of the hypothalamus-pituitary axis not only produces disorders in the immune system, but the endocrine system as well (Jankovic, 1989).

The communication between the systems is complex. At the helm is the hypothalamus, which is part of the central nervous system. From this focal point the hypothalamus sends chemical signals to the pituitary gland which in turn signals the endocrine glands (adrenals) or tissues (gonads). The endocrine glands release hormones which can feed back to the hypothalamus. Additionally, these hormones can act on the thymus, bone marrow, spleen, or lymph nodes. The thymus can, also, provide negative feedback to the hypothalamus (Grossman and Roselle, 1983; Grossman, 1984; Grossman, 1985; Emi et al., 1991; Hughes et al., 1991). As can be seen, the hypothalamus directs the pituitary, which directs the adrenal, gonads, or thymus. These glands either directly

or indirectly act on lymphocytes to alter function. Additionally, there is communication between gonads and the thymus.

The HPA controls a variety of systems and is itself influenced by these systems. The effects of sex hormones such as estradiol have been found to alter lymphocyte cellularity in various organs such as thymus and spleen (Staples et al., 1998; Sakazaki et al., 2002). Alterations in different T cell populations following estradiol treatment point to the ability to influence not only cellular population, but immune responsiveness as well. One consequence of this could be autoimmunity. It has been observed that androgens can suppress effector T cells (Grossman et al., 1991). For example, an increase in the HPA activity can lead to increased infection (Mason, 1991), whereas a decrease can lead to autoimmunity (McEwen, 1998a; McEwen, 1998b). The effects of sex hormones can be direct via receptors or indirect through cytokine or thymic hormone interaction with receptors.

General effects to immune system. The result of endocrine toxicity can lead to immune dysfunction, in addition to other abnormalities. Immune dysfunction can take the form of immunosuppression, which is an immunomodulatory effect. Immunosuppression can be local or systemic in nature. Local immunosuppression refers to organ specific effects, whereas systemic immunosuppression refers to a more broad effect. Some examples of system effects include: alterations in lymphoid organ size or weight and appearance, alterations in lymphoid tissue cell number, peripheral blood leukocyte number, and functional impairments of effector molecules eg Th cells. These systemic alterations can produce susceptibility to infection and cancer .

Estrogen and lymphoid cells. Both primary and secondary lymphoid organs exhibit sensitivity to estrogens (Ansar Ahmed and Talal, 1989; Verthelyi and Ahmed, 1998; Donner et al., 1999). Estrogens alter immune response either by direct action on specific lymphoid cells or either indirectly via lymphoid targets such as the HPA. T cells, while requiring physiological estrogen levels for maintenance and function, high estrogen concentrations produce immunosuppressive effects. T cell homeostasis can be altered following prolonged exposure.

Lymphoid cells produce cytokines that transmit chemical directions to effector cells. Estrogens can cause altered cytokine production (Fox et al., 1991; Li et al., 1993). For instance, estrogen can stimulate cytokine secretion of interferon (IFN) gamma and several interleukins. Increased cytokine production has been linked to autoimmunity (Haas et al., 1998). Alternatively, estrogens have been found to suppress IL-2 and IL-2 receptor (McMurray et al., 2001). Estrogens can inhibit proper development of primary lymphoid sites thereby influencing development of T cells within the site. Altered selection processes during T cells development is one example. Alterations to sensitive processes like selection may contribute to autoimmunity (Holladay and Smialowicz, 2000). Bypassing immunological tolerance promotes the production of autoreactive immune cells. As previously discussed, the removal of such cells is an important process. This normally occurs through apoptosis. Estrogen can interfere with the natural apoptosis process. Consequently, estrogen can alter effector functions. Specifically, estrogen has been shown to be suppressive toward T cell mediated responses (Pfeifer and Patterson, 1986; Salem et al., 2000) and suppress lymphoproliferation in general (Herrera

et al., 1992). In addition to T cells, B cells are, also, targets. Estrogen has been found to, also, have differential effects on B cells depending on the dose. B cells can be stimulated or suppressed. Estrogens have been found to stimulate antibody production which could promote autoimmunity (Verthelyi and Ahmed, 1998). Natural killer (NK) cells are non-T and non-B cell lymphocytes. NK cells exposed to suprapharmacological levels of estrogen have been shown to have reduced activity (Seaman and Gindhart, 1979; Seaman et al., 1979; Kalland, 1980). Given that NK cells function to remove tumors and viruses, a reduction in these activities would greatly compromise innate immunity (Brutkiewicz and Sriram, 2002).

Estrogen and myeloid cells. Myeloid cells consist of macrophage and granulocytes (neutrophils, eosinophils, basophils). Estrogens can target macrophage either directly or indirectly. Myelotoxicity can be manifested in different ways (Fried et al., 1974; Boorman et al., 1980). Typically, estrogens cause inhibition of degranulation or release of lysosomal enzymes. Estrogens have been demonstrated to inhibit nitric oxide production as well. Conversely, estrogens can stimulate some activities such as IL-1 and IL-6 secretion (Gulshan et al., 1990). Estrogens have been found to stimulate the reticuloendothelial system (macrophage and mononuclear cells) and peritoneal macrophage (Gulshan et al., 1990; Olsen and Kovacs, 1996). The progression from monocyte to macrophage has been shown to be estrogen sensitive (Mor et al., 2001).

Developmental stage and Immunological Consequences of Estrogens

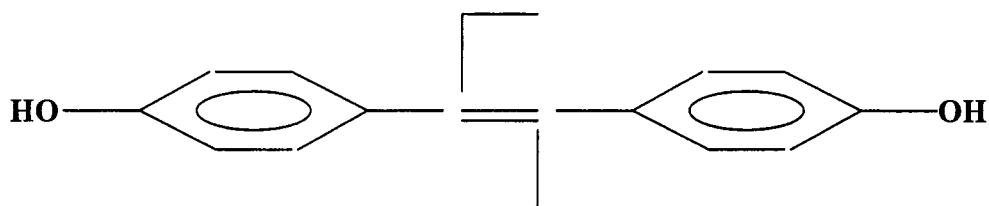
Perinatal exposure to androgens and estrogens have been found to permanently alter tissues, by altering the microenvironment of developing lymphocytes and thus their function . One possible consequence of altered function is autoimmunity (Noller et al., 1988; Wingard and Turiel, 1988). T cell developmental studies have demonstrated the ability of estrogens and estrogen-like chemicals to alter the course of development. Development has been found to be blocked at the DN to DP transition, resulting in decreased mature SP populations (Silverstone et al., 1994; Lai et al., 2000; Okasha et al., 2001). Prothymocytes have been shown to be sensitive targets, causing reduction in cell numbers, as well (Holladay et al., 1993).

Fetal and early postnatal exposure periods in vertebrates are highly sensitive to the effects of endocrine disruptor, producing impairments later. Such effects are permanent and irreversible. Exposure to such chemicals can magnify aberrant responses producing autoimmunity and hypersensitivity (Holladay and Smialowicz, 2000). Alterations in T cells subsets and functionality have been demonstrated to be altered (Calemine et al., 2002).

Diethylstilbestrol

Structure. Diethylstilbestrol is a stilbene derivative. The structure is not like steroidal estrogens and is therefore a nonsteroid (see Fig. 1 below). As an estrogen like compound, DES functions like estrogens to alter cell function.

Diethylstilbestrol (DES)



17 beta estradiol (E2)

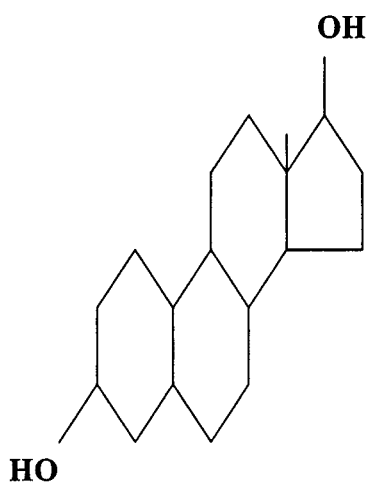


Figure 1. Structure of Diethylstilbestrol

History. Diethylstilbestrol (DES) is a synthetic estrogen that was prescribed in the United States between the 1940's and early 1970's to reduce the risk of spontaneous abortions in pregnant females (Smith and Smith, 1949; Marselos and Tomatis, 1992). DES was, also, prescribed to women with uneventful pregnancies. As a result, approximately 5-10 million Americans were exposed to DES. Prenatal DES exposure has been shown to produce reproductive toxicity and more recently, immunotoxicity in humans (Noller et al., 1988; Giusti et al., 1995; Baird et al., 1996).

Reproductive/ Teratogenic consequences. Most studies regarding DES toxicity have been focused on reproductive toxicity and teratogenicity. Prenatal exposure of females has shown an increased risk of reproductive tract abnormalities including abnormal uterine shape, defects in the fallopian tubes, or structural abnormalities in the vagina or uterus (Treffers et al., 2001). Uterine malformities including hypoplastic cavity, T shaped uterus, and constriction bands, have been observed (Scully et al., 1974). Fallopian tube defects such as shortened, sacculated, or convoluted tubes have been observed. Structural abnormalities of the vagina can take the form of adenosis and transverse fibrous ridges. Particularly, the incidence of vaginal adenosis is high (Kaufman et al., 1986). In addition to these gross and microscopic abnormalities of the vagina, cervical abnormalities such as erosion, eversion, or hypoplastic cervix collar have been found (DeCherney et al., 1981). Such abnormalities can cause an increase in infertility, spontaneous abortions, ectopic pregnancy, preterm delivery, and decreased birth weight (Scully et al., 1974).

In prenatally exposed males, urogenital abnormalities have been documented. Alterations in reproductive tract structures produce lesions. Some common abnormalities are epididymal cysts, hypoplastic testes, and cryptorchidism (Gill et al., 1977). Other alterations that have been reported include altered semen quality (Gill et al., 1979; Whitehead and Leiter, 1981).

Immunological consequences. Alterations to the immune system by DES exposure has been observed in both murine and human species. For instance, rodents have been shown to exhibit the following: myelotoxicity, suppressed cell mediated immunity, thymic atrophy, and reduced natural killer cell activity. In humans, prenatal exposure has been shown to alter T cell function (Luster et al., 1978; Ways et al., 1987) and natural killer cell function (Kalland, 1980; Ford et al., 1983). Given that immune suppression or enhancement can lead to immune dysfunction, DES exposure can lead to significant immunological consequences.

One form of immune dysfunction is cancer. Cancer is a multistage event. DES has been found to form adducts with DNA (Gladek and Liehr, 1989). Several genotoxic activities have been noted including: unscheduled DNA synthesis (Tsutsui et al., 1984) and sister chromatid exchange (Rudiger et al., 1979; Hilland Wolff, 1983).

DES exposure can occur on two levels, that of the mother and the developing embryo/fetus. Postnatal exposure, such as with the mothers, has been shown to increase the risk of breast cancer (Greenberg et al., 1984). Cancerous changes have been documented in both daughters and sons. In exposed daughters, cancer development has been well characterized. DES has been shown to cause clear cell carcinomas of the

cervix and/or vagina (Herbst et al., 1972; Scully et al., 1974; Herbst and Anderson, 1990). Other cancers that have been observed in females include malignant teratoma of the ovary, as well as, pituitary adenoma, and adenosquamous cell carcinoma (Ways et al., 1987; Burke et al., 2001). In exposed sons, testicular cancer has been observed (Gill et al., 1977).

A second form of immune dysfunction is seen in autoimmune disease. Such disorders have been noted in both murine species and in humans. In murine species, DES exposure during development in a predisposed subject lead to enhanced or induced disease postnatally (Holladay, 1999). Similarly, prenatal exposure to DES has been shown to increase the incidence of diseases such as Hashimoto thyroiditis (Noller et al., 1988).

DES and the thymus

In the adult thymus, the ability of estrogen exposure to trigger thymic atrophy has, also, been observed in murine models (Okasha et al., 2001; Calemme et al., 2002; Do et al., 2002). Furthermore, DES administration in vivo has been demonstrated to be immunomodulatory, affecting the thymocytes by inducing apoptosis and altering proliferation (Calemme et al., 2002). Additionally, lymphocyte responsiveness to mitogens has been found to be suppressed following DES exposure (Luster et al., 1984; Calemme et al., 2002; Utsuyama et al., 2002).

In the developing thymus, similar effects are observed. Fetal thymic organ culture studies have demonstrated that estrogens like DES can block thymocyte development by cell cycle arrest and apoptosis (Lai et al., 1998). Additionally,

prothymocytes are particularly sensitive to DES and produces reduced numbers. Studies in adult mice have produced similar cellular reductions in not only thymus but bone marrow as well. Stem cell precursors have, also, been demonstrated to be reduced (Holladay et al., 1993). Although the mechanisms behind immunomodulation are under investigation, little is still known.

Specific Aims

The ability of endocrine disruptors such as DES to negatively impact the functionality of immune cells has become a topic of much concern. Many studies from our lab and others have shown the ability of estrogens like DES to induce thymic atrophy and apoptosis (Do, y et al. 2002, Okasha, SA et al. 2001; Calemene, JB et al. 2002). Additionally, estrogens have been shown to alter T cell development (Silverstone, AE et al. 1994 ; Lai, ZW et al. 1998). The mechanism behind these observations is unclear. In the current study, we will test the central hypothesis that estrogens such as DES would alter the T cell differentiation in the thymus by inducing apoptosis and that such an effect could compromise the peripheral immune functions. To this end, we will address the following specific aims:

1. Determine the ability of DES to induce apoptosis in aT cell leukemia cell line.
2. Determine the role of apoptosis in development of T cells in the thymus.
3. Determine whether DES could induce alterations in positive and/or negative selection of T cells.
4. Determine the role of thymic stromal cells in DES-induced apoptosis in T cells of the thymus.

Chapter II. Diethylstilbestrol Can Induce Apoptosis in Malignant Human T Cells Through Regulation of Intrinsic and Extrinsic Pathways

Abstract

Estrogens have been shown to induce apoptosis in normal immune cells. In the current study, we investigated the ability of diethylstilbestrol (DES), a synthetic estrogen to induce apoptosis in human Jurkat T leukemia cells. Treatment of wild-type (WT) Jurkat cells *in vitro* with μM concentrations of DES induced dose-dependent decrease in cell viability and increase in apoptosis. We investigated the mechanism of apoptosis induction using FADD-deficient (FADD^{-/-}), Bcl-2 over expressing (Bcl-2⁺⁺⁺) and caspase-8 deficient (C8^{-/-}) Jurkat cells. Interestingly, FADD^{-/-} Jurkat cells showed significant resistance to apoptosis at lower concentrations of DES compared to WT Jurkat cells. This, combined with the cleavage of caspase-8 and -10 as well as partial blocking of apoptosis by these caspases inhibitors, suggested the involvement of death receptor pathway. Jurkat cells treated with DES also exhibited mitochondrial pathway of apoptosis as evidenced by dose-dependent loss of mitochondrial membrane potential, cytochrome *c* release and caspase 9 cleavage. Moreover, Bcl-2⁺⁺⁺ Jurkat cells were sensitive to apoptosis with increasing concentrations of DES. Further studies revealed that the mitochondrial pathway may be recruited independently and/or through cross-talk involving the death-receptor pathway through Bid cleavage.

Examination of the estrogen receptor (ER) isoform on WT Jurkat cells revealed the presence of ER beta not alpha. However, DES-induced apoptosis was ER-independent. DES treatment upregulated the expression of several death receptors and cognate ligands. DES treatment also activated SAPK/JNK, suggesting its role in apoptosis. Together, these data suggest that estrogens may serve as novel anti-cancer agents to treat certain malignancies of the immune system.

Introduction

In recent years, reproductive hormones like estrogen have been shown to exert pleiotropic effects on the immune system. Immune cells express estrogen receptors (ERs) through which estrogen may regulate immune cell function and differentiation (Couse and Korach, 1999). The importance of ER in T cell maturation is evident from studies in mice with ER deficiency that display thymic hypoplasia (Yellayi et al., 2000). Moreover, thymic atrophy and apoptosis in T cells has been observed with estrogen treatment (Do et al., 2002; Hirahara et al., 1994; Okasha et al., 2001; Silverstone et al., 1994).

Estrogens exert effects on target cell physiology through the ER isoforms alpha and beta which constitute ligand-dependent transcription factors (Kumar et al., 1987). There are two major groups of estrogens, endogenous and exogenous. Exogenous estrogens called xenoestrogens such as phytoestrogens mediate estrogen-like functions. Exogenous estrogens have many uses, from oral contraceptives and hormone replacement to cancer therapy (Ruggiero and Likis, 2002). Endogenous estrogens play important roles in the development and functions of a variety of tissues and cellular metabolism (Katzenellenbogen and Katzenellenbogen, 1996).

Diethylstilbestrol (DES), a stilbene derivative, is a synthetic estrogen with estrogenic activity and was used extensively throughout the United States and Europe between the 1940's and 1970's to treat pregnant women. It is estimated that ~5-10 million women were exposed to DES during pregnancy or in utero (Giusti et al., 1995). Exposure to DES in this manner has resulted in elevated risk for breast cancer in DES-

mothers and cervicovaginal cancers in DES-daughters (Bornstein et al., 1987; Cutolo et al., 1995). In addition to cancer, autoimmunity may be more common in pregnant women treated with DES (Melnick et al., 1987).

From the late 1950s through the early 1980s DES was also the most widely used chemohormonal agent against prostate cancer (Scherr and Pitts, 2003). However, DES treatment was associated with high levels of cardiovascular toxicity, because of which the treatment became unpopular. Currently, use of DES has rekindled interest as an alternative treatment of prostate cancer and test the concept of “androgen deprivation without estrogen deprivation” (Scherr and Pitts, 2003). DES has also been shown previously to induce apoptosis in prostate cancer cells (Robertson et al., 1996). Despite the recent evidence that estrogens including DES can induce apoptosis in immune cells (Calemine et al., 2002; Do et al., 2002; Mor et al., 2001; Okasha et al., 2001), the precise mechanism by which they induce apoptosis is not clear. Also, whether DES can induce apoptosis in transformed T cells has not been previously investigated. In the current study, therefore, we used Jurkat, a human transformed T cell line to test whether DES could induce apoptosis in malignant immune cells and if so, we wished to further characterize the mechanism(s) of apoptosis. The data suggested that DES can induce apoptosis in Jurkat cells through cross-talk involving the death-receptor and mitochondrial pathways of apoptosis.

Materials and Methods

Cell lines

Jurkat T leukemia cell wild-type (WT), caspase 8-deficient Jurkat, FADD-deficient Jurkat (kindly provided by Dr. Blenis, Harvard), and Bcl-2 overexpressing Jurkat (kindly provided by Dr. J. Cidlowski, NIH) were used in this study. Cells were maintained in RPMI-1640 supplemented with 10% fetal calf serum, 10 mM Hepes, 1mM glutamine, and 50 µg/ml gentamycin. All experiments were performed in serum-free medium.

DES exposure

Diethylstilbestrol (Sigma, St. Louis, MO) was dissolved in DMSO (Sigma, St. Louis, MO). Cells received a single dose of DES at various concentrations such as 1, 10, 25, or 50 µM or vehicle.

Cell preparation and determination of cellularity

Cells were harvested, washed, and resuspended in PBS. The viable cells were counted using trypan blue dye exclusion.

Detection of apoptosis using TUNEL staining or Caspase 3/7 activity

Apoptosis was detected using TUNEL assay using FITC-dUTP as described (Kamath et al., 1999) and the cells were analyzed by flow cytometry. In some experiments, caspase 3/7 activity was used as an endpoint for apoptosis determination.

The Apo-One homogeneous caspase-3/7 assay purchased from Promega, Madison, WI. Assay was performed per the manufacturer's instruction. For caspase inhibitor studies, 50 μ M of inhibitors such as caspase 2 (Z-VDVAD-FMK), caspase 8 (Z-IETD-FMK, caspase 9 (Z-LEHD-FMK), or caspase 10 (Z-AEVD-FMK) purchased from R&D Systems, Minneapolis, MN were added to cell cultures in the presence or absence of DES and apoptosis detected as described above. In some experiments, cells were incubated with Nok-1 antibody (BD Pharmingen, San Diego, CA) prior to treatment with DES and apoptosis detected as described above. Estrogen receptor antagonists tamoxifen (Sigma, St. Louis, MO) or ICI 182,780 (Astra Zeneca, Macclesfield, UK) were incubated with Jurkat cells prior to DES treatment and apoptosis was detected as described above.

Mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\Psi_m$) was detected using DiOC₆ staining as described earlier (Zamzami et al., 1995). Briefly, 1×10^6 cells were treated with DES or vehicle for 24 hrs. Prior to harvest, cells were treated with DiOC₆ (40 nM in PBS) for 15 min and harvested. Cells were analyzed by flow cytometry.

Immunoblotting

DES-treated or vehicle-treated cells were lysed, probed by specific antibody and visualized by ECL. In brief, cells were washed in PBS and lysed by freeze-thaw cycle followed by sonication cycle. Following protein concentration determination by BCA protein assay kit (Pierce, Rockville, IL), samples were denatured by boiling in SDS

sample buffer containing 8% 2- mercaptoethanol. Aliquots containing 5µg of protein were subjected to SDS-PAGE on a 12% acrylamide gradient gel, transferred to nitrocellulose, and probed with anti- caspase 2 (Alexis, San Diego, CA), anti- caspase 3, anti- caspase 8, anti-caspase 9, anti-caspase 10, anti-PARP (Cell Signaling Laboratories, Beverly, MA), anti-Bid (R&D Systems, Minneapolis, MN), anti- cytochrome c (BD Pharmingen, San Diego, CA), anti- JNK (Cell Signaling Laboratories, Beverly, MA), anti-phospho-JNK (Cell Signaling Laboratories, Beverly, MA), and anti-beta actin (Sigma, St. Louis, MO) antibodies. Blots were visualized using ECL (Amersham Biotechnology, Piscataway, NJ).

RT-PCR

Cells were treated with DES at 25 µM for 4 hrs. Following exposure, RNA was isolated using RNeasy Kit (Qiagen, Valencia, CA). The following primers were obtained from Operon (Alameda, CA):

Fas: 5'AAGGGATTGGAATTGAGGAAGAC3' and
5'AGACAAAGCCACCCCAAGTTAGAT3'

FasL: 5'CCCCTCCAGGCACAGTTCTTCC3' and
5'TTGTGGCTCAGGGGCAGGTTGTTG3'

ERα: 5'CCCGCCGGCATTCTACAGG3' and
5'ATCGGGGGCTCAGCATCCAACAAG3'

ERβ: 5'CACCTGGGCACCTTTCTCCTTTAG3'
and 5'GCTCGTCGGCACTTCTCTGTCTC3'

βactin: 5'AAGGCCAACCGTGAAAAGATGACC3' and
5'ACCGCTCGTTGCCAATAGTGATGA3'

cDNA expression array

Cells were treated with DES at 25 μ M for 0, 6, or 12 hrs. Following exposure, RNA was isolated using RNeasy Kit (Qiagen, Valencia, CA) and concentration determined by Abs 260/280 reading. RNA content was further analyzed by gel electrophoresis. RNA samples were subjected to GE Array Q series Human Apoptosis Gene Array (SuperArray, Fredrick, MD). Assay was performed per the manufacturer's instruction. Membranes were visualized by chemiluminescence and analyzed by GEArray Analyzer.

Statistical Analysis

Each experiment using flow cytometry was repeated at least twice with consistent results and representative data were plotted. In other experiments, DES-treated group was compared to vehicle controls using ANOVA followed by Dunnett's test and $p < 0.05$ was considered to be statistically significant.

Results

DES induces apoptosis in Jurkat cells in a time- and concentration-dependent manner

To determine the effect of DES treatment on transformed T cells, Jurkat wild-type (WT) cells were treated with 1, 10, 25, or 50 μ M DES or vehicle and 24 hrs later, cells were harvested and viable cell count was determined by trypan blue dye exclusion (Fig 1A) and apoptosis by TUNEL (Fig 1B). DES caused significant decrease in cell viability at 25 or 50 μ M but not at 1 or 10 μ M concentrations (Fig 1A). In contrast, DES induced dose-dependent apoptosis at all concentrations tested (Fig 1B). These data suggested that at higher concentrations, DES may also induce necrosis in addition to apoptosis or that the cells that had undergone apoptosis were transiting into necrosis. When caspase 3/7 activity was measured, it was noted that concentrations of 10 μ M or greater of DES caused significant induction of caspase 3/7 activity that peaked at 24 hrs post-treatment (Fig 1C). However, at the highest dose tested, caspase 3/7 activity was demonstrable as early as 1 hr post-treatment. When the time course of apoptosis induction was studied by TUNEL at 50 μ M DES, significant apoptosis was detected at the earliest time-point (4 hrs) tested and the levels of apoptosis continued to increase with time (Fig 1D).

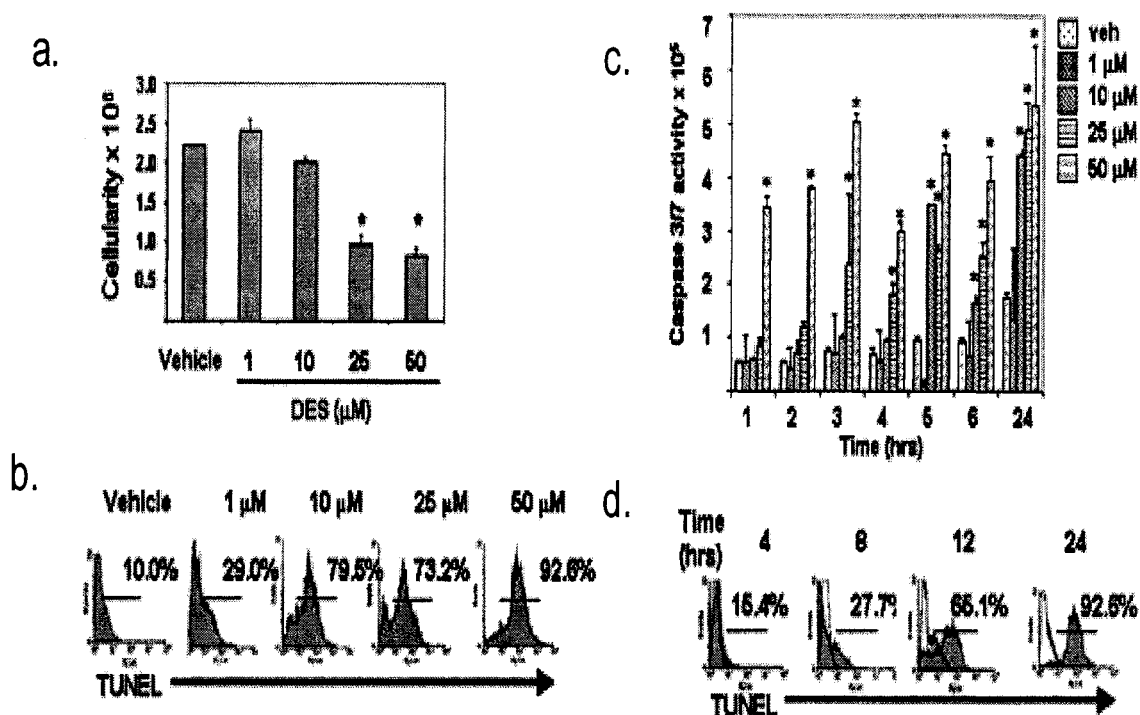


Figure 1. Effect of DES on cell viability and apoptosis in WT Jurkat cells. A) Wild-type Jurkat cells were treated with 1, 10, 25 or 50 μM DES or vehicle and harvested 24 hrs later. Cell viability was determined by trypan blue exclusion. Vertical bars represent the mean \pm SEM of triplicate counts. * Denotes statistically significant differences when compared with vehicle treated cells ($p < 0.05$). B) Cells were treated with 1, 10, 25, or 50 μM DES or vehicle for 24 hrs and analyzed by TUNEL. The percentage of apoptotic cells has been depicted in each histogram. C) Cells were treated with 1, 10, 25, or 50 μM DES or vehicle for 24 hrs and analyzed for apoptosis by caspase 3/7 activity assay. Vertical bars represent the mean \pm SEM. * Denotes statistically significant differences when compared to vehicle control ($p < 0.05$). D) Cells were treated with 50 μM DES (filled histogram) or vehicle (empty histogram) for 4, 8, 12, or 24 hrs and analyzed by TUNEL. The percentage of apoptotic cells has been depicted in each histogram.

DES induces alterations in mitochondrial stability leading to apoptosis

To determine the effect of DES on mitochondria, Jurkat WT cells were treated with 1, 10, 25, or 50 μ M DES or vehicle and 24 hrs later, cells were analyzed for changes in $\Delta\Psi_m$. DES triggered a decrease in $\Delta\Psi_m$ at all concentrations, when compared to the vehicle controls (Fig 2A). To determine the levels of various mediators of apoptosis involving mitochondria, Jurkat WT cells were treated with 50 μ M DES and 4, 6, 8, 10, or 12 hrs later, protein samples were analyzed by immunoblot. Cytochrome *c* release was observed as early as 4 hrs following DES treatment (Fig 2B). Additionally, cleavage products of both PARP and caspase 3 were also seen starting at 4 hrs post-DES treatment. Together, these results indicated that DES treatment induces mitochondrial dysfunction resulting in the release of cytochrome *c* and promoting apoptosis.

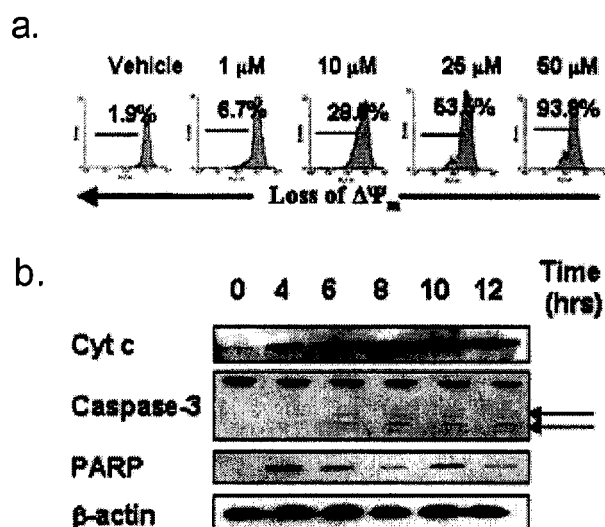


Figure 2. Effects of DES on mitochondrial function in WT Jurkat cells. A) Cells were treated with 1, 10, 25 or 50 μM DES or vehicle and harvested 24 hrs later. Cells were stained with DiOC₆. The percent decrease in mitochondrial membrane potential ($\Delta\Psi_m$) has been depicted in each histogram. B) WT Jurkat cells were treated with 50 μM DES for 0, 4, 6, 8, 10, or 12 hrs. Following protein isolation, 5 μg of protein was subjected to SDS-PAGE, transferred to nitrocellulose membrane and hybridized with Abs against cytochrome c, PARP and β -actin. Lanes without arrows show the proform and those with arrows indicate cleaved form.

DES induced apoptosis involves both extrinsic and intrinsic pathways

To study the role of death receptor pathway in DES-induced apoptosis, we tested the effect of DES on FADD^{-/-} Jurkat cells. As before, these cells were treated with 1, 10, 25, or 50 μ M DES or vehicle and 24 hrs later, the cells were harvested and apoptosis was determined by TUNEL assay, as well as, by DiOC₆(3) staining. When comparisons were made between FADD^{-/-} Jurkat cells (Fig 3A and 3B) and WT Jurkat cells (Fig 1B and 2A) for susceptibility to apoptosis induced by DES using TUNEL and DiOC₆ assays, the data showed that FADD^{-/-} Jurkat cells were more resistant to apoptosis when compared to the WT Jurkat cells at concentrations of 1 and 10 μ M. For example, at 1 μ M, FADD^{-/-} Jurkat cells were completely resistant to apoptosis as detected by TUNEL when compared to vehicle controls (Fig 3A) while Jurkat WT cells showed 29% apoptosis (Fig 1B). Similarly, at 10 μ M, FADD^{-/-} Jurkat cells showed 45% apoptosis (Fig 3A) while Jurkat WT cells showed 79.5% apoptosis by TUNEL (Fig 1B). Also, when changes in $\Delta\Psi$ m were measured, FADD^{-/-} Jurkat cells showed more resistance to loss of $\Delta\Psi$ m at 10 and 25 μ M DES when compared to the WT Jurkat cells (Fig 3B vs Fig 2A), thereby suggesting that the mitochondrial pathway of apoptosis was regulated, at least in part, by signaling through FADD. It should be noted that at higher concentrations of DES such as 50 μ M, the FADD^{-/-} Jurkat cells showed similar levels of apoptosis as the Jurkat WT cells by TUNEL and DiOC₆ assays. Together, the above data suggested the following: 1) Death receptor pathway, specifically FADD, plays a role in DES-induced apoptosis, particularly at lower concentrations. 2) Loss of $\Delta\Psi$ m in Jurkat WT cells may be

regulated, at least in part, by signaling through FADD. 3) At higher concentrations of DES, death receptor pathway is dispensable and that the activation of mitochondrial pathway alone is sufficient to induce apoptosis.

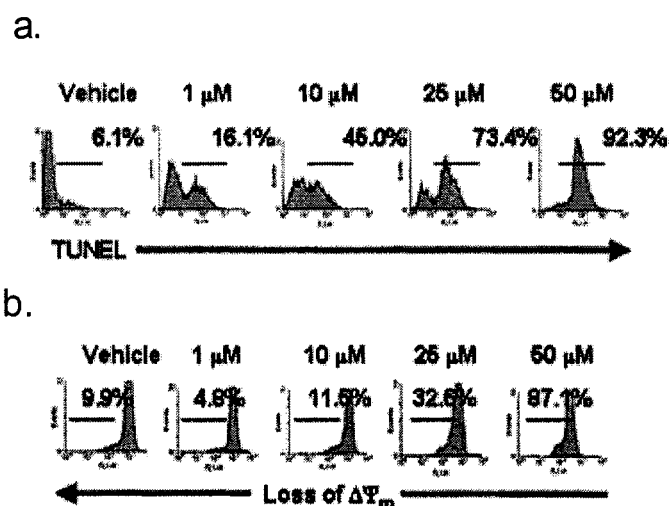


Figure 3. Effect of DES on apoptosis and mitochondrial membrane potential loss in FADD-deficient Jurkat cells. A) Cells were treated with 1, 10, 25 or 50 μM DES or vehicle for 24 hrs and analyzed by TUNEL. The percentage of apoptotic cells has been depicted in each histogram. B) Cells were treated with 1, 10, 25 or 50 μM DES or vehicle, harvested 24 hrs later, and stained with DiOC₆. The percent decrease in loss of mitochondrial membrane potential ($\Delta\Psi_m$) has been depicted in each histogram.

Having previously observed that DES caused a loss of mitochondrial stability, we sought to determine if overexpression of Bcl-2 would inhibit apoptosis. We, therefore, treated Bcl-2 overexpressing (Bcl-2⁺⁺⁺) Jurkat cells with 1, 10, 25, 50 μ M DES or vehicle and analyzed apoptosis by TUNEL. Data shown in Fig 4 indicated that Bcl-2⁺⁺⁺Jurkat cells were relatively more resistant to apoptosis at lower concentrations (1 and 10 μ M) when compared to the WT cells (Fig 1B). However, at higher concentrations of DES (25 and 50 μ M), the Bcl-2⁺⁺⁺Jurkat cells were as sensitive as the WT Jurkat cells (Fig 4 vs Fig 1B). Together, these data suggested that at lower concentrations of DES, hyper-expression of Bcl-2 may provide protection against DES-induced apoptosis while at higher doses, Bcl-2 overexpression fails to block apoptosis possibly due to increased induction of proapoptotic Bcl-2 family members which overcome the anti-apoptotic action of Bcl-2.

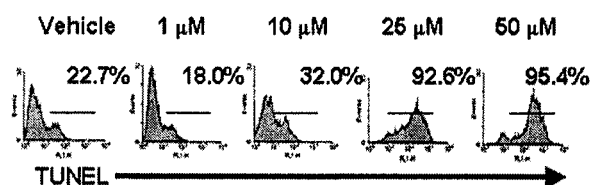


Figure 4. Effect of DES on Bcl-2 over-expressing Jurkat cells. Bcl-2 over-expressing Jurkat cells were treated with 1, 10, 25 or 50 μ M DES or vehicle for 24 hrs, harvested, and analyzed by TUNEL. The percentage of apoptotic cells has been depicted in each histogram.

Role of Caspase 8 in DES induced apoptosis

Caspase 8 acts as an initiator of apoptosis in the death receptor pathway. To investigate its role, we used caspase 8 deficient ($C8^{-/-}$) Jurkat cells. These cells were treated as described above with 1, 10, 25, or 50 μ M DES or vehicle for 24 hrs and apoptosis was determined by TUNEL or DiOC₆. $C8^{-/-}$ Jurkat cells were resistant to DES-induced apoptosis at 1 μ M concentration when compared to the vehicle controls, while at higher doses, it was sensitive to apoptosis and the level of sensitivity was somewhat similar to that seen using similar doses against Jurkat WT cells (Fig 5A vs Fig 1B). Interestingly, the $C8^{-/-}$ Jurkat cells were more resistant to loss of $\Delta\Psi_m$ when compared to the Jurkat WT cells at all concentrations of DES tested (Fig 5B vs Fig 2A). Together, these data suggested a role for caspase 8 in DES-induced apoptosis as well as in regulating the loss of $\Delta\Psi_m$.

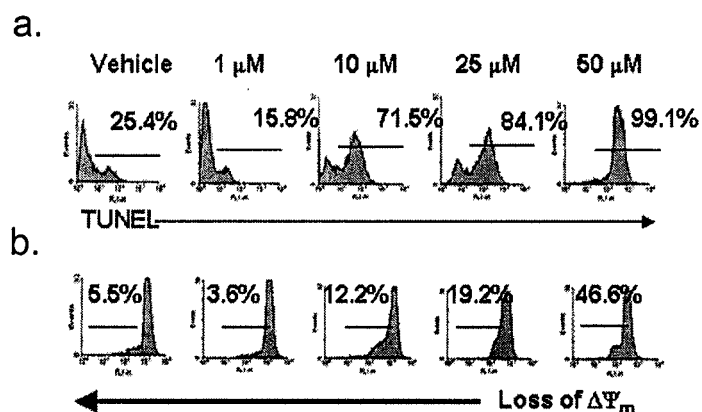


Figure 5. Apoptosis and mitochondrial membrane potential loss in DES-treated Caspase 8-deficient Jurkat cells. A) Cells were treated with 1, 10, 25 or 50 μ M DES or vehicle for 24 hrs, harvested, and analyzed by TUNEL. The percentage of apoptotic cells has been depicted in each histogram. B) Cells were treated with 1, 10, 25 or 50 μ M DES or vehicle for 24 hrs, harvested, and stained with DiOC₆. The percent decrease in loss of mitochondrial membrane potential ($\Delta\Psi_m$) has been depicted in each histogram.

To further examine the role of caspases in DES induced apoptosis, specific caspase activation was determined following DES treatment by immunoblot. Fig 6A shows the cleavage of caspases 2, 8, 9, and 10 following 50 μ M DES treatment. The data also showed that cleavage of specific caspases occurred over a temporal gradient. To elucidate the functional importance of each caspase in promoting apoptosis, Jurkat WT cells were incubated with caspase inhibitors. The data revealed that caspase 2, 8, 9, and 10 inhibitors effectively reduced the caspase 3/7 activity thereby suggesting their involvement in DES-induced apoptosis (Fig 6B). The addition of caspase inhibitors alone did not alter the caspase 3/7 activity.

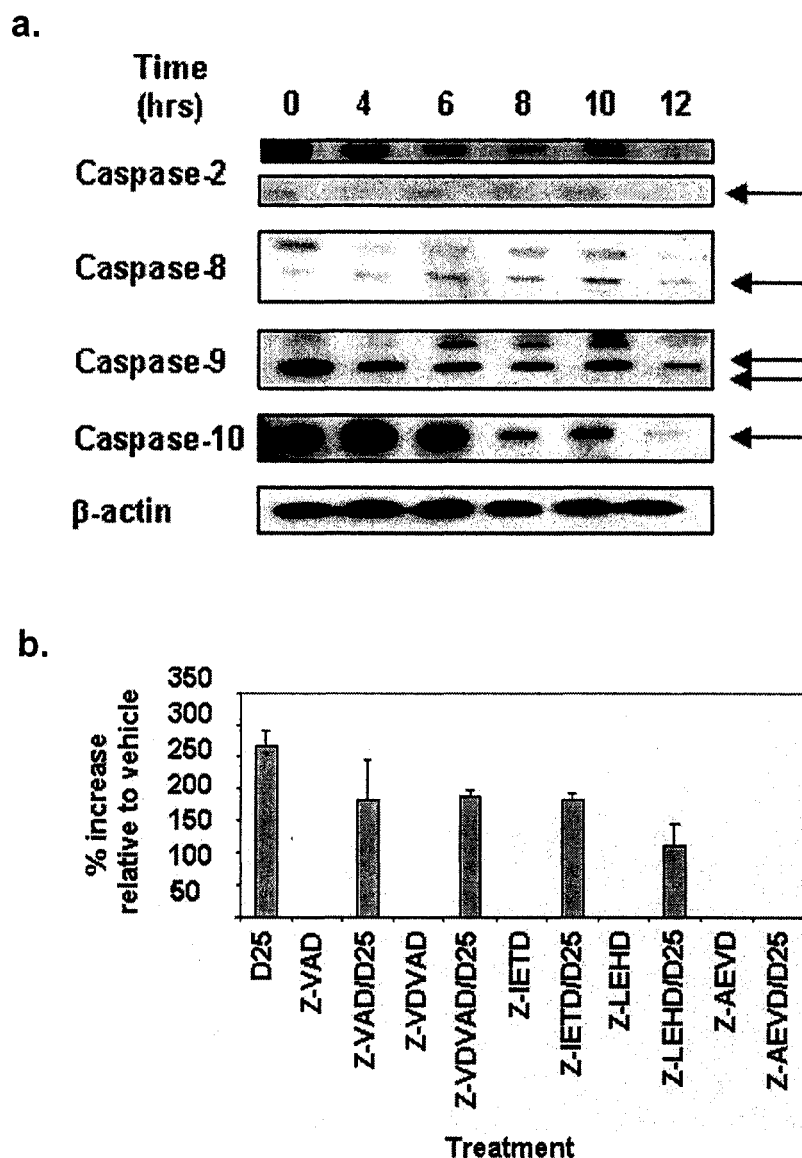


Figure 6. DES induces apoptosis by activating multiple caspases. A) WT Jurkat cells were treated with 50 μ M DES for 0, 4, 6, 8, 10, or 12 hrs. Following protein isolation, 5 μ g of protein was subjected to SDS-PAGE, transferred to nitrocellulose membrane and hybridized with specific antibodies. Lanes with arrows indicate cleaved forms of caspases. B) WT Jurkat cells were treated with 25 μ M DES in the presence or absence of various caspase inhibitors for 24 hrs and caspase 3/7 activity was determined. Data were expressed as percent increase in caspase 3/7 activity when compared to vehicle control. Vertical bars represent the mean \pm SEM of triplicate cultures. * Denotes statistically significant difference when compared to DES treatment alone ($p < 0.05$).

DES induces Bid cleavage linking both extrinsic and intrinsic pathways of apoptosis

It has been shown that the death receptor pathway can communicate with the mitochondrial pathway through cleavage of Bid (Li et al., 1998; Luo et al., 1998). To this end, Jurkat WT cells were treated with 50 μ M DES for 0, 4, 6, 8, 10, or 12 hrs, after which they were harvested and protein expression for Bid was detected by immunoblot. The antibody that we used detected the proform of Bid. Thus, any decrease in its expression was indicative of Bid cleavage. As seen from Fig 7, DES treatment caused a significant decrease in Bid, beginning at 6 hours, indicative of Bid cleavage. Moreover, the decrease in expression proceeded in a time-dependent manner.

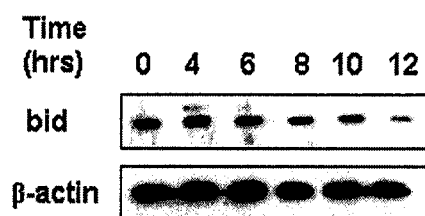


Figure 7. Induction of Bid in DES-treated WT Jurkat cells. WT Jurkat cells were treated with 50 μ M DES for 0, 4, 6, 8, 10, or 12 hrs. Following protein isolation, 5 μ g of protein was subjected to SDS-PAGE, transferred to nitrocellulose membrane, and hybridized with Bid specific antibody that detects the proform. Beta actin was used as an internal control.

Role of estrogen receptor(ER) in DES-induced apoptosis

We next examined the mechanism by which DES triggers apoptosis in Jurkat cells, specifically addressing whether DES was acting through ER. To this end, we determined the expression of ER in Jurkat cells using RT-PCR. Untreated Jurkat cells were found express the ER beta but not alpha isoform (Fig 1A). Next, we considered the possibility that treatment with DES may alter the expression levels of ER alpha or beta isoforms. To test this, Jurkat cells were treated with DES and analyzed for ER expression levels. The data showed that following DES treatment, Jurkat cells exhibited a slight increase in expression of ER beta but failed to express ER alpha (Fig 8A). Next, we tested the role of ER in DES-induced apoptosis using a caspase 3/7 activity assay. Both ICI 182,780 and tamoxifen, two well established ER antagonists failed to inhibit apoptosis induced by DES (Fig 8B), thereby suggesting that the induction of apoptosis by DES was independent of direct interaction between DES and the ER.

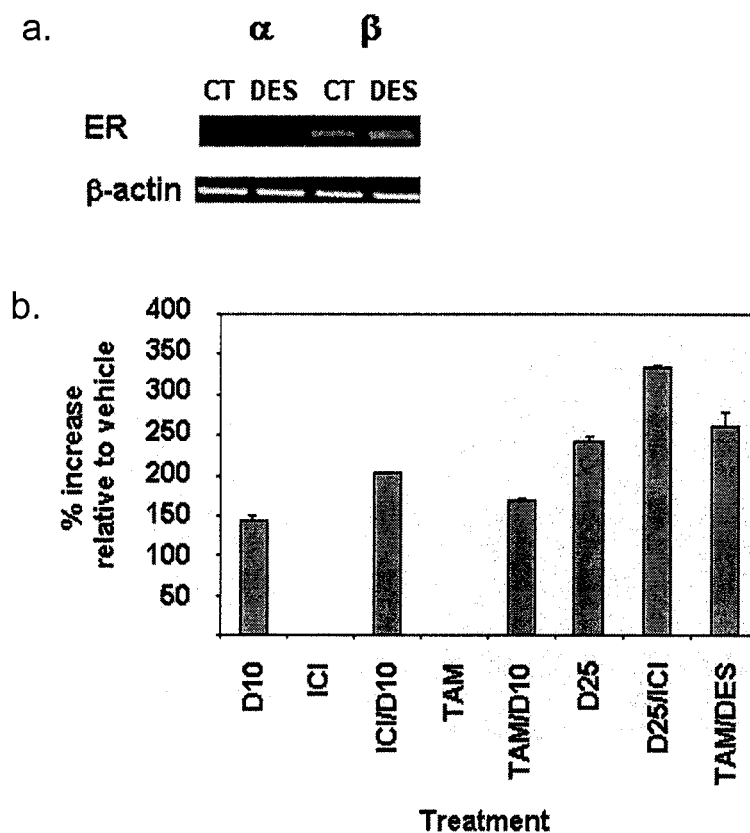


Figure 8. Characterization of ER expression in Jurkat cells. A) Jurkat cells were treated with 25 μ M DES or vehicle (CT) for 4 hrs and harvested. Following RNA isolation, RT-PCR was used to detect ER mRNA transcripts. Beta actin was used as an internal control. B) Jurkat cells were treated with 1 μ M ICI 182,780 or 1 μ M Tamoxifen for 1 hr prior to treatment with 10 or 25 μ M DES. The cells were harvested 24 hrs later and apoptosis was determined by caspase 3/7 assay. Data were expressed as percent increase in caspase 3/7 activity when compared to vehicle control. Vertical bars represent the mean \pm SEM of triplicate cultures.

Role of Fas and Fas ligand (FasL) in DES-induced apoptosis

Because estrogen-induced apoptosis may involve Fas and FasL (Mor et al., 2001; Mor et al., 2003), we addressed their role in the apoptosis of Jurkat cells. First, Jurkat cells were treated with DES and the expression of Fas and FasL was determined by RT-PCR. Analysis of mRNA revealed that Jurkat cells expressed both Fas and FasL and that upon DES treatment, Fas and FasL expression increased slightly (Fig 9A). To determine the role of Fas-FasL interactions in DES-induced apoptosis, Jurkat cells were pretreated with Nok-1 antibody against FasL, prior to DES treatment, and Caspase 3/7 activity was measured. The data showed that Nok-1 Ab failed to block DES-induced apoptosis (Fig 9B). At a similar concentration, Nok-1 could inhibit trimeric FasL-induced apoptosis in Jurkat cells (data not shown).

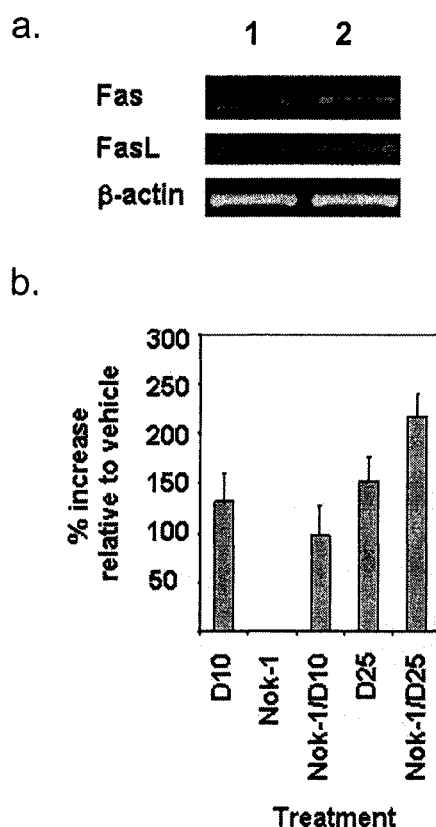


Figure 9. Effect of DES on Fas and FasL expression in Jurkat cells. WT Jurkat cells were treated with 25 μ M DES (lane 2) or vehicle (lane 1) for 4 hrs and harvested. Total RNA was extracted. (A) RT-PCR was performed to determine Fas and FasL expression, with beta actin as an internal control. (B) Cells were pretreated with NOK-1 (anti-FasL Ab) 1 hr before treatment with 10 (D10) or 25 (D25) μ M DES. Apoptosis was determined by caspase 3/7 assay. Data were expressed as percent increase over vehicle control. Caspase 3/7 activity was determined by fluorescence. Vertical bars represent the mean \pm SEM of triplicate cultures. * Denotes statistical significant differences when compared to DES treatment alone ($p < 0.05$).

Role of other TNF/TNFR gene family members in DES-induced apoptosis

To test other possible TNF and TNFR gene family involvement, we used microarray analysis. The data shown in Table 1 indicated that many members of TNF/TNFR were found to be upregulated during the course of DES treatment. It was interesting to note that studies at 6 and 12 hours post-DES treatment revealed differential induction of several of the TNF/TNFR genes thereby suggesting an additional level of complexity in the induction of death receptors/ligands.

Table 1. Effect of DES on gene expression profile in WT Jurkat Cells.*

Time	Gene Family	Gene Name	Fold increase	Gene bank#
6 hr	TNF	Fas	3.5	X63717
		LT-b	3.3	NM_002341
		TNF-b	3.0	D12614
	TNFR	CD40	4.2	NM-001250
		TRAIL-R3	4.0	AF016267
		TRAIL-R	3.8	AF016266
		TRAIL-R4	2.0	AF021232
	ATM/p53	Chk2	4.3	NM_007194
		p63	3.4	X69910
12 hr	TNF	LT-b	2.9	NM_002341
		TNF-b	14.4	D12614
	TNFR	TNFR1	8.1	M33294
		TRAIL-R	5.2	AF016266
		TRAIL-R3	3.7	AF016267
		LTbR	7.9	L04270
	ATM/p53	Chk1	2.5	AF016582
		Rpa3	3.9	L07493
		Gadd45a	3.7	M60974
		HUS1	2.5	NM_004507
		Mdm2	2.9	Z12020

*WT Jurkat cells were incubated with 25 μ M DES or vehicle for 6 or 12 hrs. cDNA microarray analysis for apoptotic genes was carried out as described in Methods. Genes showing 2 fold or greater increase in expression when compared to vehicle controls were depicted.

Role of SAPK/JNK pathway in DES induced apoptosis

We next examined if exposure to DES would trigger SAPK/JNK pathway which is known to play a role in apoptosis (Faris et al., 1998). Exposure of cells to 50 μ M DES resulted in SAPK/JNK phosphorylation that was observed 4 hrs post-DES exposure (Fig 10).

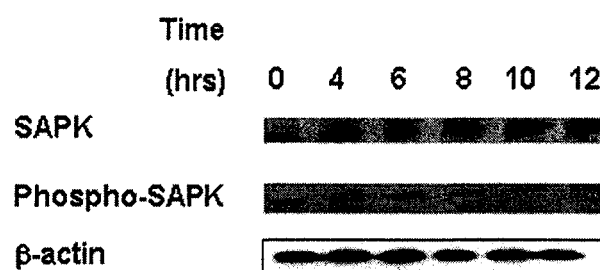


Figure 10. Effect of DES on SAPK activation. WT Jurkat cells were treated with 50 μ M DES for 0, 4, 6, 8, 10, or 12 hrs. Following protein isolation, 5 μ g of protein was subjected to SDS-PAGE, transferred to nitrocellulose membrane, and hybridized with antibodies against SAPK and phosphorylated SAPK. Beta actin was used as an internal control.

Discussion

Endogenous and exogenous estrogens are well known for their immunosuppressive properties, although the precise mechanisms have not been characterized. Recent studies from our laboratory and elsewhere suggested that estrogens may induce apoptosis in T cells which may trigger thymic atrophy (Calemine et al., 2002; Do et al., 2002; Okasha et al., 2001). We have also demonstrated that E2-induced thymic atrophy and apoptosis may be regulated by Fas-FasL interactions inasmuch as, these effects were markedly decreased in mice deficient in Fas and Fas ligand (Do et al., 2002). A recent study investigated the relationship between the level of FasL expression and thymus cell number using ovariectomized female rats, and suggested that estrogen-induced thymic atrophy may be mediated by estrogen-induced FasL expression (Mor et al., 2001). Whether estrogens can induce apoptosis in transformed T cells has not been previously investigated. DES has been shown previously to induce apoptosis in prostate cancer cells (Robertson et al., 1996). Moreover, the use of DES to treat prostate cancer has been looked into with considerable interest (Scherr and Pitts, 2003). The current study has significant implications in understanding the effects of DES on malignancies of the immune system, specifically those derived from T cells. Our studies demonstrate for the first time that transformed T cells can be killed by DES and suggest the potential role of estrogens in the regulation of malignancies of the immune system. Moreover, the current study also sheds light on the possible molecular pathways of apoptosis induced by DES in transformed T cells that can be extrapolated to normal T cells.

Recent studies have indicated that apoptosis may proceed mainly through two pathways: death-receptor pathway and the mitochondrial pathway (Hengartner, 2000; Rathmell and Thompson, 2002). In the current study, we noted that Jurkat cells deficient in FADD or caspase 8 cells were partially resistant to induction of apoptosis by DES particularly at lower doses whereas at higher concentrations, they were as sensitive as the WT Jurkat cells. These data suggested the involvement of death receptor pathway of apoptosis at least at lower concentrations of DES. The fact that C8^{-/-} Jurkat cells were more sensitive to apoptosis than FADD-deficient Jurkat cells as detected by TUNEL, suggested that other death receptor caspases such as caspase 10 may play a role. Caspase 10 has been suggested as an initiator caspase similar to caspase 8 in the death receptor pathway (Wang et al., 2001). The involvement of caspase 10 was supported by cleavage of caspase 10 in DES-treated WT Jurkat cells and partial blocking of apoptosis by inhibitor of caspase 10. DES treatment also triggered the mitochondrial pathway of apoptosis in WT Jurkat cells. This was evident from the loss of $\Delta\Psi_m$, cytochrome c release, cleavage of caspase 9 and the ability of caspase 9 inhibitor to block apoptosis to a significant extent, in DES-exposed WT Jurkat cells. Furthermore, the FADD-deficient cells showed significant loss of $\Delta\Psi_m$ although to a lesser degree than the wild-type Jurkat cells. These data suggested that DES was triggering the mitochondrial pathway, at least in part, independently. However, there was clear evidence for cross-talk between the death receptor and the mitochondrial pathways as evidenced by cleavage of Bid, and more importantly, by the observation that FADD- and caspase 8-deficiency led to increased resistance to loss of $\Delta\Psi_m$.

It is well-established that estrogen regulates cellular responses through binding to its receptor, the estrogen receptor (ER). In the nucleus, estrogen modulates the expression of estrogen-responsive genes through the action of the ER at the transcriptional level. However, more recently, there is evidence to support receptor-independent function of estrogen or its analogues. For example, estrogen activates the MAP kinase cascade in brain cells derived from mice lacking ER- α expression (Singh et al., 2000). Similarly, in ER-deficient human breast cancer cell lines, estrogen was shown to stimulate Akt activation (Tsai et al., 2001). In the current study, we found that Jurkat cells constitutively expressed only the beta isoform, which was further upregulated with DES treatment. DES has the ability to bind both isoforms of the estrogen receptor, α and β , with high affinity (Kuiper and Gustafsson, 1997; Nikov et al., 2001). Thus, DES appears to bypass direct interactions with the receptor in our system and induce apoptosis. The actual mechanism by which DES triggers ER-independent apoptotic activity remains to be determined.

SAPK or JNK constitute a family of novel kinases that bind to the c-Jun transactivation domain and phosphorylate Ser63 and Ser73 (Kyriakis et al., 1994). Unlike the MAP kinases, the SAPK/JNK are strongly activated by cellular stresses (Derijard et al., 1994). It has been shown that overexpression of SAPK induces apoptosis and that interference with activation of SAPK protects against apoptosis (Xia et al., 1995). Our data showed that JNK/SAPK is phosphorylated in the early stages of DES exposure. JNK/SAPKs have also been shown to be activated by TRAIL in parallel to induction of

apoptosis in human T and B cell lines downstream of FADD and caspases (Herr et al., 1999). Inasmuch as, in the current study, DES treatment led to upregulation of TRAIL, it is possible that this may have contributed to activation of SAPK pathway. There are many possible targets of the JNK signaling pathway that may affect the mitochondria, including members of the Bcl-2 group of apoptotic regulatory proteins. The presence of activated JNK/SAPK could be targeting the mitochondria directly.

In the current study, caspase 2 was cleaved in WT Jurkat cells following DES treatment. Caspase 2 has been suggested to be both autocatalytic and have the ability to cleave cytosolic Bid protein, thereby activating the mitochondrial pathway (Guo et al., 2002). Interestingly, caspase 2 has also been suggested to be able to directly act on the mitochondria (Guo et al., 2002). Studies on the activation of caspase 2 have produced controversial results. Caspase 2 activation has been suggested to occur down stream of apoptosome formation (O'Reilly et al., 2002), yet others have suggested the activation to occur upstream of the mitochondria (Guo et al., 2002). Though the specific nature of caspase 2 activation is as yet unclear, our studies do implicate this caspase in DES-induced apoptosis as evidenced by the partial decrease in apoptosis following addition of inhibitor of caspase 2.

The intrinsic or the mitochondrial pathway of apoptosis is regulated by at least 20 Bcl-2 related proteins in mammalian cells (Cory et al., 2003). Bcl-2, Bcl-x_L and Bcl-w, protect cells from a wide range of cytotoxic insults. In contrast, other Bcl-2 relatives promote apoptosis and these include Bax, Bak, Bok, Bik, Bad and Bim. Previous studies have suggested that Bcl-2 overexpression in Jurkat cells can provide protection against

mitochondrial pathway of apoptosis triggered by hypoxia (Weinmann et al., 2004). In this system, unlike DES-induced apoptosis, death receptor pathway was not induced. In the current study, we noted that Bcl-2 overexpressing cells, although partially resistant to DES-induced apoptosis, particularly at 1 and 10 μ M, became highly sensitive to apoptosis at higher doses, potentially due to the increased expression of pro-apoptotic molecules, such as Bad, and possibly decreased expression of anti-apoptotic molecules, such as Survivin and XIAP. Further studies are necessary to address these pathways. In summary, the current study demonstrates that DES can induce apoptosis in Jurkat cells by activating both the intrinsic and extrinsic pathways of apoptosis. While at higher doses, DES can independently activate the intrinsic pathway, at lower concentrations, the extrinsic pathway seems to clearly engage in cross-talk with the intrinsic pathway. The findings that DES can induce apoptosis in transformed human T cells suggests the possible role for estrogens in regulating malignancies of the immune system.

Chapter III. Induction of Apoptosis in T cells from Murine Fetal Thymus Following Perinatal Exposure to Diethylstilbestrol

Abstract

Perinatal exposure to diethylstilbestrol (DES) is known to cause thymic atrophy in mice although the precise mechanism remains unclear. In the current study we investigated whether perinatal exposure to DES would trigger apoptosis in thymocytes. To this end, C57BL/6 pregnant mice were injected intraperitoneally (ip) on gestational day (gd) -15 and -16 with 5µg/kg DES. Analysis of thymi harvested from mice on gd-17, gd-19 and postnatal day (PD) 1, showed a reduction in thymic cellularity on gd-17, increase on gd-19 and no significant change on PD-1. Additionally, DES treatment significantly altered the proportion and absolute number of T-cell subsets, particularly on gd-17 and -19. Apoptosis was increased in DES-treated thymocytes when compared to the controls and was seen only on gd-17 but not on gd-19 or PD-1. Moreover, DES-treated gd-17 thymocytes had increased DEVDase activity. Microarray analysis of 96 apoptotic genes in gd-17 thymocytes revealed that exposure to DES increased the expression of several apoptotic genes primarily belonging to tumor necrosis factor (TNF) and TNF receptor (TNFR) family. Taken together, these results suggest that DES-induced thymic atrophy following perinatal exposure may result, at least in part, from increased apoptosis mediated by death receptor pathway involving TNF family

members.

Introduction

Estrogens have been shown to mediate a wide range of toxic effects on the immune system (Ahmed 2000). Specifically, the thymus has been found to be sensitive to the toxic effects of estrogens (Okasha et al. 2001; Do et al. 2002). Previous studies from our laboratory and elsewhere have shown that estrogens can cause thymic atrophy in adult mice by inducing apoptosis in T cells involving Fas-FasL interactions (Mor et al. 2000; Do et al. 2002; Okasha et al. 2001; Mor et al. 2001). Diethylstilbestrol (DES) is a synthetic estrogen that was widely used to support pregnancy from 1941- 1974. In the USA, an estimated 5-10 million women received DES. Exposure to DES in humans has been associated with an increased risk for breast cancer in "DES mothers" and a lifetime risk of cervicovaginal cancers in "DES-daughters"(Giusti, Iwamoto, and Hatch 1995). Exposure to DES has also been linked to a wide range of abnormalities in DES sons and daughters including immune system disorders, psychosexual effects and reproductive disorders (Giusti, Iwamoto, and Hatch 1995).

Fetal thymus has been shown to be very sensitive to DES-induced toxicity which may result, at least in part, from its effect on stem cells (Holladay et al. 1993). In murine fetal thymic organ cultures, DES has been demonstrated to block thymocyte development by cell cycle arrest and apoptosis, thereby suggesting a direct effect of DES on the thymus as well (Lai et al. 2000). Thus, prenatal exposure to DES may alter the T cell differentiation in the thymus and T cell repertoire in the periphery, which in turn could have significant immunological consequences in the adult. Studies in humans suggested that prenatal exposure to DES can trigger increased autoantibody production and altered

delayed type hypersensitivity (DTH) reaction (Forsberg 2000; Noller et al. 1988).

Thymocytes, along with other immune cells, have been shown to express the estrogen receptor (ER). While ER alpha has been shown to be required for thymic development as well as atrophy induced by estrogens, the role of ER beta remains unclear (Kuiper and Gustafsson 1997; Staples et al. 1999). ER beta is known to be expressed in humans and rat thymus but not in mouse thymic tissue (Couse et al. 1997)

DES has been found to induce a variety of genes depending on the tissue (Matsuno et al. 2004; Terasaka et al. 2004). Some of the induced genes have been found to have a putative estrogen response element (ERE) in the promoter. One could speculate that given the variety of genes affected by DES, that thymic atrophy may also be under the regulation of many genes that are altered by DES, including those involved in the regulation of apoptosis. Thymocytes have been demonstrated to express high levels of Fas and their interactions with Fas ligand (FasL) expressed on stromal cells may play a critical role during development (Kishimoto, Surh, and Sprent 1998; Castro et al. 1996). An example of this occurrence is during thymic selection (Kurasawa, Hashimoto, and Iwamoto 1999). It is interesting to note that estrogen treatment increases the expression of FasL through the binding of ER to the ERE motif expressed on the FasL promoter (Mor et al. 2000). Thus, such a mechanism may trigger estrogen-induced thymic apoptosis and atrophy (Do et al. 2002).

Inasmuch as apoptosis contributes to deletion of the majority of T cells in the thymus, this active regulatory mechanism could be a potential target for immunotoxicants currently known to destroy thymic tissue. In the current study, we investigated the

ability of perinatal DES exposure to induce apoptosis and alter regulatory apoptotic genes in thymocytes. We provide direct evidence that prenatal exposure to DES induces thymic atrophy that correlates with increased apoptosis induction in thymocytes.

Material and Methods

Animals

Timed pregnant (vaginal plug=day 0) C57BL/6 mice were purchased from the National Institute of Health (Bethesda, MD). All animals were housed in polyethylene cages equipped with filter tops and wood shavings. Each animal cage had rodent chow and tap water *ad libitum*. Mice were housed at a constant temperature ($23\pm 2^{\circ}\text{C}$) with a 12h-light:12h-dark lighting schedule.

DES exposure and sample collection

DES was obtained from Sigma, MO. DES was dissolved and diluted in corn oil. Time kinetic studies were carried out on pregnant mice treated intraperitoneally (i.p.) with $5\mu\text{g/kg}$ DES or the vehicle control (corn oil) on gestational day (gd) 15 and 16. To this end, 2 pregnant mice were injected either with DES or vehicle. From each pregnant mouse, we obtained an average of 8-10 pups. Thymi from pups were harvested and viable cellularity enumerated.

Cell preparations

Thymi were removed and placed in RPMI-1640 medium (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Grand Island, NY), 10 mM Hepes, 1 mM glutamine, $40\mu\text{g/ml}$ gentamicin sulfate, and $50\mu\text{M}$ 2-mercaptoethanol. Single cell suspensions were prepared from thymi using

homogenization. Cells were pelleted by centrifugation (1300 rpm, 7 min, 4 °C) and resuspended in 0.83% ammonium chloride lysis buffer (Sigma, St. Louis, MO) to lyse the erythrocytes. Cells were again washed twice in medium. Viability of cells was determined on a hemocytometer by trypan blue dye exclusion, using an inverted phase contrast microscope. The data on thymic cellularity were depicted as the total number of viable thymocytes/pup.

Detection of T cell subsets by flow cytometry

Harvested thymocytes (1×10^6) were washed with phosphate-buffered saline (PBS) (Invitrogen, Grand Island, NY). In double-staining studies, 0.5 μ g of FITC-anti-CD4 and PE-anti-CD8 mAbs (BD Pharmingen, San Diego, CA) were simultaneously added to each sample. Cells were then fixed with 1% paraformaldehyde and flow cytometric analysis was performed by a FACScan (Becton Dickinson, Franklin Lakes, New Jersey) using the Cyclops operating software (Cytomation, Ft. Collins, CO).

Detection of apoptosis using the TUNEL Assay

Thymocytes were analyzed for apoptosis using the TUNEL assay, in which FITC-dUTP nick-end labeling is used to detect apoptosis related DNA fragmentation (Boehringer Mannheim, Indianapolis, IN). As described earlier (Kamath et al. 1997), thymocytes (1×10^6) from vehicle- or DES-treated mice were cultured for 24 hrs in RPMI with 10% FBS. This approach allows for better detection of drug-induced apoptosis in the thymus because apoptosis-committed cells can die without being rapidly cleared by

phagocytic cells (Kamath et al. 1997; Camacho, Nagarkatti, and Nagarkatti 2004). The cells were washed twice with PBS and fixed with 4%-paraformaldehyde for 30 min at room temperature. The cells were again washed with PBS, permeabilized 2 min on ice with 0.1% Triton X-100 in 0.1% sodium citrate, washed again with PBS and incubated with FITC-dUTP for 1 hr at 37°C. Fluorescence was measured by flow cytometry.

Analysis of DEVDase activity

Perinatal samples from pups were obtained at gd-17, -19, and postnatal day (PD)1 following DES treatment of the pregnant mother. Freshly isolated thymocytes were assayed for DEVDase activity following in vitro culture using the ApoOne Homogeneous Caspase 3/7 Assay obtained from Promega (Madison, WI). Manufacture's protocol was used to determine activity. The data from replicate pools were depicted as mean activity \pm SEM.

Microarray Analysis

Microarray analysis was performed as described in detail elsewhere (Fisher, Nagarkatti, and Nagarkatti 2004). Briefly, thymi from gd-17 and -19 pups exposed to DES or vehicle were harvested and RNA was extracted using TRIzol reagent (GibcoBRL, Carlsbad, CA). Apoptotic Q series microarrays from SuperArray (Bethesda, MD) were used to determine apoptotic gene induction. Manufacture's protocol was used to perform the assay. The list of 96 genes screened is provided at the manufacturer's website: http://www.superarray.com/product_q.php

Statistical Analysis

Results are presented as the mean \pm SEM of replicate pools per treatment group.

Statistical analyses were performed using the Student's t-test and $p < 0.05$ was considered to be statistical significant.

Results

Altered thymic cellularity and T cell subsets following perinatal exposure to DES

A dose of 5 µg/kg DES was administered into pregnant C57BL/6 mice on gd- 15 and -16 and thymic cellularity was determined on gd-17, -19, and on postnatal day (PD) 1 in fetal and neonatal mice (Fig. 1). Thymic atrophy was demonstrated on gd-17 but not on gd-19 and PD-1. In fact, on gd-19, the DES-treated group had significantly increased numbers of thymocytes/pup when compared to the vehicle-treated mice.

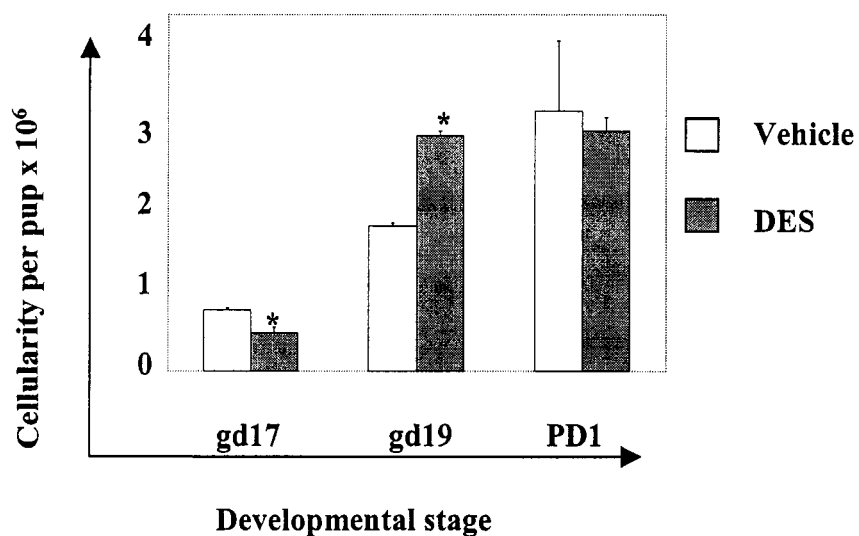


Figure 1. Thymic cellularity after perinatal DES-exposure. C57BL/6 pregnant mice were injected with 5 μ g/kg DES or the vehicle on gd-15 and -16. On gd-17, -19, and PD-1 post-DES treatment, the thymi were removed and cellularity determined. Asterisks (*) indicate statistically significant differences in the mean cellularity of DES-exposed thymocytes when compared to the vehicle controls within each group ($p < 0.05$). Data were represented as the mean thymic cellularity per pup \pm SEM.

We also examined the percentage of T cells expressing CD4/CD8 markers in the thymus of DES-exposed fetuses and newborns. The results from a representative experiment (Fig 2A) and data from multiple experiments (Fig 2B) are presented. The double-negative (CD4-CD8-; DN) T cells, which form the predominant subset at gd-17, showed no significant alteration post-DES treatment. However, the percentage of the double-positive (CD4+CD8+ or DP) T cells and single positive CD4 (CD4+CD8- or SPCD4+) T cells decreased significantly post-DES treatment. The percentage of single-positive CD8 (CD4-CD8+ or SPCD8) T cells remained unchanged following DES treatment on gd-17. On gd-19, DES-exposure caused an increase in the percentage of SPCD8 T cells, while other T cell subsets were not altered. On PD-1, DES failed to mediate alterations in the proportions of T cell subsets.

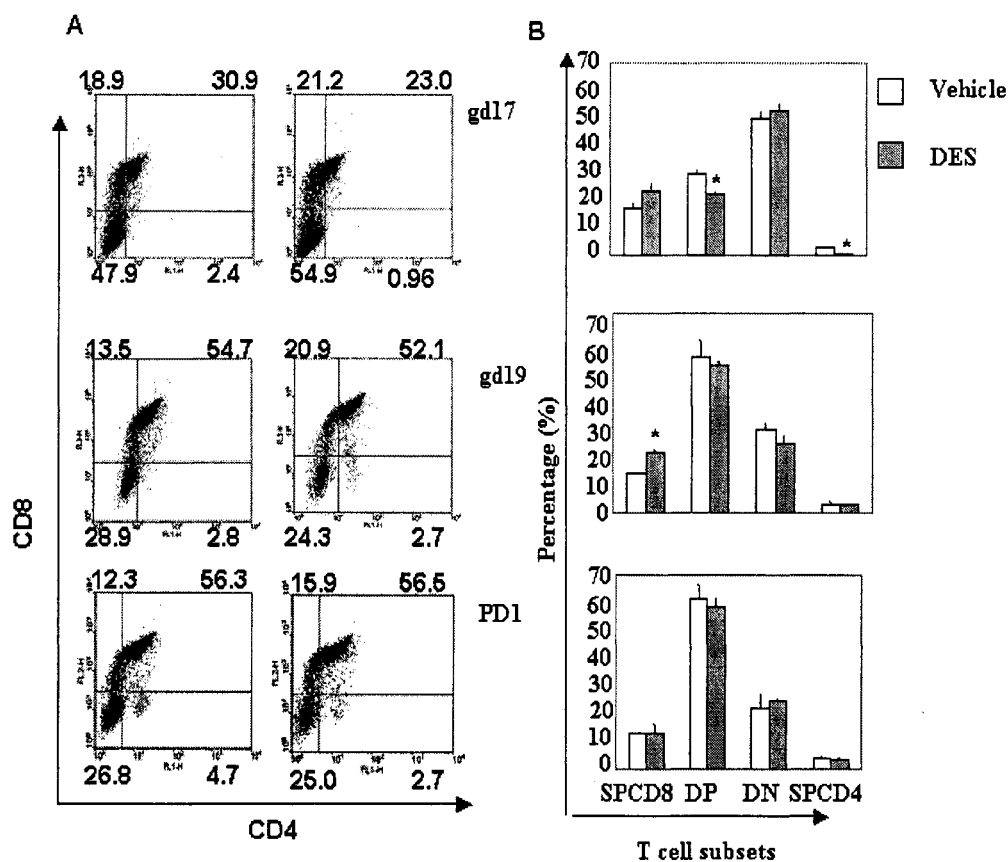


Figure 2. Effects of perinatal exposure to DES on thymic T-cell subsets. C57BL/6 pregnant mice were injected with 5 μ g/kg DES or the vehicle on gd-15 and -16. On gd-17, -19, and PD-1 post-DES treatment, fetal and neonatal thymocytes were double-stained with FITC-anti- CD4 and PE-anti- CD8 mAbs. Representative dot-plots showing the percentage of T-cell subsets in the thymus are depicted (A) and data collected from replicate pools have been plotted (B). Asterisks (*) indicate statistically significant differences when T-cell subsets from DES-exposed thymocytes were compared to vehicle controls within each group ($p < 0.05$).

When the overall cellularity for each T cell subset was calculated per pup (Fig 3), it was reduced in DES treated groups for DP T cells but not for other T cell subsets, on gd-17. On gd-19, an increase absolute cellularity was noted in all T cell subsets. On PD-1, no significant alterations were observed in total cellularity for all T cell subsets following DES-exposure except for SPCD4 T cells which exhibited a modest decrease, when compared to the vehicle control (Fig. 3). Together, these data indicated that most significant DES-induced changes in the total numbers of T cell subsets can be observed on gd-17 and gd 19.

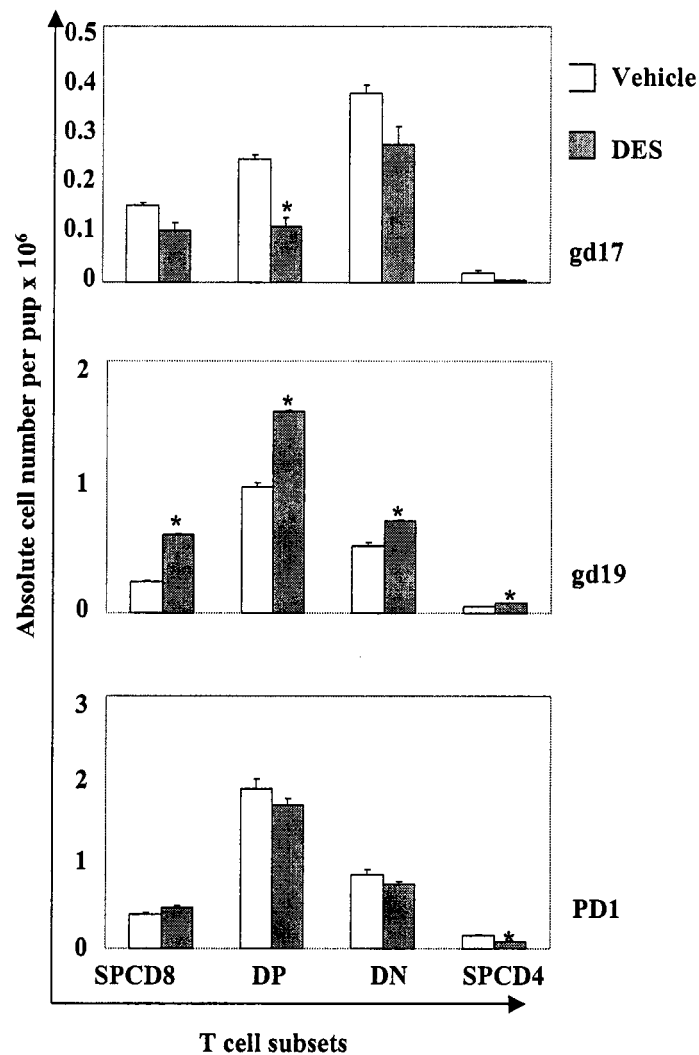


Figure 3. Absolute cellularity of T cell subsets in the fetal and neonatal thymus following DES treatment. C57BL/6 pregnant mice were injected with 5 μ g/kg DES or the vehicle on gd-15 and -16. On gd-17, -19, and PD-1 post-DES treatment, fetal and neonatal thymocytes were isolated and enumerated for absolute cell numbers. Based on the percentages of T cell subsets (Fig. 2), the absolute numbers of various subpopulations were calculated and depicted. Data were represented as the mean T cell subset cellularity per pup \pm SEM.

Detection of apoptosis in developing thymus after perinatal DES-exposure

Thymi from mice exposed to DES perinatally were assayed for apoptosis using the TUNEL method. In this assay, freshly isolated thymocytes were incubated in medium for 24 hrs *in vitro* then stained by TUNEL. This approach allows greater detection of DES-induced apoptotic cells because apoptosis-committed cells can die without being rapidly cleared by phagocytic cells (Kamath et al. 1997). The results from a representative experiment have been shown in Fig. 4. Thymocytes from Gd-17 but not gd-19 or PD-1 mice, exposed to DES, showed significantly higher percentage of apoptosis, when compared to the vehicle-treated groups. These data correlated well with DES-induced thymic atrophy that was seen only on gd-17, but not on other days.

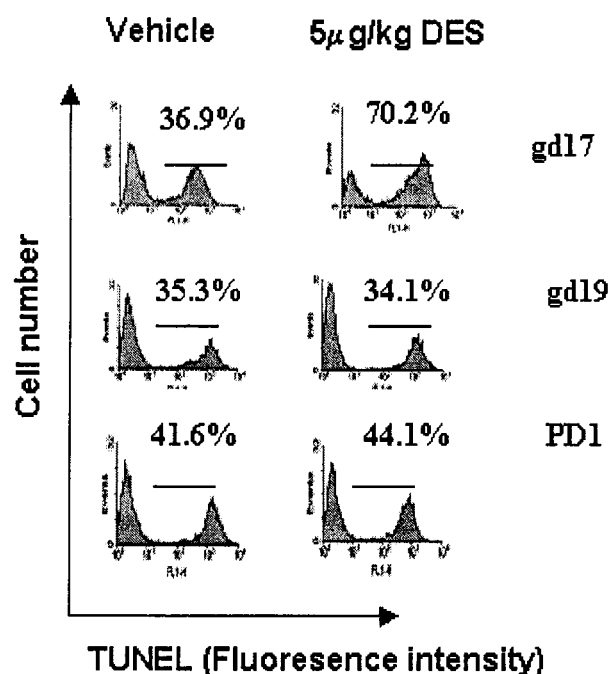


Figure 4. Induction of apoptosis in fetal and neonatal thymus following perinatal DES-treatment. C57BL/6 pregnant mice were injected with 5 μg/kg DES or the vehicle on gd-15 and -16. On gd-17, -19 and PD1 post-DES treatment, fetal and neonatal thymocytes were isolated, cultured for 24 hrs and then stained with FITC-dUTP for apoptosis detection. Percentage of apoptotic cells is represented in each representative histogram.

Detection of DEVDase in thymocytes following perinatal exposure to DES

Caspase activation is an early event in apoptosis, marked by classic caspase-3 activation (Gurtu, Kain, and Zhang 1997). We therefore determined caspase-3/-7 activity following perinatal DES-treatment. As shown in Fig. 5, significant increases in caspase-3/-7 activity in gd-17, but not gd -19 or PD1 thymocytes were seen following perinatal exposure to DES. These data were consistent with apoptosis induction seen on gd-17 but not on other days.

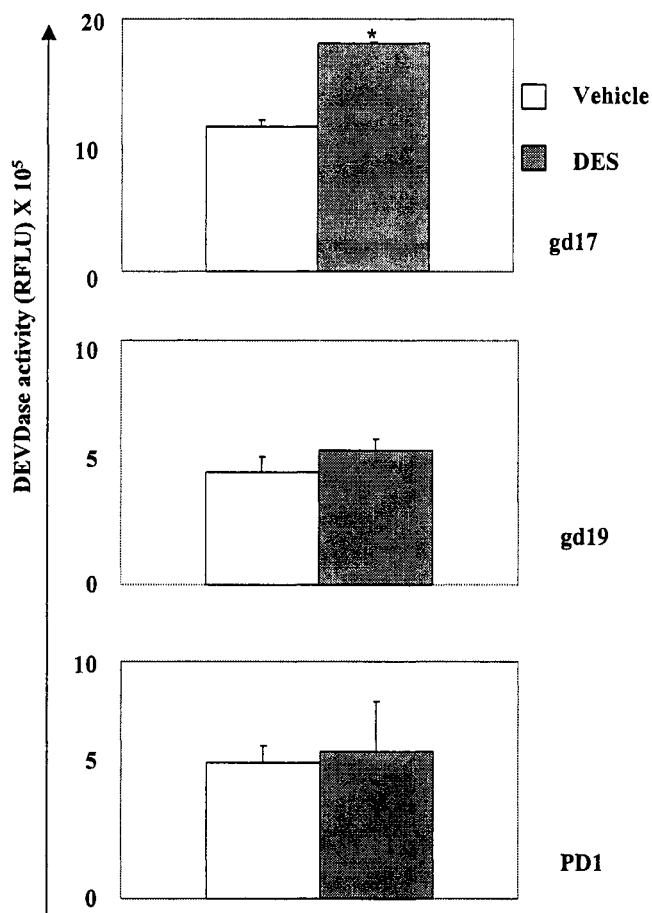


Figure 5. Perinatal DES-treatment increases DEVDase activity in the thymus.

C57BL/6 pregnant mice were treated with 5 μ g/kg DES or the vehicle on gd-15 and -16. On gd-17, -19 and PD1 post-DES treatment, fetal and neonatal thymocytes were isolated to assess caspase-3/-7 activity as described in the Materials and Methods section. Data from 2 replicate pools were represented as the mean activity \pm SEM. Asterisks (*) indicate statistically significant differences between DES-treated and control groups ($p < 0.05$).

Expression of apoptotic genes in the developing thymus following perinatal exposure to DES

We determined if DES could alter the expression of apoptotic genes in the thymus. To this end, microarray analysis of 96 apoptotic genes was carried out following DES treatment on thymocytes from gd-17 and -19 mice. On gd-17 following DES treatment, several tumor necrosis factor (TNF) and tumor necrosis factor receptor (TNFR) family member were upregulated as shown in Table 1. However on gd-19, we observed that the mRNA profile was no different from control, as indicated by no significant increase in gene expression (data not shown). Taken together, these data showed that the expression of several apoptotic genes was upregulated in thymocytes on gd-17.

TABLE 1

 Effect of DES on gene expression profile in developing thymocytes

gd17	TNF	FasL	2.4	NC_000067
		CD27L	6.2	NM_011617
		4-1BBL	2.7	NM_009404
		Apo3L	4.2	NM_033042
		HVEML	2.7	NM_019418
		4-1BB	5.8	NM_17071
	TNFR			
	ATM/p53	p53	2.0	BC005448

*Pregnant mice were treated with 5 µg/kg DES or vehicle on gd-15 and -16. Thymocytes were harvested from fetuses on gd-17. cDNA microarray analysis for apoptotic genes was carried out as described in Methods. Genes showing 2 fold or greater increase in expression when compared to vehicle controls were depicted.

Discussion

Perinatal exposure to DES in humans is known to cause significant alterations in the immune system leading to increased susceptibility to autoimmunity and infections (Adam et al. 1985; Noller et al. 1988). Previous studies in experimental animals have suggested that prenatal exposure to DES causes thymic atrophy and that this may result from toxicity against stem cells (Holladay et al. 1993). Using fetal thymic organ cultures, it was shown that DES inhibits thymocytes at different stages of development as well as induce apoptosis (Lai et al. 2000). However, whether *in vivo* prenatal exposure to DES would trigger apoptosis in thymocytes has not been reported previously. To this end, the current study demonstrates that DES promotes significant apoptosis induction in developing thymocytes, which in turn may be responsible for causing thymic atrophy.

During thymic development, T cells undergo a selection process to produce cells that are self-MHC restricted and self-tolerant, involving DN, DP and SP stages of differentiation (von Boehmer et al. 1989; Kisielow and von Boehmer 1995). In the current study, we analyzed the effect of DES on T cell differentiation at different stages of development. Our data showed that following DES exposure on gd-15 and 16, significant thymic atrophy could be detected at gd-17. Additionally, apoptosis of thymocytes could be detected only at gd-17 but not on gd-19 or PD-1. These data indicate that thymocytes are more sensitive during the early selection process. This finding is consistent with other studies that have found that during later stages of T cell development, the effects of DES are less pronounced (Holladay et al. 1993). Our studies also show that at gd-17, approximately 30% of the thymocytes are DP in the control

groups. DN T cells represented approximately half of the thymocytes and SP^{CD8} approximately 20%. Very few SP^{CD4} thymocytes were observed. Following exposure to DES, gd-17 thymocytes were altered most significantly in the percentages of DP and SP^{CD4} populations that were reduced significantly. Moreover, on gd-17, the absolute numbers of DP T cells decreased following DES exposure while that of all other T cell subsets remained unaltered. On gd-19, DES induced an increase in the proportion of SP^{CD8} T cells with no significant effect on the percentages of other T cell subsets. However, on gd-19, due to an increase in the total number of thymocytes/pup found following prenatal DES exposure, the absolute numbers of all T cell subsets were found to be significantly increased. While the precise reason as to why DES-treatment increases the number of thymocytes on gd-19 is not clear, it can be speculated that this may result from increased seeding of T cell progenitor cells to the thymus to compensate for the DES-induced loss of thymocytes on gd-17. Moreover, on gd-19, DES was no longer effective in triggering apoptosis which may also have contributed to increased numbers of thymocytes. On PD-1, the proportion and absolute numbers of all T cell subsets in DES-exposed animals had reverted to control levels except for a modest decrease in the total numbers of SP^{CD4} T cells. Thus, it appears that the DES effect was short-lived and that the total thymic cellularity returned to normal levels by PD-1. However, it should be noted that in our studies, we exposed the mice to DES on gd-15 and -16 only. Thus, it is not clear whether exposure of pregnant mice beyond gd-16 to DES would have caused immunotoxic effects that would be evident in the pups even after birth.

Previous studies have shown that the ability to detect drug-induced apoptosis in thymocytes *in vivo* is difficult due to rapid clearance of apoptotic cells by phagocytes (Camacho, Nagarkatti, and Nagarkatti 2004; Kamath et al. 1997). We have shown that *in vitro* culture allows for the detection of apoptosis without interference of phagocytosis of apoptotic cells (Kamath et al. 1997). In addition, the ability of DES to induce apoptosis in thymocytes was corroborated by demonstrating that gd-17 thymocytes had increased levels of DEVDase activity.

Microarray analysis of apoptotic genes at gd-17 showed that DES treated thymocytes upregulate several TNF and TNFR family members. Interestingly, FasL was among one of the upregulated transcripts. At the gd-19 stage, the gene profile was not significantly different from that of control. These results correlated well with the induction of apoptosis at gd-17 and lack of at gd-19. Fas/FasL interactions play a critical role in the thymus during T cell development (Castro et al. 1996). Fas has been observed during specific developmental stages of thymocytes such as the DN stage (Ogasawara et al. 1993). Given this, it is clear that there are specific developmental stages that are sensitive to Fas-mediated apoptosis (Fleck et al. 1998). Our data suggested that increased FasL expression in DES-exposed thymocytes may play a role in DES-induced apoptosis in thymocytes. Given that the estrogen responsive element (ERE) has been found in the FasL gene promoter (Mor et al. 2000), it can be speculated that DES may act through ERE to induce FasL gene expression. These findings are consistent with previous studies from our laboratory demonstrating that Fas-FasL interactions play a critical role in estrogen-induced apoptosis in thymocytes (Do et al. 2002).

T cells that mature in the thymus undergo positive and negative selection. These processes select self-MHC restricted T cells and delete autoreactive cells, thereby allowing tolerant cells to be sent to the periphery (Palmer 2003; Sebzda et al. 1999; Saito 1998; Saito and Watanabe 1998). The finding that prenatal exposure to DES can lead to thymic atrophy through induction of apoptosis on gd-17, and increased thymocyte number on gd-19, suggests that DES may interfere with the T-cell selection processes in the thymus, which could alter the T-cell repertoire in the periphery. Such alterations during development may influence postnatal immune responses particularly because T cells are long-lived. Moreover, continuous exposure to DES during the entire gestation period may have more pronounced effect on T cell differentiation in the thymus and consequently in the periphery that could continue to persist in post-natal life.

Chapter IV. Diethylstilbestrol Alters Positive and Negative Selection of T Cells in the Thymus And Modulates T Cell Repertoire in the Periphery

Abstract

Prenatal exposure to diethylstilbestrol (DES) is known to cause altered immune functions and increased susceptibility to autoimmune disease in humans. In the current study, we investigated the effects of DES on T cell differentiation in the thymus using the HY-TCR transgenic mouse model in which the female mice exhibit positive selection of T cells, while the male mice show negative selection. In female HY-TCR Tg mice, exposure to DES showed more pronounced decrease in thymic cellularity when compared to male mice. Additionally, female mice also showed a significant decrease in the proportion of double-positive (DP) T cells in the thymus and HY-TCR-specific CD8⁺ T cells in the periphery. Male mice exhibiting negative selection also showed decreased thymic cellularity following DES-exposure. Moreover, the male mice showed increased proportion of double-negative (DN) T cells in the thymus and decreased proportion of CD8⁺ T cells. The density of expression of HY-TCR on CD8⁺ cells was increased following DES exposure in both females and males. Finally, the proliferative response of thymocytes to mitogens, and that of peripheral lymph node T cells to male H-Y antigen was significantly altered in female and male mice following DES-treatment.

Taken together, these data suggest that DES alters T cell differentiation in the thymus by interfering with positive and negative selection processes, which in turn modulates the T cell repertoire in the periphery.

Introduction

Diethylstilbestrol (DES) is a synthetic estrogen that was used in the US between 1940's and 1975 to prevent spontaneous abortion. It is estimated that approximately 5-10 million expectant mothers and developing fetuses were exposed to DES. The long term consequences of DES exposure to mothers include increased risk of breast cancer, while DES-exposed daughters have shown increased risk of cervicovaginal cancer (Giusti et al., 1995). Other abnormalities in DES daughters and sons have also been reported which include immune system disorders, psychosexual effects, and reproductive abnormalities (Giusti et al., 1995). Studies regarding consequences to the immune system following DES exposure have shown that prenatal exposure alters immune functions in T cells in addition to other immune cells (Ford et al., 1983; Ways et al., 1987). Increased incidence of autoimmunity has also been observed following prenatal DES exposure, in adult life (Noller et al., 1988). Reports such as these suggest that the immunological consequences of DES-exposure may be far reaching.

The thymus is the site of T cell development in which T cells differentiate into functionally mature T cells that are self-MHC restricted and self-tolerant. Developing T cells in the thymus must transition through several developmental stages. All developing T cells start at the double negative (DN) stage in which lineage is established. This stage is followed by a double positive (DP) stage that is marked by the expression of both CD4 and CD8. DP cells undergo positive and negative selection to become single positive (SP) expressing either CD4 or CD8 (Shortman and Wu, 1996; Sebzda et al., 1999).

Positive selection ensures that the TCR will interact with self-MHC thereby becoming MHC-restricted, whereas negative selection ensures tolerance to self-peptides when presented on self-MHC (Kisielow and von Boehmer, 1995). During development, thymic T cells are subject to elimination. Apoptosis in developing T cells can be initiated due to the lack of survival signals at all stages. These survival signals ensure that proper development of T cells occurs (Kisielow and Miazek, 1995; Levelt and Eichmann, 1995; Falk et al., 2001).

Previous studies from our laboratory and elsewhere have shown that estrogens can trigger thymic atrophy in adult mice by inducing apoptosis in T cells involving Fas-FasL interactions (Mor et al., 2001; Okasha et al., 2001; Do et al., 2002). Also, in fetal thymic organ culture studies, DES exposure was found to inhibit thymocyte differentiation at various stages of development and to induce cell cycle arrest and apoptosis thereby suggesting that DES can directly act on T cell differentiation (Lai et al., 2000). In addition, DES has been shown to be toxic to stem cell precursors, which may, at least in part, contribute to thymic atrophy (Holladay et al., 1993). In chapter 3, we observed that thymic effects following perinatal exposure were specifically seen prior to gestational day 19. Specifically, we observed a reduction in thymic cellularity and increase in apoptosis following perinatal exposure to DES. T cell selection is known to occur prior to gestational day 19. Together, such studies have suggested that prenatal exposure to DES may alter positive and negative selection of T cells in the thymus leading to altered T cell repertoire in post-natal life.

In the present study, we investigated whether exposure to DES would alter T cell repertoire by using the HY-T cell receptor (TCR) transgenic system in which mice express a TCR transgene specific to male HY antigen. In this model, thymocytes expressing the HY-transgenic TCR are positively selected in female mice and negatively selected in male mice (Kisielow et al., 1988; Teh et al., 1988). We find that both female and male HY-TCR transgenic mice are sensitive to DES- induced thymic atrophy and that these mice exhibit significant alterations in positive and negative selection of T cells upon DES exposure.

Materials and Methods

Mice

HY-TCR transgenic (HY-TCR-Tg) mice C57BL/10AiTac- TgN, ~ 6 weeks of age were purchased from Taconic Farms (Germantown, NY). All animals were housed in polyethylene cages equipped with filter tops and wood shavings. Each animal cage had rodent chow and tap water ad libitum. Mice were housed in an environment of constant temperature ($23 \pm 2^\circ \text{C}$) and on a 12h-light:12 h-dark lighting schedule.

DES exposure and sample collection

DES was obtained from Sigma, MO. DES was dissolved and diluted in corn oil. Animals were treated subcutaneously (sc) with 1 mg/kg DES or the vehicle (corn oil) on day 0 and on day 3, thymi, spleens, and lymph nodes were harvested and cell number enumerated as described (Okasha et al., 2001; Do et al., 2002).

Cell preparation

Following sacrifice, thymi or peripheral lymphoid organs were harvested and placed into RPMI-1640 medium supplemented with 10% FBS, 50 μM β -mercaptoethanol, and 40 $\mu\text{g/ml}$ gentamicin sulfate. Single cell suspensions were prepared, pelleted by centrifugation (1300 rpm, 7 min, 4°C), and resuspended in 0.83% ammonium chloride (Sigma, St. Louis, MO) to lyse the erythrocytes. Cells were further washed twice in medium. Cell viability was determined on a hemocytometer by trypan blue dye using an inverted phase contrast microscope. For calculating total organ

cellularity, the data were expressed as total numbers of cells/mouse \pm standard error of mean (SEM).

Detection of phenotypic markers by flow cytometry

Isolated cells (1×10^6) were washed with phosphate-buffered saline (PBS; Invitrogen, Grand Island, NY) and incubated 30 min on ice with the following monoclonal antibodies (mAbs): PE-CD8 (BD Pharmingen, San Diego, CA), CyChrome-CD4 (BD Pharmingen, San Diego, CA), and FITC-T3.70 (anti-HY-TCR) (eBioscience, San Diego, CA). After incubation with the antibodies, cells were washed once with PBS and fixed in 1% paraformaldehyde. Flow cytometric analysis was performed using a Coulter FC500. HY-TCR-deficient cells were excluded from the analysis.

Proliferation Response

Briefly, 5×10^5 cells (thymocytes, splenocytes, or lymph nodes) from vehicle (corn oil) or DES-treated mice were stimulated with Con A ($2 \mu\text{g/ml}$) or anti-CD3 ($5 \mu\text{g/ml}$) for 48 hrs. In other experiments, 5×10^5 lymph node cells from female or male DES-treated mice were stimulated with 5×10^5 irradiated normal male splenocytes as a source of H-Y antigen, for 4 days. Following incubation, cells were pulsed with $2 \mu\text{Ci}$ ^3H -thymidine during the final 8 hrs of incubation. Cells were harvested using an automated cell harvester (Skatron, Sterling, VA). The amount of radioactivity was determined using a scintillation counter and the mean counts per minute (c.p.m.) \pm SEM of triplicate cultures was calculated.

Statistical significance

Results are presented as the mean \pm SEM of replicate pools consisting of 6 mice per treatment group. Statistical analysis was performed using the Student's t test and $p < 0.05$ was considered to be statistically significant.

Results

Both female and male HY-TCR-Tg mice are sensitive to the thymic atrophy induced by DES

To test whether exposure to DES would induce thymic atrophy in HY-TCR-Tg mice, 1 mg/kg DES or the vehicle was injected into male and female mice and 3 days later, the thymic cellularity was measured. As shown in Fig. 1, female HY-TCR-Tg mice showed an average 47% reduction in thymic cellularity following exposure to DES while male HY-TCR-Tg mice showed 32% reduction in thymic cellularity when compared to the controls.

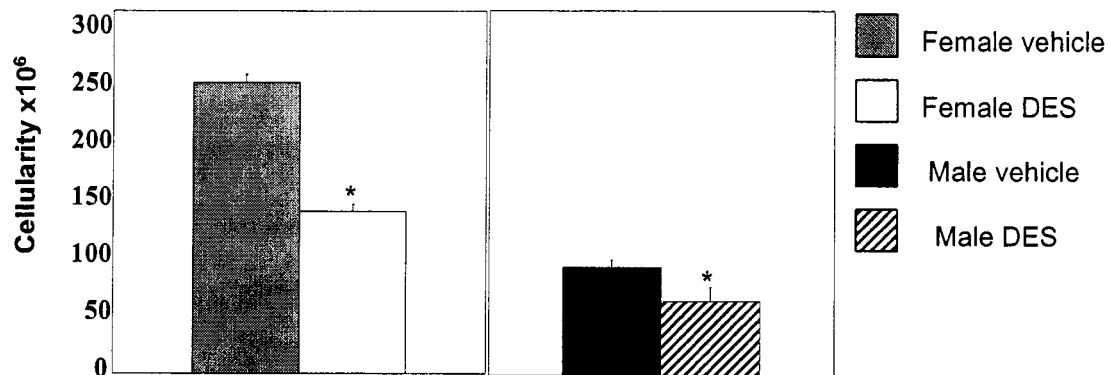


Figure 1. Effects of DES exposure on the thymus of female and male HY-TCR-Tg mice. HY-TCR-Tg mice were treated with either 1 mg/kg DES or vehicle and 72 hours later, thymi were harvested to determine viable cellularity. Asterisks (*) indicate statistically significant differences in the mean cellularity of DES-exposed thymocytes when compared to the vehicle controls within each group ($p < 0.05$). Vertical bars represent mean \pm SEM of 6 individual mice

DES alters the proportion of T-cell subsets in female and male HY-TCR-Tg mice

Having observed a reduction in thymic cellularity in DES-treated HY-TCR-Tg mice, we next determined the effect on T cell subsets in the thymus. Fig. 2A, C show representative experiments on percentages of T cell subsets while Fig. 2B, D depict data pooled from multiple experiments. As seen from Fig 2A,C, vehicle-treated HY-TCR-Tg female mice showed development of both double-positive (DP) and single positive (SP)CD8 T cells, which is expected due to positive selection of transgenic SPCD8 T cells in these mice. In contrast (Fig 2C,D), the vehicle-treated male HY-TCR-Tg mice were virtually devoid of DP T cells and had few SPCD8 T cells due to negative selection as reported previously (Fisher et al., 2005). Following DES-treatment of female HY-TCR-Tg mice, there was a significant decrease in the percentage of DP T cells and an increase in SPCD4 T cells with no significant alterations in the other T cell subsets (Fig. 2A,B). In evaluating the HY-TCR-Tg males, we found that the DN population increased significantly, while the SPCD8 decreased after DES treatment with no significant alterations seen in other subpopulations (Fig. 2C, D). Together, these results suggested that DES may alter T cell differentiation involving positive and negative selection.

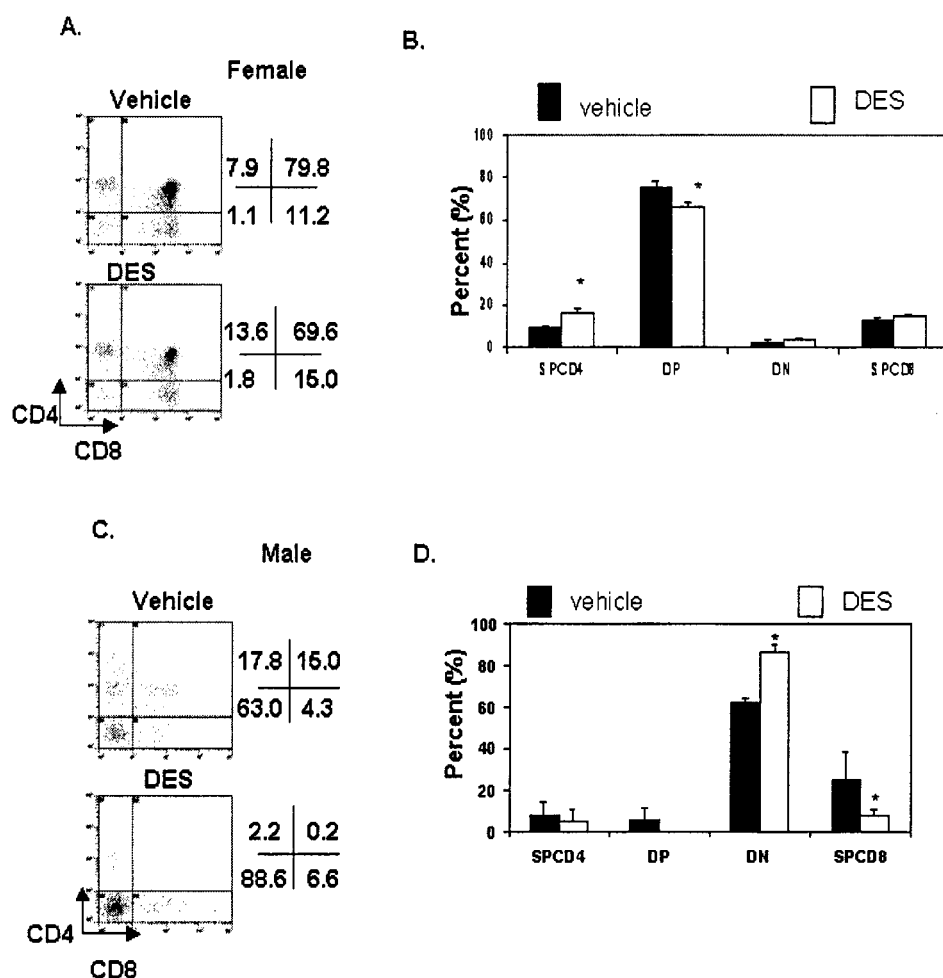


Figure 2. Effects of DES exposure on thymocyte subpopulations in female and male HY-TCR-Tg mice. HY-TCR-Tg mice were treated with either 1 mg/kg DES or vehicle and 72 hours later, thymi were harvested and triple stained with FITC-anti-HY-TCR, PE-anti-CD8, and Cy-chrome-anti-CD4 antibodies. Cells expressing HY-TCR were gated and further analyzed. The percentages of T cell subsets in the 4 quadrants has been indicated. Panels A and C depict representative data from female and male mice respectively. Panels B and D show pooled data from 6 individual female and male mice respectively.

Effect of DES on HY-TCR expression in CD8+ cells

Given that thymic T cell subsets were altered following DES exposure, we next examined the expression of the HY-TCR on thymocytes (Fig. 3). Upon examination, the HY-TCR^{hi} expression in females increased from 43.9 % to 54.6%, while that of HY-TCR^{lo} decreased from 58.4% to 44.1%. In the males, the HY-TCR expression also increased from 62.0 % to 94.8% following DES-treatment while, HY-TCR^{lo} cells decreased from 38.2% to 5.2%. Thus, the male mice showed more pronounced changes when compared to the female mice. Because increases in TCR expression can alter T cell selection (Dave et al., 1998; Dave et al., 1999), the above data suggested a mechanism by which DES may alter the T cell selection process.

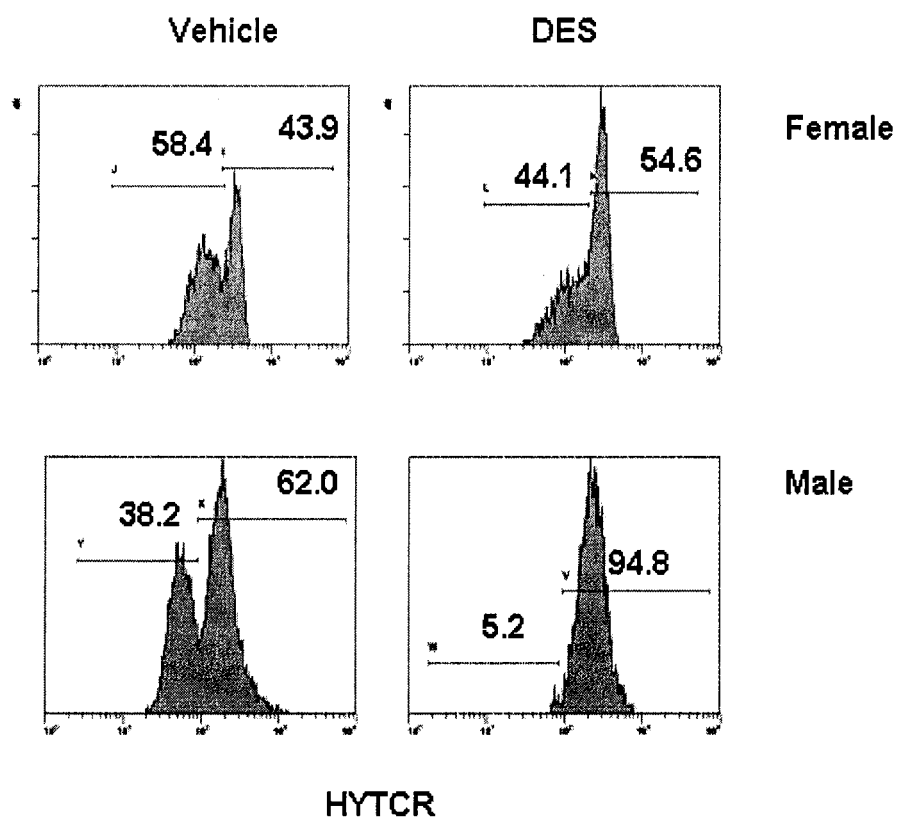


Figure 3. Effects of DES exposure on HY-TCR expression in female and male HY-TCR-Tg mice. HY-TCR-Tg mice were treated with either 1 mg/kg DES or vehicle and 72 hours later, thymi were harvested and stained with FITC-anti-HY-TCR. Histograms indicating percentages of HY-TCR^{hi} and HY-TCR^{low} cells are shown.

Effect of DES on the proliferative response in HY-TCR-Tg female and male thymocytes

To determine if DES also altered the activation of male and female HY-TCR thymocytes, we next tested their ability to respond to Con A or anti-CD3 mAb. We found that in DES-treated thymocytes, the response to both anti-CD3 and Con A was significantly reduced in females by 18.0% and 26.0% respectively (Fig. 4). In the males the mitogen response to anti-CD3 or Con A was also reduced by 23.0% and 22.0% respectively, when compared to the vehicle controls.

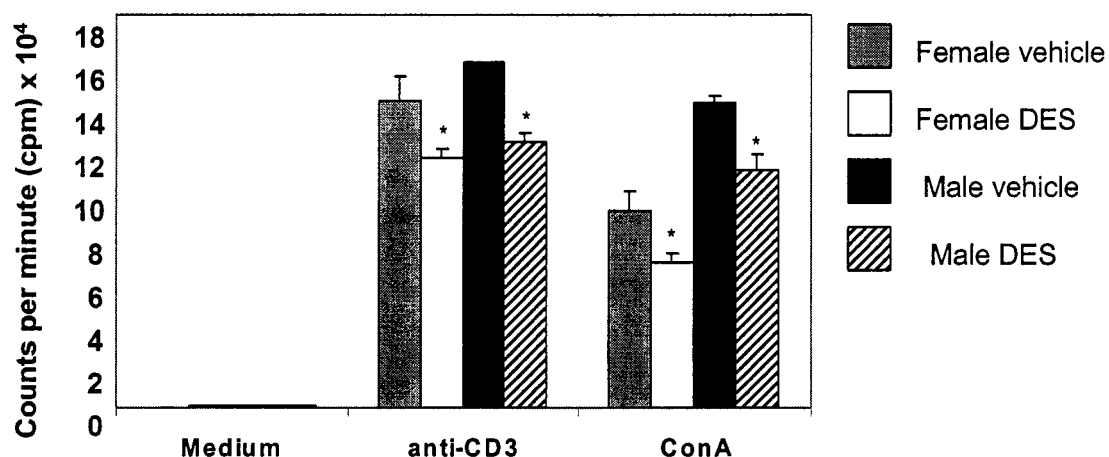


Figure 4. Effect of DES on proliferative response of thymocytes from HY-TCR-Tg female and male mice to T cell mitogens. Thymocytes were harvested from HY-TCR-Tg mice treated with either 1 mg/kg DES or vehicle, 72 hours post-treatment. 5×10^5 thymocytes were cultured *in vitro* for 48 hours with 2 μ g/ml ConA, 5 μ g/ml anti-CD3 mAbs, or in 10% RPMI medium alone. Thymocytes were pulsed with 2 μ Ci of 3 H-thymidine during the final 8 hours of culture. Data are represented as the mean c.p.m. \pm SEM of three replicate cultures per each treatment group. Statistically significant differences ($p < 0.05$) between treatment groups are indicated by asterisks.

DES does not significantly alter splenic cellularity

Exposure of HY-TCR-Tg mice to DES caused a no significant reduction in splenic or lymph node (data not shown) cellularity in either HY-TCR-Tg females or males when compared to the controls (Fig 5).

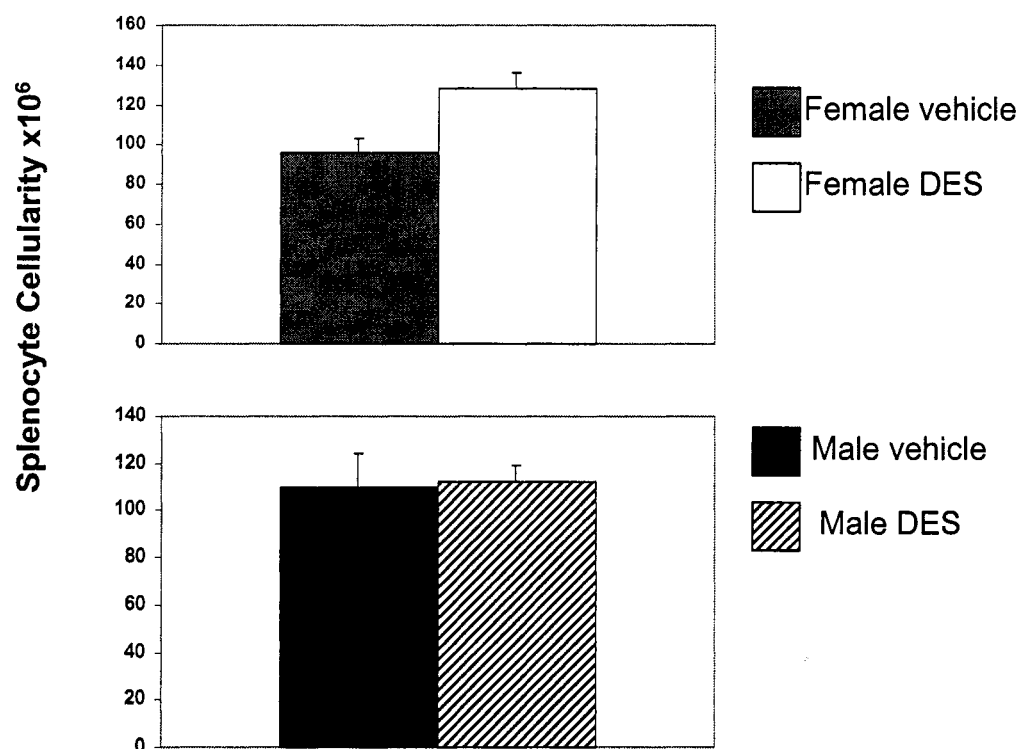


Figure 5. Effects of DES exposure on the spleen cellularity of female and male HY-TCR Tg mice. HY-TCR-Tg mice were treated with either 1 mg/kg DES or vehicle and 72 hours later, the spleens were harvested to determine viable cellularity. Data represent mean \pm SEM of 6 mice/group.

DES-induced T cell subset alterations in thymus affect the peripheral splenic T cells of female and male HY-TCR-Tg mice

Next, we investigated the effects of DES on the proportion of peripheral T-cells in female and male HY-TCR-Tg mice by staining the cells for CD4 and CD8. The cells that did not stain for CD4 and CD8 were considered as non-T cells. The data from representative experiments have been shown in Fig. 6A,C and data from multiple experiments have been depicted in Fig. 6B,D. In female HY-TCR-Tg mice exposed to DES, there was an increase in the percentage of non-T cell population, and a decrease in SPCD8 and SPCD4 subpopulations in the spleen (Fig. 6A,B). Similar results were observed in the lymph nodes (data not shown). In the splenocytes of male HY-TCR-Tg mice, significant changes were seen only in the SPCD8 population which decreased significantly when compared to the controls (Fig. 6C,D). It should be noted that in vehicle-treated male HY-TCR-Tg mice, the proportions of SPCD4 and CD8 T cells was dramatically reduced when compared to similar cells from vehicle-exposed female HY-TCR-Tg mice, consistent with the observation that in male mice there is negative selection of T cells while in female mice, there is positive selection. Together, these data showed that DES-exposure alters the proportion of T cells and non-T cells in the periphery.

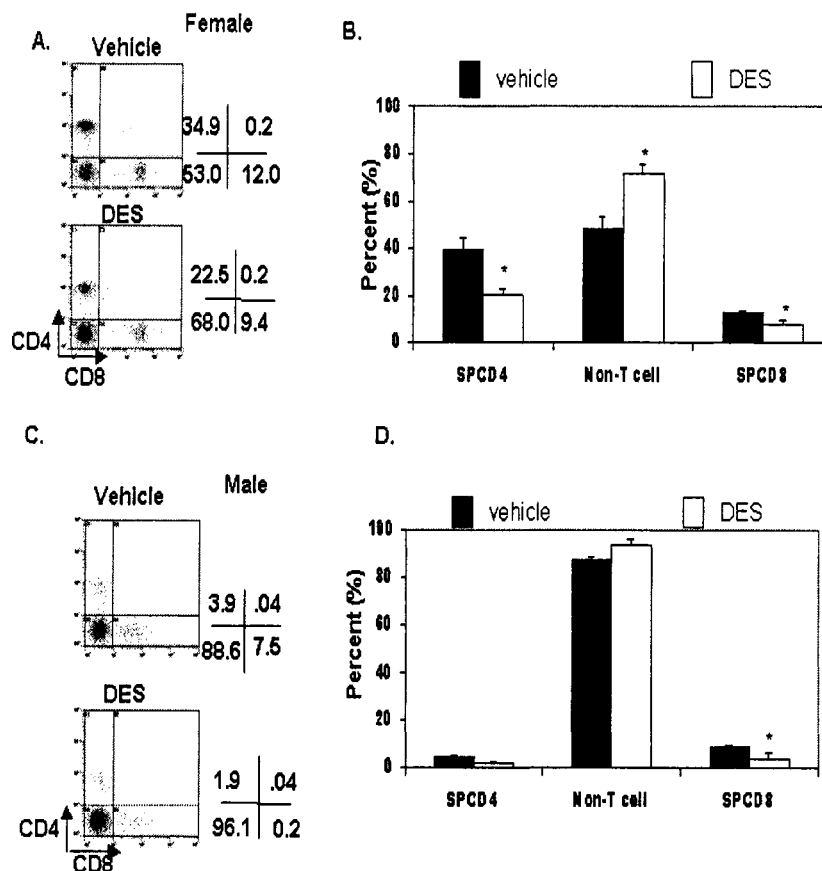


Figure 6. Effect of DES on T cell subpopulations in the splenocytes. Female and male HY-TCR-Tg mice were treated with either 1 mg/kg DES or vehicle and 72 hours later, spleens were harvested from HY-TCR-Tg mice and triple-stained with FITC-anti-HY-TCR, PE-anti-CD8, and Cy-chrome-anti-CD4 antibodies. Cells not expressing HY-TCR were excluded. Representative dot plots showing percentages of CD4⁺ and CD8⁺T cell subsets in female and male mice are depicted for each treatment group (Panels A and C). Multiple experiments for female and male mice are represented in panels B and D, respectively. Data represent mean \pm SEM of 6 mice/group. (*) indicates statistically significant differences between treatment groups.

The peripheral T cell response against H-Y antigen is altered in female HY-TCR-Tg mice

The HY-TCR specifically recognizes the male H-Y antigen. In female HY-TCR-Tg mice, male antigen is a foreign antigen, while in the male, the H-Y antigen is a self-antigen. Because the antigen is non-self in female mice, an immune response is mounted, unlike in the male mice (Kisielow et al., 1988; Maile et al., 2005). To this end, we cultured the LN T cells from male or female mice exposed to vehicle or DES with irradiated male splenocytes as a source of HY-Ag and studied the T cell proliferation as described (Fisher et al., 2005). As seen in Fig. 7, female HY-TCR-Tg mice treated with DES showed a significant increase in responsiveness to the male H-Y antigen when compared to the controls. Conversely, T cells from male HY-TCR-Tg mice, upon DES-exposure, did not exhibit a significant change in proliferative response when compared to the controls.

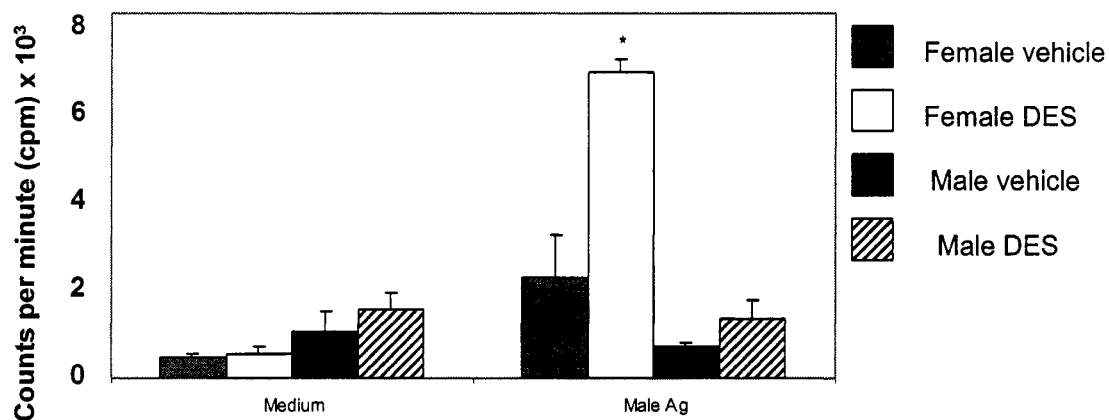


Figure 7. Proliferative response to male H-Y antigen in female and male HY-TCR-Tg mice. HY-TCR-Tg mice were treated with either 1 mg/kg DES or vehicle and 72 hrs later, lymph nodes were harvested. The lymph node cells (5×10^5) were stimulated with 5×10^5 irradiated normal male splenocytes or media for 5 days. Data are represented as the mean c.p.m. \pm SEM of three replicate cultures per each treatment group. Statistically significant differences ($p < 0.05$) between treatment groups are indicated by asterisks.

Discussion

Prenatal exposure to DES has been shown to increase the susceptibility to infections and autoimmunity (Noller et al., 1988; Giusti et al., 1995). In the current study, we tested the hypothesis that DES exposure alters T cell selection process in the thymus using the HY-TCR-Tg mouse model. Unlike normal mice in which it is difficult to study positive and negative selection, HY-TCR-Tg mice permit such a study because male and female HY-TCR-Tg mice selectively exhibit negative and positive selection, respectively. The hypothesis that DES may alter the process of thymocyte selection is based on the ability of estrogens and DES to induce apoptosis in thymocytes (Okasha et al., 2001; Do et al., 2002). Moreover, observations in fetal thymic organ cultures have shown effects of DES on thymic T cell differentiation (Lai et al., 1998; Lai et al., 2000). In the current study, we found that though both female and male HY-TCR-Tg mice were sensitive to DES-induced thymic atrophy, female HY-TCR-Tg mice were more sensitive relative to male HY-TCR-Tg mice. Moreover, DES treatment caused significant alterations in the proportion of T cell subsets as well as the density of TCR expression. Also, the ability of T cells from the thymus and periphery to respond to mitogens and HY-antigen, respectively, was also significantly altered.

Consistent with the fetal thymic organ culture studies, which demonstrated impaired T cell development (Lai et al., 1998; Lai et al., 2000), we found altered T cell development in the HY-TCR-Tg model. Previous studies have demonstrated significant increases in DN populations and reductions in the DP population following DES exposure (Holladay et al., 1993; Silverstone et al., 1994). The data we present here are

consistent with the model alterations in DN and DP populations. Because in female HY-TCR-Tg mice, the T cells primarily undergo positive selection, a decrease in DP T cells suggested that exposure to DES may cause a decrease in positive selection. Decreased positive selection may cause decreased generation of SPCD8 cells in the periphery. This was in fact confirmed by the decrease in the proportions of SPCD8 T cells in the spleens of female mice exposed to DES.

In HY-TCR-Tg male mice, there is negative selection of T cells in the thymus due to the presence of male H-Y antigen. This was confirmed in the current study by demonstrating that in vehicle-treated HY-TCR-Tg male mice, there was total deficiency of DP T cells as well as decreased proportions of SPCD8 T cells. This was also reflected in the periphery where we found decreased proportions of SPCD8 T cells in the spleens of vehicle-treated male mice when compared to vehicle-exposed female mice. Interestingly, following DES treatment, there was an increase in the proportions of DN T cells and a decrease in the percentage of SPCD8 T cells in the thymus. This suggested that in male mice exposure to DES may block the differentiation of DN T cells into DP T cells.

The DP stage in T cell differentiation is important because thymocytes express the TCR complex. It is from this stage that further development proceeds, based upon the interaction between the TCR and MHC:peptide. While low affinity interactions may promote positive selection, high affinity binding can trigger negative selection. Thus TCR expression plays a critical role in the T cell selection process (Dave et al., 1999). Upon examination of the HY-TCR expression in the thymus of both male and female

HY-TCR-Tg mice, we found that there was dramatic increase in the density of the HY-TCR. This may promote increased clonal deletion and apoptosis as evidenced by thymic atrophy seen following exposure to DES.

While studies on positive and negative T cell selection in the thymus of normal mice is difficult to dissect, the use of HY-TCR-Tg model clearly allows to investigate these pathways independently. Recently, we have used this model to study the effect of environmental contaminants such as TCDD on T cell selection process (Fisher et al., 2005). Thus, the HY-TCR-Tg mice may serve as a novel and useful model to study the effect of drugs/chemicals on T cell differentiation in the thymus. In summary, the current study suggests that developmental exposure to DES may alter the T cell positive and negative selection thereby causing altered T cell repertoire in the periphery. This may account for altered immune response seen in individuals prenatally exposed to DES.

Chapter V. Diethylstilbestrol-Induced Apoptosis in the Thymus Requires T Cell-Stromal Cells Interactions

Abstract

Exposure to diethylstilbestrol (DES), a synthetic estrogen, has been shown to cause thymic atrophy and apoptosis. However, the precise nature of cells in the thymus targeted by DES, is not clear. To this end, we investigated the effect of DES on thymic stromal cells and T cells found in the thymus, referred to as thymocytes. Co-culture of purified thymocytes and stromal cells with DES in vitro induced apoptosis in thymocytes but not in stromal cells. Interestingly, thymocytes or stromal cells cultured alone with DES failed to undergo apoptosis. Exposure to DES caused upregulation in FasL expression in stromal cells but not in thymocytes. In contrast, Fas expression on thymocytes was not significantly altered by DES. Culture of FasL⁺ stromal cells with Fas⁻ thymocytes or FasL⁻ stromal cells with Fas⁺ thymocytes, in the presence of DES, failed to induce apoptosis in thymocytes. Taken together, these data suggested that DES-induced apoptosis in thymocytes requires contact between FasL⁺ stromal cells and Fas⁺ thymocytes.

Introduction

Estrogens are known to adversely affect the immune system by inducing thymic atrophy (Calemine et al., 2002; Do et al., 2002) and impairing T cell function (Salem et al., 2000; Calemine et al., 2002) in experimental animals. In prenatally exposed mice, estrogen has been shown to alter development and differentiation of T cells (Silverstone et al., 1994; Lai et al., 1998). Such alterations in the thymus may produce prolonged immunomodulation in the periphery.

Diethylstilbestrol (DES) is a synthetic estrogen that was used in the US between 1940's and 1975 to prevent spontaneous abortion. It is estimated that approximately 5-10 million expectant mothers and developing fetuses were exposed to DES. The long term consequences of DES exposure to mothers has been increased risk of breast cancer, while DES-exposed daughters have shown increased risk of cervicovaginal cancer (Giusti et al., 1995). In addition, prenatal DES exposure has also been shown to increase the incidence of autoimmunity in humans during adult life (Noller et al., 1988). Together, such studies suggest that DES may alter the T cell differentiation in the thymus.

The thymic stromal cells represent a mixture of various cell types including fibroblasts, macrophages, and dendritic cells (Boyd et al., 1993). The stromal cells play critical roles in extravasation, lymphopoiesis, and T cell selection. Studies on thymic stromal cells have suggested that contact between differentiating T cells and stromal cells is important for induction of apoptosis (Gao et al., 1996; Sharova et al., 2001). Stromal cells are known to express FasL (French et al., 1997), while thymocytes express Fas (Castro et al., 1996). Blocking of Fas expression in the thymus has been shown to inhibit

apoptosis (Hershberger et al., 1998). The promoter of FasL gene has an estrogen-responsive element (Mor et al., 2003), thereby suggesting that estrogens may induce thymic atrophy by upregulating FasL on stromal cells (Mor et al., 2001). Thymus, including the stromal cells have also been shown to express the estrogen receptor (ER) alpha (Kuiper et al., 1997; Kohen et al., 1998). Although ER beta has also been found to be expressed in the thymus of certain species, it is not expressed in the mouse (Couse et al., 1997; Kuiper and Gustafsson, 1997). It has been suggested that expression of ER is important for thymic development and induction of atrophy caused by estrogen (Staples et al., 1999).

Previous studies have shown that estrogens including DES are immunomodulatory, inducing thymic atrophy, altering T cell development, and triggering apoptosis (Silverstone et al., 1994; Lai et al., 1998; Okasha et al., 2001; Calemme et al., 2002; Do et al., 2002). Also, studies from our laboratory demonstrated that estrogen-induced apoptosis in the thymus may involve Fas-FasL interactions inasmuch as mice deficient in these molecules were more resistant to apoptosis in the thymus (Do et al., 2002). Additionally, previous data in chapters 3 and 4 revealed that developing T cells (thymocytes) undergo apoptosis in the thymus following DES exposure. Exposure to DES was found to increase FasL expression in the thymus. Furthermore, stromal cells are known to express FasL. However, whether estrogens mediate their apoptotic effects directly on T cells or indirectly via their action on thymic stromal cells, remains unclear. In the current study, we tested the hypothesis that DES may induce the expression of FasL on thymic stromal cells thereby inducing apoptosis in Fas⁺ T cells that come in

contact with stromal cells. Our data support this hypothesis and demonstrate that stromal cell-T cell interactions involving FasL and Fas are necessary for induction of apoptosis in T cells.

Materials and Methods

Mice

Female, six week old C57Bl/6 mice (wild-type, WT) were purchased from the National Institutes of Health (Bethesda, MD). Female B6Smn.C3H-Fas^{gld} (gld, FasL-defective) and B6.MRL-Fas^{lpr} (lpr, Fas-deficient) mice on C57BL/6 background were purchased from Jackson Laboratory (Bar Harbor, ME) and used at 6 weeks of age. All animals were housed in polyethylene cages equipped with filter tops and wood shavings. Each animal cage had rodent chow and tap water ad libitum. Mice were housed in an environment of constant temperature (23±2° C) and on a 12h-light:12 h-dark lighting schedule.

Cell preparation

Thymi were harvested from groups of 4 mice and gently fragmented in RPMI-1640 medium supplemented with 10% FBS, 50 µM β-mercaptoethanol, and 40 µg/ml gentamicin sulfate. Stromal cells were isolated from the thymus as described (Gray et al., 2002). Briefly, the cells were allowed to settle by gradient and supernatant removed. The remaining cells were digested with a mixture of 20% collagenase D and 10% DNase to collect stromal cells. Cells were pelleted by centrifugation (1300 rpm, 7 min, 4°C) and resuspended in 0.83% ammonium chloride (Sigma, St. Louis, MO) to lyse the erythrocytes. Cells were further washed twice in medium. Cell viability was determined on a hemocytometer by trypan blue dye exclusion, using an inverted phase contrast microscope. To prepare T cells from the thymus, we used a stomacher, a machine that

prepares single cells out of the thymus by mechanically disrupting the organ in tissue culture medium. Such T cells were >95% pure and will be referred to as thymocytes.

DES exposure

DES (Sigma, MO) was dissolved and diluted in DMSO. Stromal cells and thymocytes were co-cultured at 2:1 ratio (He et al., 2004) or cultured individually and treated with various concentrations of DES for 8 hrs. In other experiments, mice were treated subcutaneously with the vehicle or DES (1 mg/kg body weight) dissolved in corn oil.

FasL expression analysis

FasL expression on stromal cells was determined as follows. Thymocyte and stromal cells were co-cultured with vehicle or DES as described above. The cells were washed with phosphate-buffered saline (PBS) (Invitrogen, Grand Island, NY). To detect FasL expression on stromal cells, we stained the cells for FasL and CD45 and analyzed those cells that were negative for CD45. Briefly, cells were double-stained with 0.5 μ g of the biotin-anti-CD45.2 (BD Pharmingen, San Diego, CA) and PE-anti-FasL (BD Pharmingen, San Diego, CA) mAbs. Cells stained with biotin-anti-CD45.2 were secondarily stained with FITC-streptavidin (BD Pharmingen, San Diego, CA). Cells were then fixed with 1% *p*-formaldehyde and fluorescence was measured by flow cytometry.

Fas expression analysis

Fas expression on thymocytes was determined as follows: Thymocytes and stromal cells were co-cultured with vehicle or DES as described above. To detect Fas expression on stromal cells, we stained the cells for Fas and CD45 and analyzed those cells that were positive for CD45. Briefly, cells were incubated with 0.5 μ g of the biotin-anti-CD45.2 (BD Pharmingen, San Diego, CA) and PE-anti-Fas (BD Pharmingen, San Diego, CA) mAbs. Cells stained with biotin-anti-CD45.2 were secondarily stained with FITC-streptavidin (BD Pharmingen, San Diego, CA). Cells were then fixed with 1% paraformaldehyde and fluorescence was measured by flow cytometry.

TUNEL analysis

Thymocyte and stromal cell co-cultures (2×10^6) were washed with PBS. To study apoptosis in T cells, we carried out double-staining studies, in which cells were first incubated with 0.5 μ g of the biotin-anti-CD45.2 (BD Pharmingen, San Diego, CA) and then with PE-streptavidin (BD Pharmingen, San Diego, CA). Cells were then fixed with 1% paraformaldehyde, washed with PBS, and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate on ice for 2 min. Cells were washed again with PBS and incubated with FITC-dUTP, TUNEL reagent (Boehringer Mannheim, Indianapolis, IN), for 1 hr at 37°C. Fluorescence was measured by flow cytometry. In some in vivo studies, WT mice were injected with 1mg/kg body weight DES and 3 days later, thymocytes were harvested cultured in vitro for 24 hrs and analyzed for apoptosis by TUNEL as described (Do et al., 2002). This in vitro culture allows for better detection of drug-induced apoptosis in the

thymus because apoptosis-committed cells can die without being rapidly cleared by phagocytic cells (Do et al., 2002).

Confocal Immunofluorescence Microscopy

Samples of isolated cell preparations of thymocytes or stromal cells were washed with PBS, adhered to glass slides, and fixed with 4% paraformaldehyde. Cells were washed with PBS, blocked with mouse CD16/CD32 antibodies (Pharmingen, San Diego, CA), and again washed. Cells were stained with the following primary and secondary antibodies: anti-CD45.2 (Pharmingen, San Diego, CA) followed by Streptavidin Alexa Fluor 555 (Molecular Probes, Eugene, OR), anti-FasL (Oncogene, Boston, MA) followed by Cy2 mouse anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), or anti-Fas (Pharmingen, San Diego, CA) followed by Cy5 goat anti-hamster IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) and washed. Following primary staining, cells were stained with secondary antibodies, washed with PBS, and mounted. Slides were analyzed by confocal microscopy.

Calculation of statistical significance

For in vivo studies, groups of 4-5 mice were used and data were analyzed using Student's *t* test and a *p*-value of ≤ 0.05 was considered significant. Results are presented as mean \pm SEM.

Results

Analysis of thymic stromal cell enrichment

Stromal cell isolation and enrichment was carried out using a combination of density gradient and enzyme digest as described in Methods. Figure 1 shows microscopic evaluation of the enrichment. The larger cells represent the stromal cells and the smaller represent T cells referred to as thymocytes (Fig. 1A). The enrichment process yielded approximately 84% pure stromal cells. When thymocytes were evaluated for CD45 expression, ~95% of the population were CD45+ cells (Fig. 1B). Confocal staining for CD45 and morphological analysis showed CD45+ cells to be thymocytes (small, rounded) and CD45- cells to be stromal cells (large, irregular) (Fig. 1C). Also, in cocultures, T cells were found to form conjugates with stromal cells (Fig 1C).

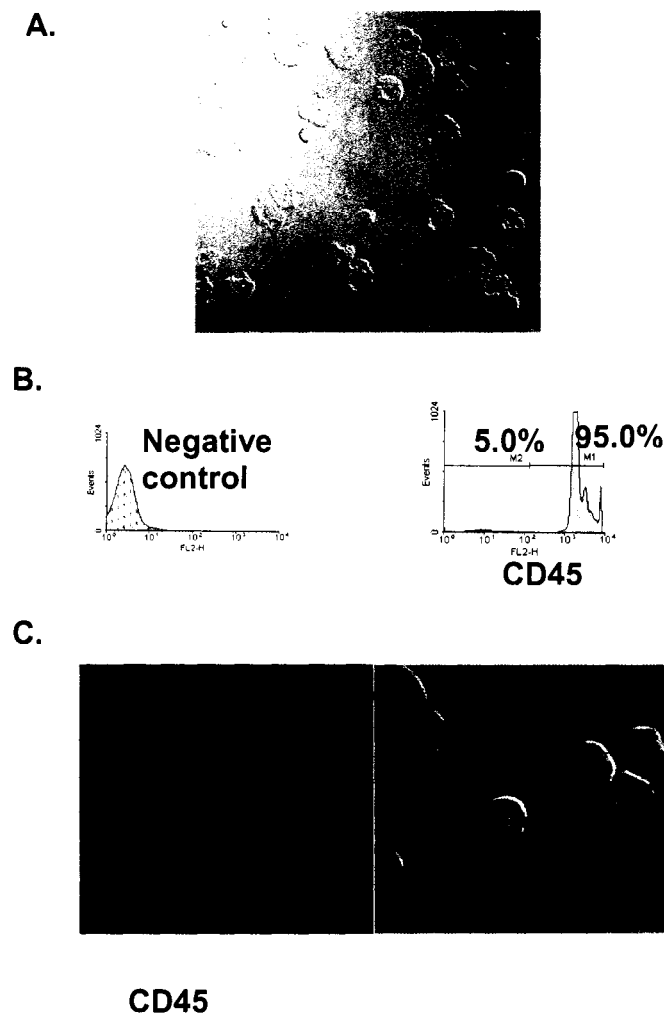


Figure 1. Stromal cell enrichment. In panel A, enriched stromal cells were analyzed visually by microscopy. Majority of the large, granular and irregular cells represent stromal cells. In panel B, thymocytes were analyzed for CD45 expression by flow cytometry, and in panel C, coculture of stromal cells and thymocytes (T cells) were analyzed by confocal microscopy after staining for CD45. The arrows indicate stromal (S) and T (T) cells. These data are representative of 4 experiments.

Contact between thymocytes and stromal cells is required for DES induced apoptosis

First, we investigated whether administration of DES into C57BL/6 WT mice would cause thymic atrophy and apoptosis in T cells. To this end, mice were injected with 1mg/kg DES or vehicle and 3 days later, the thymocytes were harvested and analyzed. The data shown in Fig. 2 indicated that DES treatment caused significant decrease in thymic cellularity when compared to the vehicle controls (Fig. 2A). Moreover, when the thymocytes were analyzed for apoptosis, DES-exposed T cells underwent increased levels of apoptosis when compared to the controls.

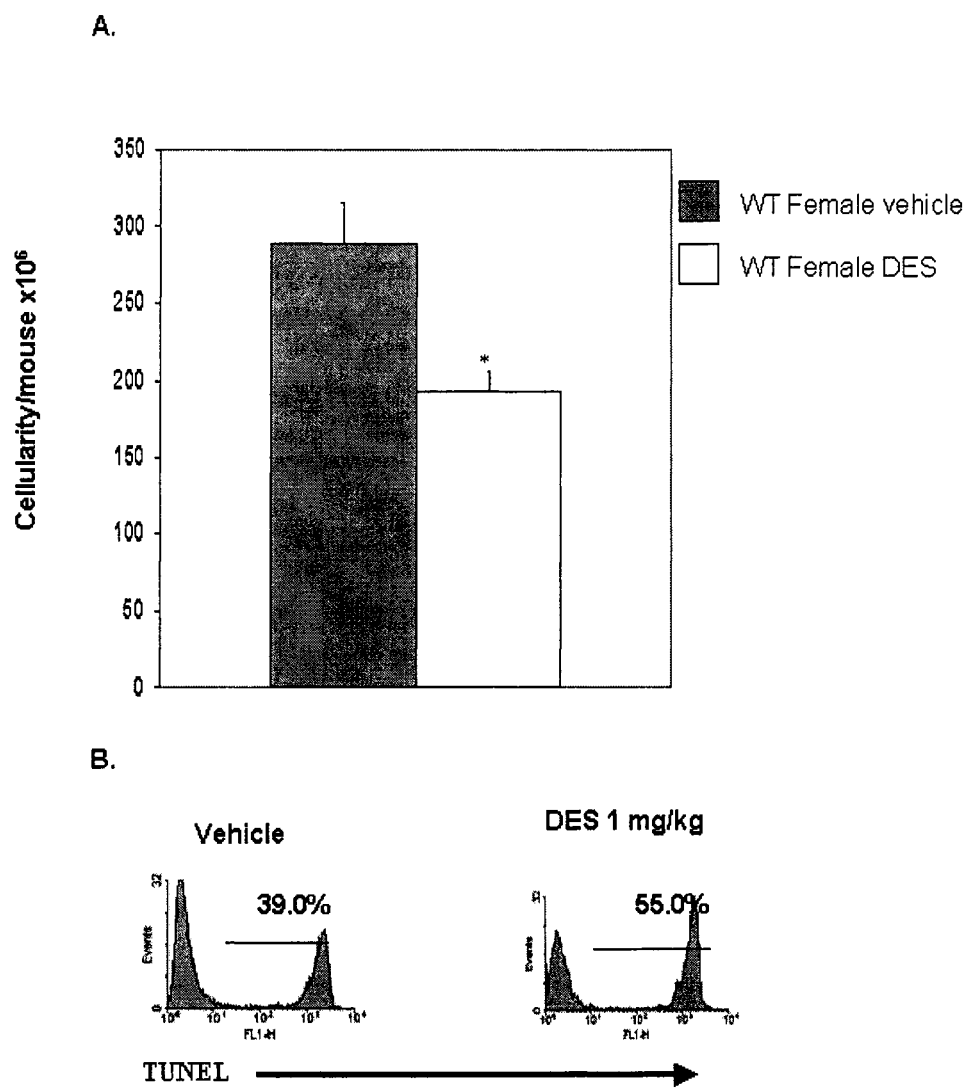


Figure 2. DES induces thymic atrophy and apoptosis in vivo. (A) WT mice were treated with DES (1 mg/kg) or vehicle. Three days later, thymocytes were harvested and cellularity determined by trypan blue exclusion. Vertical bars represent the mean \pm SEM of 5 mice/group. * Denotes statistically significant difference when compared to vehicle treated cells. (B) Apoptosis in thymocytes was assessed by TUNEL assay. Percent apoptosis is indicated.

To investigate if DES was directly causing apoptosis in thymocytes or was mediating its effect through thymic stromal cells, cultures of stromal cells alone, thymocytes alone, or co-cultures of stromal cells and thymocytes were treated *in vitro* with various concentrations of DES. We observed that DES at all concentrations tested, failed to induce apoptosis in cultures consisting of thymocytes alone (Fig 3A) or stromal cells alone (Fig 3B). Also, when stromal cells cocultured with thymocytes were analyzed for apoptosis using CD45-deficiency as a marker, we noted that the stromal cells failed to exhibit apoptosis (Fig. 3D). However, when thymocytes (CD45+) cocultured with stromal cells were analyzed for apoptosis, (Fig. 3C), significant increase in apoptosis was detected particularly at 25 and 50 μ M of DES, when compared to the vehicle controls. These data suggested that DES does not directly induce apoptosis in thymocytes or stromal cells but does trigger apoptosis in thymocytes when such cells are co-cultured with stromal cells.

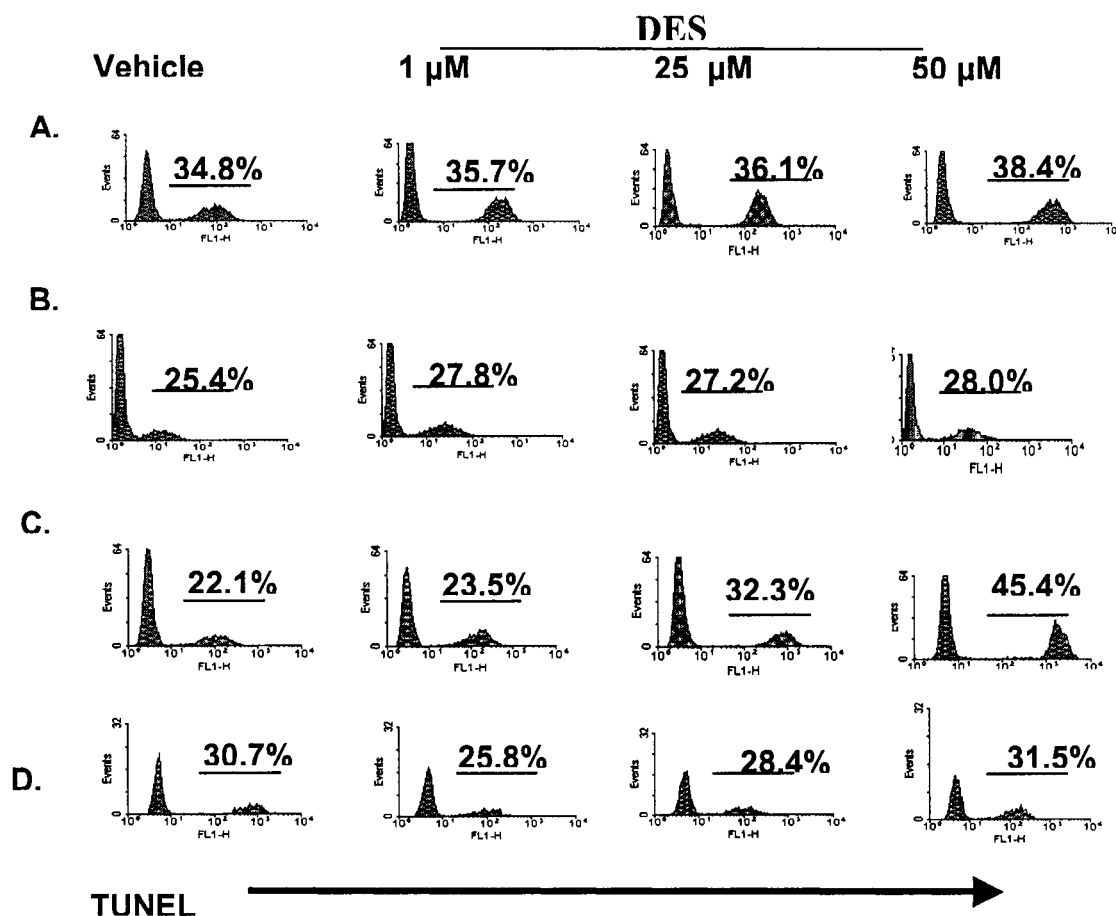


Figure 3. Stromal cell contact-induced apoptosis in thymocytes. WT stromal cells and thymocytes were cultured alone or in combination as described in methods with vehicle or 1, 25, or 50 μ M DES for 8 hrs. Cells were harvested and stained as described in Methods. Stromal cells (CD45-) or thymocytes (CD45+) were analyzed for apoptosis by TUNEL. The percentage of apoptotic cells has been depicted in each histogram. Replicate cultures for each treatment group were prepared and pooled for analysis. The panels are as follows: (A) Thymocytes alone, (B) Stromal cells alone, (C) Thymocytes after co-culture, and (D) Stromal cells after co-culture. These data are representative of 4 experiments.

DES increases the expression of FasL on stromal cells and but not Fas on thymocytes

To determine if the increase in thymocyte apoptosis observed in cocultures following DES exposure might be due to an increase in the expression of FasL on thymic stromal cells and/or Fas on thymocytes, we co-cultured thymocytes and stromal cells in the presence of DES and double-stained first with biotin-anti-45.2 followed by FITC-streptavidin and secondly with PE-anti-FasL or PE-anti-Fas Abs. Abs against CD45 were used to detect thymocytes that express CD45 and stromal cells that lack CD45. Flow cytometric analysis as shown in Fig. 4A demonstrated that majority of vehicle-exposed thymocytes expressed Fas and showed no further increase in the levels of Fas upon DES exposure. In addition, we examined the thymocytes and stromal cells exposed in vivo to DES or vehicle by confocal microscopy and found that Fas was expressed on both thymocytes and stromal cells obtained from vehicle-treated mice (Fig 4B). Moreover, the expression of Fas on these cells was not significantly altered following exposure to DES.

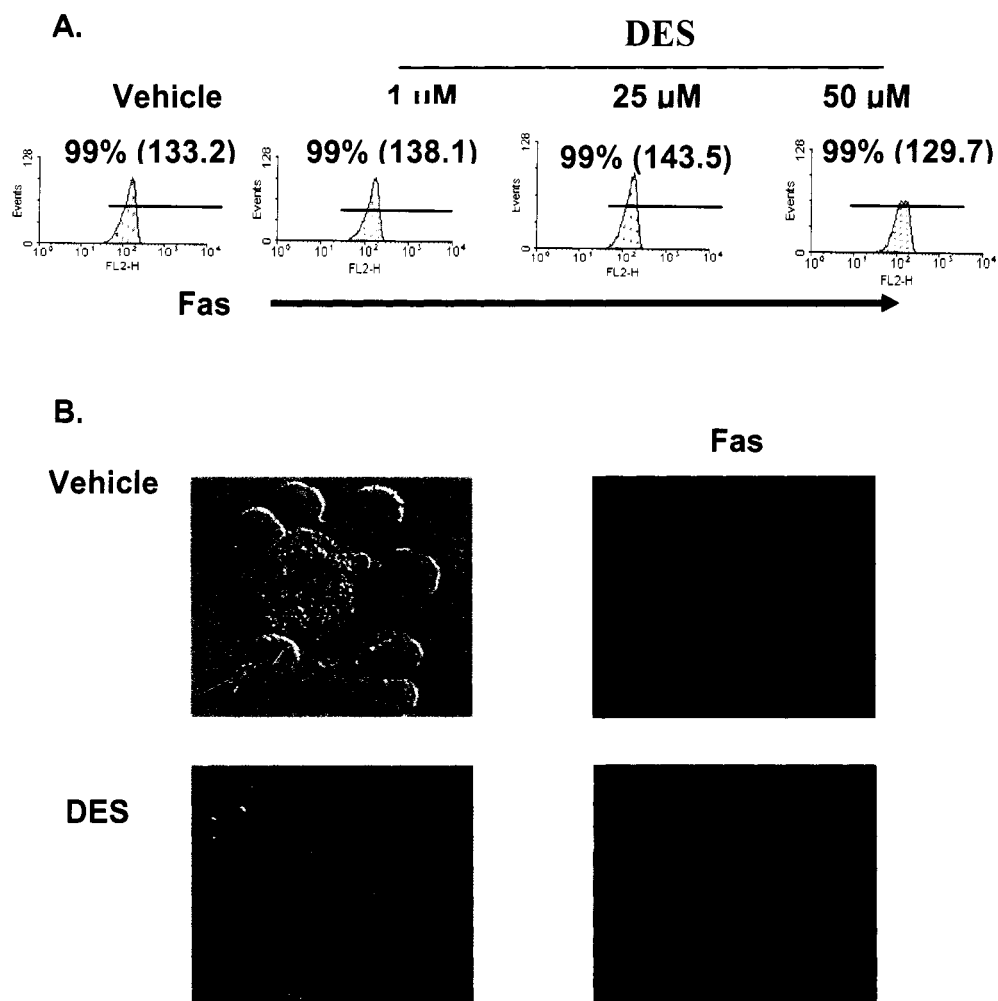


Figure 4. Effect of DES on Fas expression. WT thymocytes were cultured in combination with stromal cells as described in methods with vehicle or 1, 25, or 50 μ M DES for 8 hrs. Cells were harvested and double stained with anti-CD45 and anti-Fas mAbs as described in Methods. The CD45⁺ population was gated and analyzed for Fas expression by flow cytometry (A). The percent positive cells and mean fluorescence intensity (MFI, number in parentheses) was indicated in each histogram. In panel B, thymocytes from vehicle- or DES-injected mice were analyzed for Fas expression by confocal microscopy. The arrows indicate smaller T cells and larger stromal cells.

Similar studies were performed on FasL expression. As shown in Fig 5A, vehicle-treated stromal cells showed significant levels of FasL and this expression increased significantly following exposure in vitro to DES in terms of increase in percentage and density of expression of FasL. When stromal cells and thymocytes exposed in vivo to vehicle or DES were analyzed by confocal microscopy, it was noted that vehicle treated thymocytes failed to express FasL whereas the stromal cells expressed FasL, consistent with the previous studies showing that FasL is expressed only on stromal cells but not T cells of the thymus (Castro et al., 1996; French et al., 1996). Furthermore, FasL expression on stromal cells was significantly enhanced following exposure to DES (Fig. 5B).

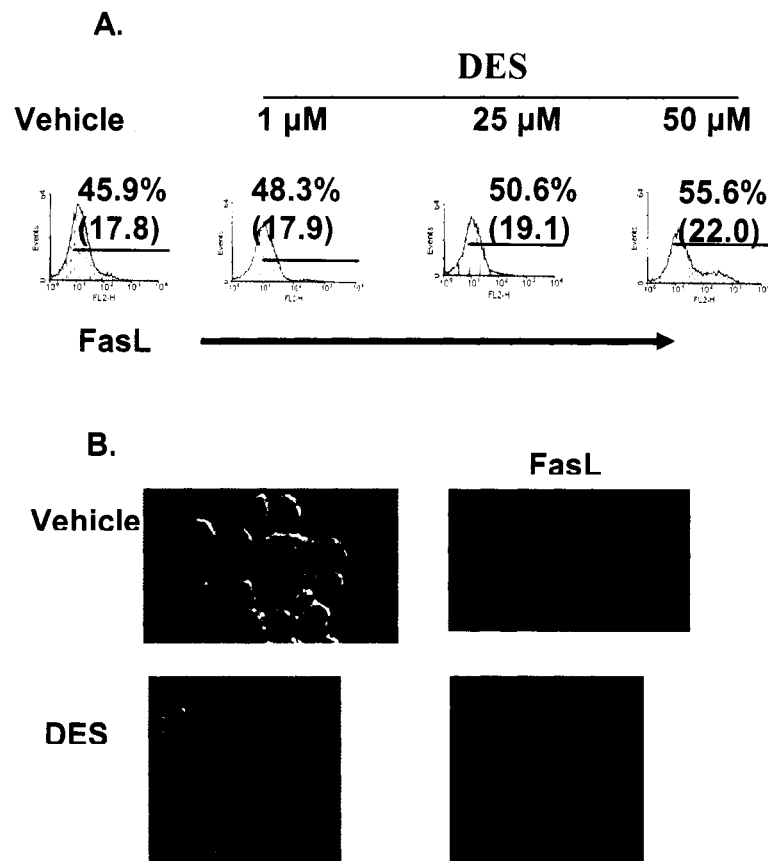


Figure 5. Upregulation of FasL on stromal cells following DES exposure. In panel A, WT stromal cells were cultured in combination with thymocytes as described in methods, with vehicle or 1, 25, or 50 μ M DES for 8 hrs. Cells were harvested and double-stained with anti-CD45 and anti-FasL mAbs as described in Methods. The CD45-negative population was gated and analyzed for FasL expression by flow cytometry. The percent positive cells and mean fluorescence intensity (MFI, number in parentheses) was indicated in each histogram. The gates were set based on use of isotype control Ab staining. In panel B, thymocytes from vehicle or DES-injected mice were analyzed for FasL expression by confocal microscopy. The arrows indicate stromal cells that are large and surrounded by smaller T cells.

Role of Fas and FasL on DES induced apoptosis

To further address the precise role of Fas:FasL interactions in DES-induced apoptosis, thymocytes and stromal cells were isolated from C57BL/6 wild-type (WT), *gld/gld* (gld), and *lpr/lpr* (lpr) mice and mixed in various combinations. Cells were then treated with DES in vitro and analyzed for apoptosis in thymocytes by gating CD45⁺ cells. When WT stromal cells were co-cultured with WT thymocytes in the presence of DES, significant increase in apoptosis was noted in thymocytes at 25 and 50 μ M DES when compared to vehicle controls (Fig. 6A). In contrast, when thymocytes from cocultures consisting of WT stromal cells + lpr thymocytes (Fig 6B), gld stromal cells + WT thymocytes (Fig 6C) or gld stromal cells + lpr thymocytes (Fig 6D) were screened for apoptosis in the presence of DES, no significant increase in apoptosis was detected, when compared to the controls. These data together demonstrated that Fas-FasL interactions involving thymocytes and stromal cells was critical in DES-induced thymic apoptosis.

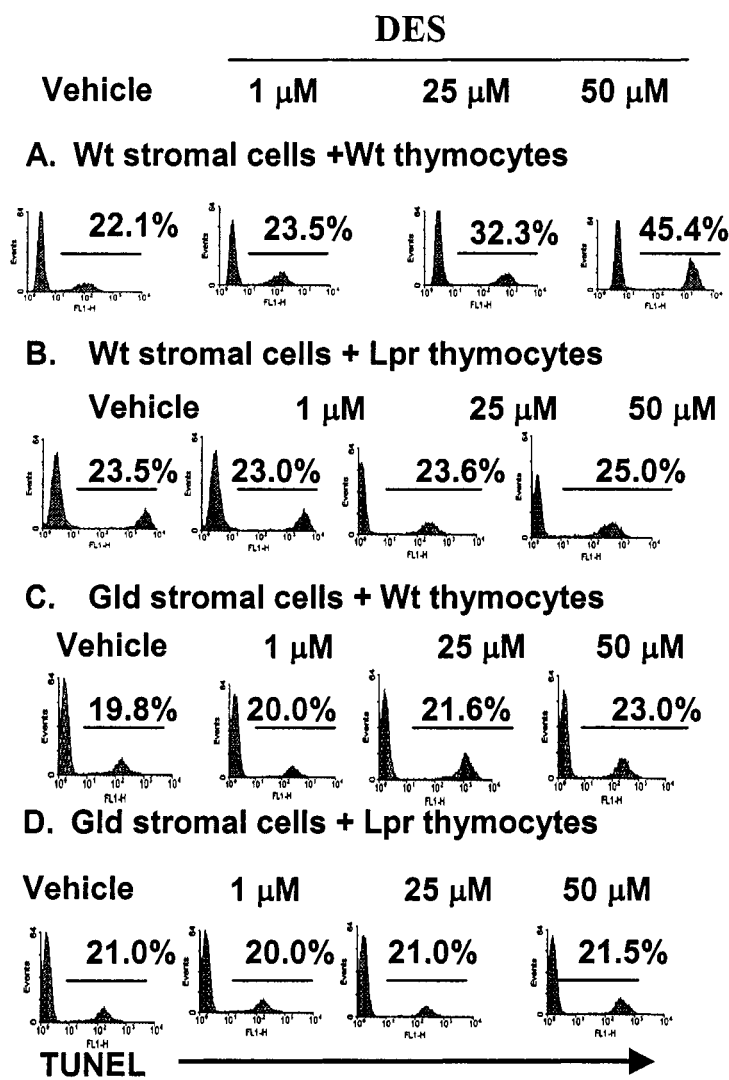


Figure 6. Interactions between Fas and FasL are required for DES-induced thymocyte apoptosis. The following co-cultures were prepared: WT stromal + WT thymocytes (A), WT stromal cells + Lpr thymocytes (B), Gld stromal cells + WT thymocytes (C), or Gld stromal cells + Lpr thymocytes (D). Cultures were incubated with vehicle or 1, 25, or 50 μ M DES for 8 hrs. Cells were harvested, stained for CD45 and analyzed for apoptosis by TUNEL. The percentage of apoptotic cells has been depicted in each histogram. These data are representative of 4 experiments.

Discussion

Estrogens including DES have been shown to induce thymic atrophy by triggering apoptosis (Calemine et al., 2002; Do et al., 2002). Previous studies from our laboratory have demonstrated that Fas-FasL interactions play a critical role in estrogen-induced apoptosis in thymocytes inasmuch as *gld* and *lpr* mice were more resistant to estradiol-induced thymic atrophy and apoptosis (Do et al., 2002). In the present study, we tested the hypothesis that DES exposure can increase stromal cell expression of FasL resulting in apoptosis of Fas-bearing thymocytes that come in contact with the stromal cells.

The thymus is a dynamic environment in which T cells develop. The stroma plays a critical role during different stages of T cell development (van Ewijk et al., 1994; Anderson and Jenkinson, 2001). Furthermore, thymocytes undergo apoptosis initiated by thymic epithelial cells (Schreiber et al., 1996), suggesting the need for contact between T cells and stromal cells. In addition, T cell-stromal cell interactions also play a critical role in positive selection of T cells.

Thymic stromal cells are known to express FasL, whereas thymocytes fail to express FasL but express Fas (Castro et al., 1996; French et al., 1996). We hypothesized that DES may primarily act on stromal cells and cause thymocytes to undergo apoptosis following cell-cell contact with stromal cells. In the current study, we found that stromal cell: thymocyte contact is critical for DES-induced apoptosis of thymocytes, as individual cultures were resistant to the toxic effects of DES. These findings were consistent with those previously suggesting a role of contact between thymocytes and

thymic epithelial cells in the induction of apoptosis in the former cells (Schreiber et al., 1996).

The precise mechanism through which exposure to DES induces the FasL expression on stromal cells is not clear. However, previous studies have suggested that FasL gene promotor has a functional ERE (Mor et al., 2003) through which DES may act. Given that stromal cells are important for many aspects of the thymocytes such as growth, development, and positive and negative selection of T cells (McGargill et al., 2000; Prockop and Petrie, 2000; Lind et al., 2001), the ability of DES to alter surface molecules on stromal cells may lead to profound consequences for developing T cells in the thymus. Upregulating FasL, a key molecule in Fas-mediated apoptosis, could interfere with developing thymocytes, as apoptosis is an important mechanism of removing cells during selection (Cairns et al., 1993; Anderson et al., 1994; Klug et al., 1998; Bouillet et al., 2002). These alterations may likely be the reason behind altered T cell development observed following DES exposure (Holladay et al., 1993; Silverstone et al., 1994; Lai et al., 1998).

Chapter VI. Conclusions

Mechanism of Apoptosis in T cell leukemia Jurkat

Tumor cells were found to be vulnerable to DES, as they exhibited reduced cell numbers and increase apoptosis after treatment as shown in chapter 2. The results suggested a complex apoptotic pathway induced by DES (Fig. 1). Apoptosis was found to involve death receptor components, as evidenced by the resistance of FADD^{-/-} tumor cells to apoptosis, in addition to the cleavage of caspase 8 and 10. FasL was found to, also, be increased at the mRNA level. Given that blocking the interaction of Fas and FasL did not alter apoptotic outcome, there may be some level of functional redundancy on TNF family and/or a possible role of the mitochondria that provides an alternate mechanism. The mitochondria was evidenced to play a role in apoptosis through detection of truncated Bid, the resistance of Bcl-2⁺⁺⁺ tumor cells, and early release of cytochrome *c*. DES may either act directly at the level of the mitochondrial or through indirect recruitment of the death receptor pathway.

The mitochondria can be a target for many proapoptotic molecules. Stress brought on by DES exposure is known to induce genotoxic damage. A typical cell response to such an assault is to repair, which would alter the cell cycle progression. The presence of cell cycle regulatory proteins, particularly p53 suggests that an additional

level of regulation is involved in apoptosis. Regulation of p53 may involve JNK, because promotor induction of p53, in addition to, FasL and TNF α is known to be promoted by JNK phosphorylation. Further investigation into the role of p53 and the cell cycle may prove insightful. Another area of interest may be in further investigation of the Bcl-2 family members. This family is composed of both proapoptotic and antiapoptotic members. Study of these members may provide further insight into the pathway of apoptosis.

Renewed interest in DES as a potential therapy for cancers eg. prostate has emerged. The ability of DES to cause apoptosis of cancer cells independent of the ER was observed in the current investigation. Medical use of DES to treat cancer centers on the ability of DES to reduce testosterone through androgen deprivation. Though our studies were not specific to medicinal use of DES, several points such be kept in mind. Our work utilized Jurkat, a T cell leukemia cell line. Jurkat is a useful modeling system for mature T cells. Secondly, the system, used in our study, is an independent system unlike normal T cells that are dependent on the body environment. Finally, the effects of DES can not be characterized as specific to cancerous cell necessarily, though we utilized a cancerous model for study.

Our studies may add to the knowledge of how endocrine disruptors affect T cells. The mechanism behind the modulation of T cells by DES is through the induction of apoptosis. Our investigation revealed a complex apoptotic pathway. The ability of DES to induce apoptosis may help to explain the mechanism behind altered immune responses in humans.

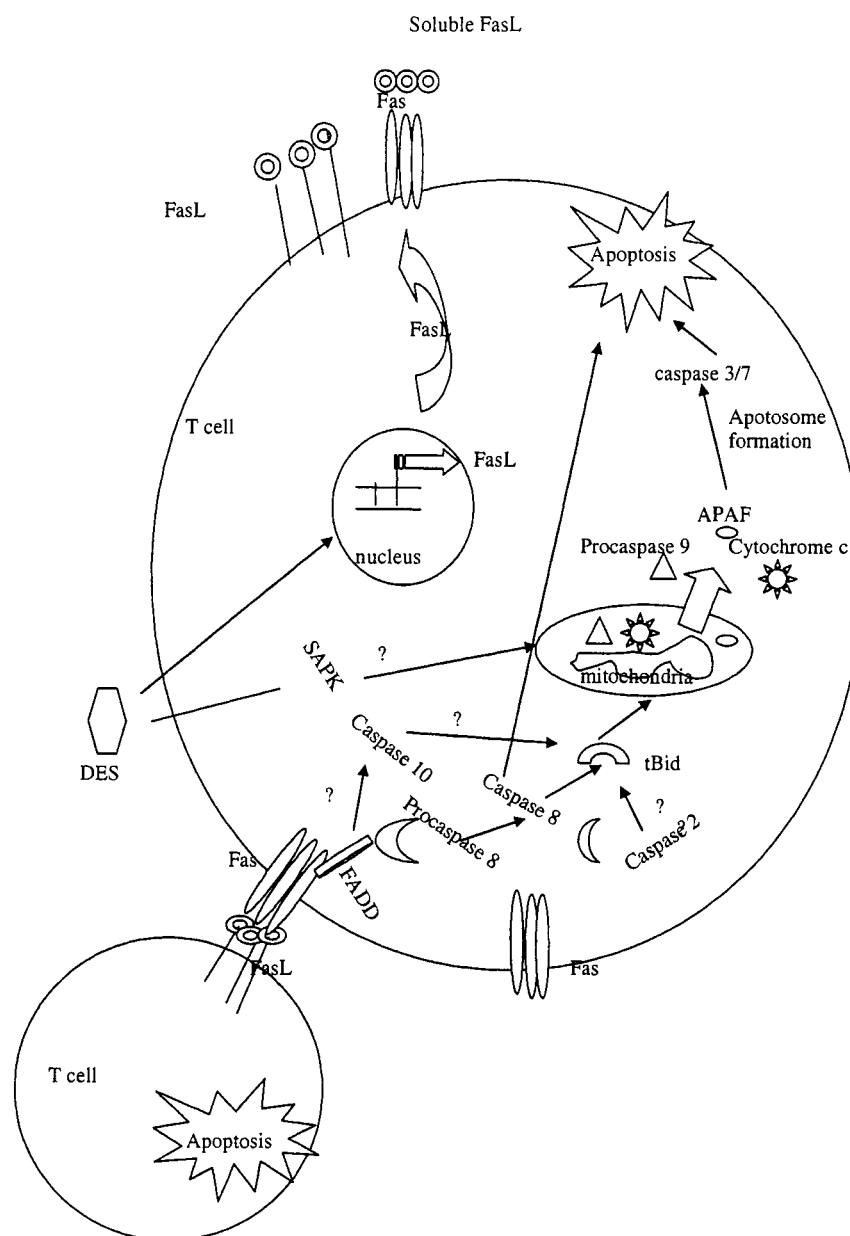


Figure 1. Model pathway of DES-induced apoptosis in T cells.

Mechanism of DES induced apoptosis in the thymus

DES treatment in utero lead to a pronounced reduction in cell number and increased apoptosis as shown in chapters 3 and 4. In reviewing the induction of apoptotic gene following DES treatment at gd17, significant increases in the TNF family were noted, suggesting that DES induced apoptosis during development may involve the death receptor pathway.

An additional mechanism may play a role in inducing apoptosis. Since the mitochondrial pathway represents a stress mediated pathway, DES could be acting via the mitochondria. The presence of p53 was detected during the study. Given that p53 can act on the mitochondria to induce apoptosis under stress, this may prove to be an addition pathway of inducing apoptosis in thymocytes.

Thymocytes are clearly more sensitive at certain periods during development, as observed in our study. Apoptosis could be detected pre-gd-19 but not thereafter. Thymocyte selection is an event that may be targeted, since it occurs early. Altered positive and negative selective processes were noted in the HY-TCR study. Additionally, the ability to proliferate in response to mitogens was reduced after DES treatment. Reduced functionality may contribute to immune defects or abnormalities in the mature response.

Further study of longterm immune effects suchas autoimmunity may prove to be valuable, given that prenatal exposure in humnas have been linked to autoimmunity. Though our study model used a short term exposure wheras human exposure was chronic throughout fetal development, further investigation may yiels a model system to study

this longterm exposure to DES in mice. It should be noted, also, that our effects were transient. Additionally, the dose regiment was lower in our study compare to the dosage that human fetuses were exposed to. This would also be a concern for future studies on longterm DES effects. A possible model to study the implication of fetal exposure to longterm immunological effects such as autoimmunity could be the NZB/NZW F1 mice. The effects perinatal exposure of DES in these mice could be monitored periodically during the first year of life. This may help in understanding the longterm immunological outcome of DES exposure.

Though we have observed the result of thymic DES exposure, the initial events that culminate in DES induced apoptosis in thymocytes remain to be clarified. The previous studies in chapters 3 and 4 have provided evidence to help clarify downstream mechanisms. The data in chapter 5 revealed a possible initiation mechanism that could lead to the induction of apoptosis (Fig. 2). The data suggested that DES may increase the expression of FasL on thymic stromal cells. Fas mediated apoptosis of thymocytes is dependent upon Fas expressing thymocytes and FasL expressing stromal cells. Thus, cell:cell contact is critical to initiating the apoptotic cascade in thymocytes following DES exposure. The increase in FasL following DES exposure is suggestive that estrogen promote Fas mediated apoptosis. Given this, the current study suggested that DES might modulate the FasL expression on the thymic stromal cells resulting in apoptosis.

T cell development is dependent on thymic stromal cells. In this study, epithelial cells (CD45-) were found to have increased expression in FasL following DES exposure. Epithelial cells play a role in positive selection of T cells in the thymus. This may

provide a mechanism by which positive selection is altered in HY-TCR mice following DES treatment. Further study into specific apoptosis of these thymic T cells may provide confirmation. Additionally, stromal cells consist of other cells such as dendritic cells. Dendritic cells express CD45⁺ and FasL, in addition to other surface markers. Dendritic cells have been found to play a role in negative selection. Analysis of these cells following DES exposure may also reveal more information on thymic T cell interactions.

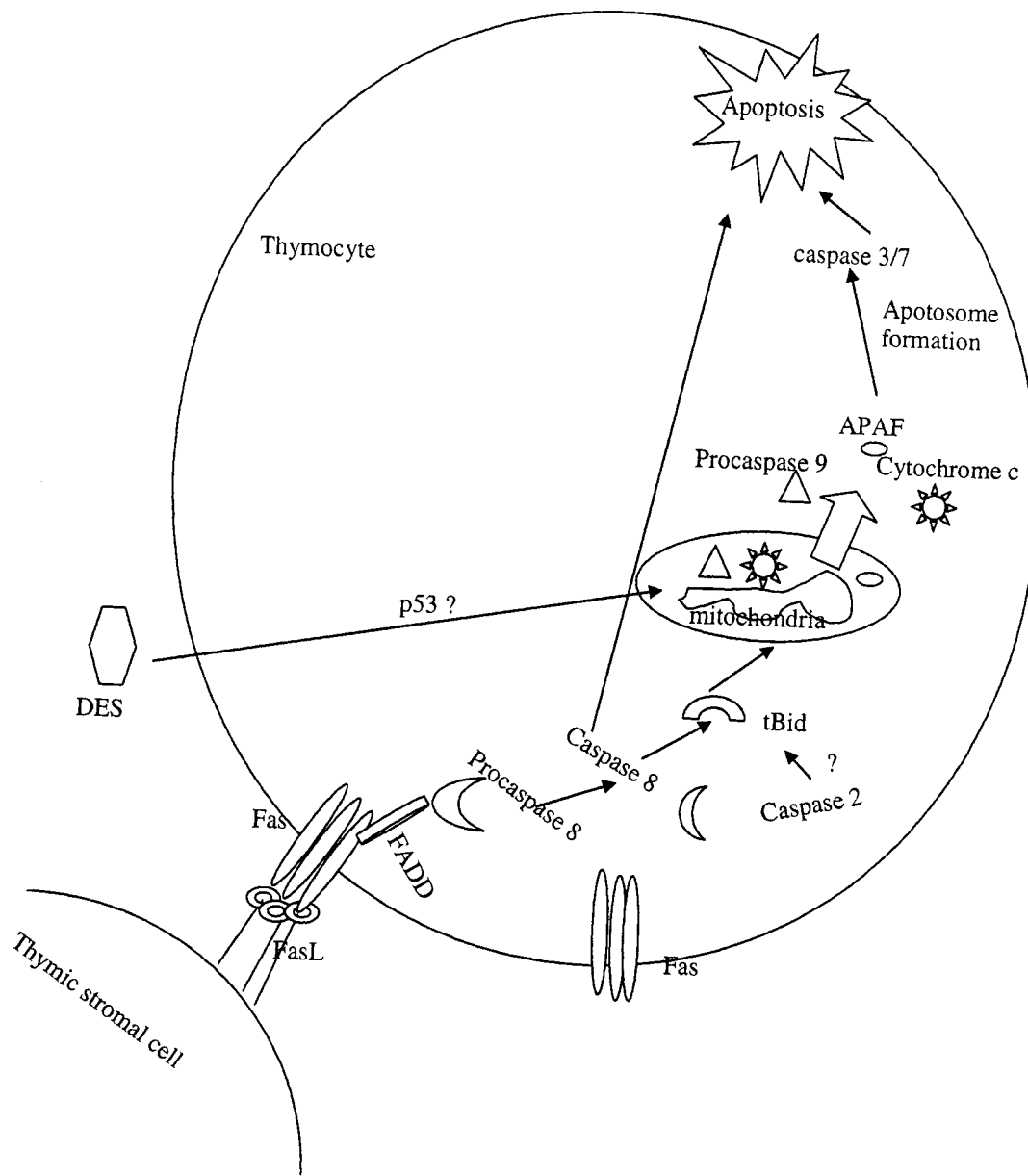


Figure 2. Model system for DES-induced apoptosis in thymocytes

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