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Genes That Regulate Apoptosis in the Mouse Thymus

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Abstract Elimination of self-reactive T lymphocytes occurs during T-cell development in the thymus by a process known as negative selection. The mechanism that drives negative selection is apoptosis. To identify genes that regulate apoptosis in the mouse thymus, a library of negatively selected T cells was constructed and, by subtractive screening, several differentially regulated genes were isolated. Two transcripts that are repressed during cell death were identified, in addition to two induced transcripts. Further experiments demonstrated that cell death in thymocytes can occur via several induction pathways and each pathway appears to be regulated by a unique cascade of genes.

Apoptosis in the immune system functions at many levels to remove unnecessary or potentially dangerous cells. Several years ago it was recognized that negative selection, the process by which self-reactive T cells are removed from the thymus, occurs by clonal deletion [White et al., 1989]. More recently, clonal deletion has been shown to arise through the specific induction of apoptosis in those cells that recognize antigen with high affinity. The first indications that apoptosis was the process that results in negative selection came from experiments showing that treatment of immature $CD4^+$, $CD8^+$ thymocytes with antibody to the CD3 component of the T-cell receptor resulted in death by apoptosis [Smith et al., 1989]. These experiments provided evidence that crosslinking the TCR on immature thymocytes results in death, an important observation given that negative selection is known to occur via interaction of the TCR with antigen. The conclusions from these experiments were strengthened by the observations that immature thymocytes may be induced to undergo apoptosis by interaction with antigen presenting cells [Swat et al., 1991; MacDonald and Lees, 1990]. Similar results were obtained with thymic organ cultures treated with antibodies to CD3. Taken together, these data strongly suggested that negative selection occurs through the induction of apoptosis.

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The experiments of Murphy et al. [1990] provided unequivocal evidence that negative selection results in apoptosis. In these experiments, transgenic mice were generated in which most T cells express the same TCR. When these animals were injected with the peptide recognized by the transgenic TCR, the ovalbumin peptide OVA 323-336, essentially all the immature thymocytes were induced to die by apoptosis, proving that antigen-specific negative selection occurs through apoptosis.

Some of the earliest experiments demonstrating the existence of an active process of cell death demonstrated that immature lymphocytes may be induced to undergo apoptosis in response to synthetic glucocorticoids such as dexamethasone [Wyllie, 1980; Cohen and Duke, 1984]. T-cell lines that behave similar to immature thymocytes also are exquisitely sensitive to glucocorticoids. These lines have been useful to many who study apoptosis in cell lines or in tumors of the immune system; however, the physiological relevance of cell death by glucocorticoids has remained elusive. Recent data from Ashwell and colleagues have shed some light on the significance of this inducer of apoptosis [Vacchio et al., 1994]. It appears that the developing thymus produces physiological levels of glucocorticoids capable of inducing apoptosis in immature thymocytes. However, as shown by several investigators [Iwata et al., 1992; Zacharchuk et al., 1990], simultaneous crosslinking of the TCR and exposure to glucocorticoids results in cell survival rather than enhanced apoptosis. Thus, those cells that have TCRs with moderate- to high-affinity interaction with either self-MHC

or antigen will be spared from death induced by endogenous glucocorticoid and hence are free to undergo the processes of positive and/or negative selection. Only those cells that have TCRs that do not recognize MHC will be killed by glucocorticoids and thus die by a form of neglect [Vacchio et al., 1994].

These data indicate that development in the thymus results from a careful balance of life and death whereby cells that react with self-MHC at a low to moderate affinity are spared and undergo positive selection. By contrast, those cells that recognize self with a high-affinity TCR interaction undergo negative section and apoptosis. Finally, cells that are neither positively nor negatively selected are prevented from surviving through the induction of apoptosis by intrathymic glucocorticoids. Such a fine tuned system provides an environment in which vast numbers of cells may circulate through the developmental pathways of the thymus, preserving only those cells that are most useful for antigen recognition in the peripheral immune system. This level of careful control is essential if the animal is to avoid the inadvertent positive selection of a self-reactive T cell and risk autoimmune disorders.

Very recent data indicate that the vast majority of cells in the thymus actually die through neglect [Surh and Sprent, 1994]. Surh and Sprent used TUNEL [Gavrieli et al., 1992] to detect the DNA strand breaks that occur with apoptosis to examine negative selection in the thymus. By the use of a TCR transgenic mouse, they were able to demonstrate that negative selection did indeed lead to death by apoptosis, agreeing with the earlier data of Murphy et al. [1990]. However, their data allowed a direct measurement of the level of apoptosis during negative selection. The data indicated this apoptosis makes a very small contribution to the overall level of small loss in the thymus. Instead, their data provide the first direct evidence that the bulk of apoptosis in the thymus is due to death by neglect, the lack of either positive or negative selection. When the experiments of Ashwell are considered, one must assume that the majority of apoptosis in the thymus is likely to be induced by endogenous glucocorticoids [Vacchio et al., 1994].

ISOLATION OF GENE INDUCED DURING NEGATIVE SELECTION

In order to determine the molecular events that mediate these complex processes in the thymus, we generated a cDNA library from thymocytes induced to undergo negative selection and, by differential hybridization, have isolated several genes that are either induced or repressed coincident with negative selection. To construct our library, we took advantage of the transgenic TCR model system described by Murphy et al. [1990]. Thymocytes were removed from these mice and RNA isolated 3, 4, or 5 h following the injection of antigen, the ovalbumin peptide 323–336 (Fig. 1a). The RNA was converted into cDNA, cloned and screened by a differential hybridization strategy, shown in Figure 1b.

apt-1 AND *apt-3* ARE REPRESSED DURING CELL DEATH

Following several rounds of screening, four clones were isolated, two of which were re-

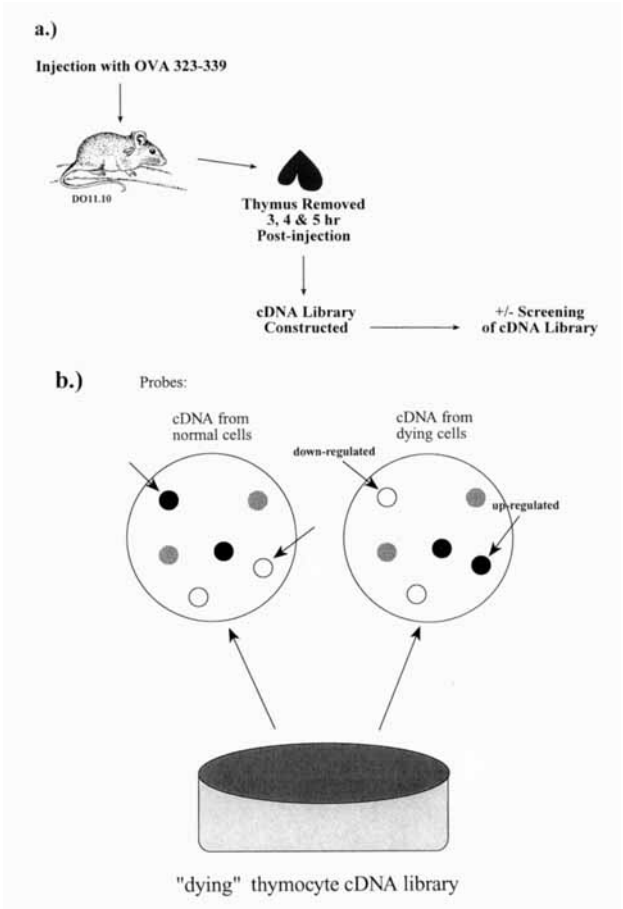

Fig. 1. Construction of a cDNA library from dying thymocytes (**a**) and subtractive screening of the cDNA library (**b**).

pressed and two of which were induced during cell death in thymocytes. In order to examine more carefully the patterns of expression of these genes, we took advantage of the T cell hybridoma, DO11.10, which bears the same TCR as the transgenic mouse used in these studies and undergoes apoptosis upon TCR crosslinking. Figure 2 shows the pattern of expression in DO11.10 cells of two genes, *apt-1* and *apt-3*, that are repressed when cells are induced to die by treatment with PMA + A23187. Similar patterns of expression were observed with the anti-TCR antibody, F23.1 as well as when DO11.10 cells were induced to die by treatment with the synthetic glucocorticoid, dexamethasone (data not shown).

nur77 IS REQUIRED FOR CELL DEATH IN T CELLS

Another gene, initially designated *apt-2*, was found to be expressed in DO11.10 cells following induction of apoptosis by exposure to the anti-TCR antibody, F23.1 (Fig. 3). We subsequently found that this pattern of expression is unique to the induction of cell death through engagement of the TCR and induction of apoptosis by glucocorticoids or ionizing radiation does not result in the expression of *apt-2*. Upon determination of the DNA sequence, *apt-2* was revealed to be a previously isolated gene known as *nur77*, first isolated from a cDNA library of serum stimulated 3T3 cells [Lau and Nathans, 1987]. This gene encodes a member of the steroid hormone receptor superfamily of zinc finger DNA binding transcription factors. Unlike most other members of this family, *nur77* has no identified intracellular ligand. The expression of *nur77* also has been demonstrated in PC12 cells when these cells are induced to differentiate into neuronal cells by treatment with nerve growth factor. Thus, *nur77* has been shown to be induced during cell proliferation and cell differentiation and, in our experiments, following the induction of apoptosis.

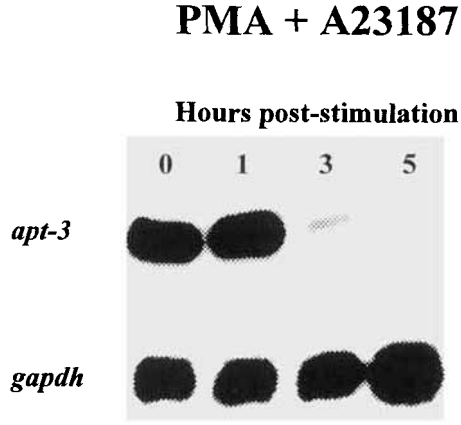

Fig. 2. Expression of *apt-3* in DO11.10 cells induced to die by treatment with PMA + A23187. Cells were treated with PMA + A23187 and RNA prepared at the indicated times following treatment.

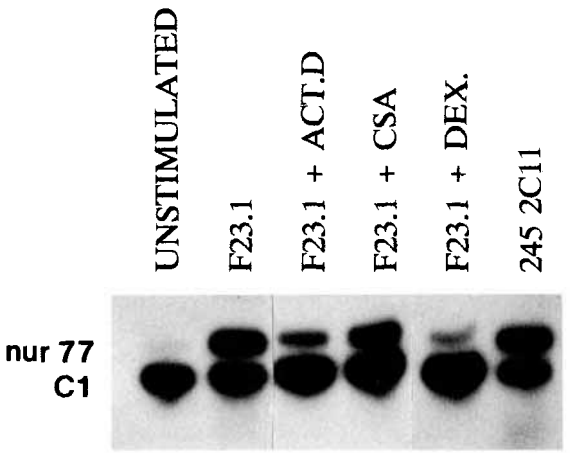

Fig. 3. Expression of *nur77* in DO11.10 cells. DO11.10 cells were treated with the anti-TCR antibody, F23.1, F23.1 in the presence of the transcriptional inhibitor actinomycin D, F23.1 in the presence of Cyclosporin A, F23.1 in the presence of dexamethasone or with antibody to the CD3 complex, 245 2C11. Cells were treated as indicated for 2 hr and RNA prepared at the indicated times following treatment.

To determine whether the expression of *nur77* was critical for the induction of apoptosis, we created an antisense construct of the *nur77* gene and, by transient transfection analysis demonstrated that *nur77* is required for cell death [Liu et al., 1994]. In these experiments, we took advantage of the fact that the nur77 promoter region was both well defined and drives high level of expression following TCR engagement. We generated a cDNA construct that used this promoter to drive the expression of the *nur77* antisense construct. This technique ensured that the antisense construct would be induced in much the same manner as the endogenous gene, providing a comparative level of both the endogenous gene, as well as the antisense form of *nur77*. Because transfection of T-cell lines is not an efficient process, it was necessary to devise a strategy to detect specifically the small percentage of cells that received the antisense vector. To accomplish this, we took advantage of a strategy originally described by Harlow and colleagues [Zhu et al., 1993], using the cell surface

marker CD20 as a co-transfection marker. By using an excess concentration of the *nur77* antisense vector relative to the CD20 vector, it is likely that every cell that expresses CD20 also received the antisense vector. Such experiments demonstrated conclusively that transfection with antisense *nur77* resulted in accumulation of $CD20^+$ cells [Liu et al., 1994]. Conversely, the transfection of DO11.10 cells with a truncated nur77 sense control resulted in barely detectable levels of $CD20^+$ cells. These data suggest that prevention of *nur77* expression by the antisense construct results in significant inhibition of the normally occurring induction of apoptosis following TCR crosslinking. We concluded that *nur77* was required for cell death induced by TCR engagement in DO11.10 cells. These data are supported by the findings of Woronicz et al. [1994], who showed that a dominant negative form of *nur77* inhibited cell death in the T-cell line 2B4. Collectively, these data suggest that *nur77* plays an important role in normal T-cell death.

apt-4 IS INDUCED DURING CELL DEATH IN T CELLS

Another gene, *apt-4,* also was isolated from the library of dying thymocytes. When DO11.10 cells are induced to die via TCR interactions, expression of this gene is induced. The pattern of *apt-4* induction is different from that observed for *nur77.* While *nur77* is expressed within 1–1.5 h following TCR crosslinking, *apt-4* mRNA is detected 4–5 h after the induction of death (Fig. 4). We have sequenced a portion of the 8-kb *apt-4* cDNA, and it does not resemble any sequence in the Genbank database.

p53 IS REQUIRED FOR RADIATION-INDUCED CELL DEATH IN THYMOCYTES

In addition to the genes we have isolated from the cDNA library made from dying thymocytes, we have shown, in collaboration with Tyler Jacks and Scott Lowe, that the p53 tumor suppressor gene is also required for some forms of thymocyte cell death [Lowe et al., 1993]. In these experiments, thymocytes from p53 null mice were employed to assess the role of p53 in the regulation of apoptosis. While thymocytes from these p53 null mice display normal death response to glucocorticoid and signals mimicking TCR stimulation, they are unable to die in response to ionizing radiation. By contrast, thymocytes from normal littermate controls are exquisitely sensitive to induction of apoptosis by ionizing radiation. Therefore, while the apoptotic program is normal in these mice, p53 is required to couple the signals generated by ionizing radiation to this pathway. These data complement, and are in complete agreement with, those of Clarke et al. [1993].

CONCLUSIONS

We have isolated several genes that are either induced (*nur77* and *apt-4*) or repressed (*apt-1* and *apt-3*) during cell death in thymocytes stimulated to undergo negative selection. One of these genes, *nur77* is induced only through TCR engagement and is a required component of this pathway [Liu et al., 1994; Woronicz et al., 1994]. Another gene, *apt-4,* also is induced by TCR engagement and preliminary data suggest that this gene, as well as *nur77,* is required for T-cell death [T. Kallinic, S. Smith, and B. Osborne, unpublished observations]. Furthermore, in separate studies, the p53 tumor suppressor gene was shown to be required for the induction of cell death in thymocytes by ionizing radiation. However thymocytes from mice lacking the p53 gene undergo apoptosis quite normally in response to signals that mimic TCR engagement as well as by dexamethasone. These data, taken together, suggest that at least several cell death pathways exist in murine T cells. As outlined in Figure 5, we propose that each induction signal, glucocorticoids, TCR engagement or exposure to radiation, results in the induction of unique signal transduction pathways and that each

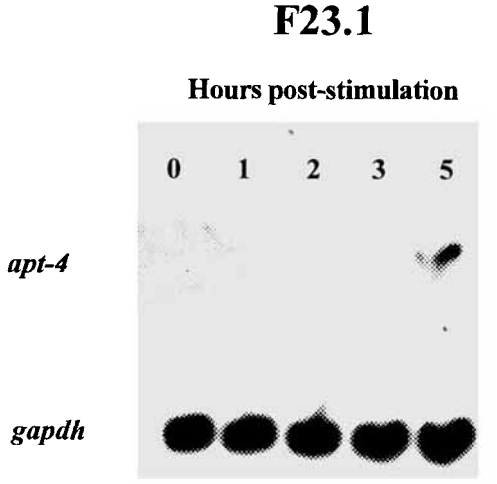

Fig. 4. Expression of *apt-4* in DO11.10 cells. DO11.10 cells were treated with PMA + A23187 and RNA prepared at the indicated times following treatment.

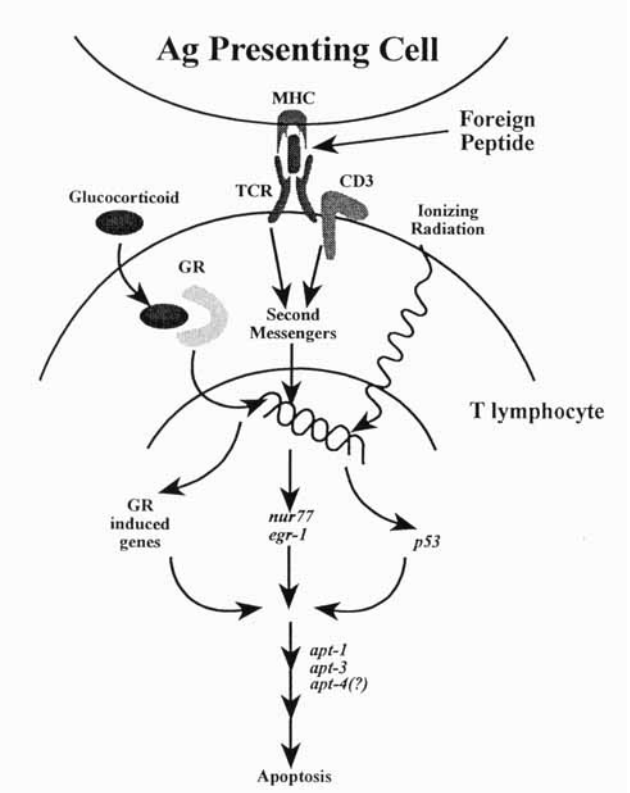

Fig. 5. Molecular pathways leading to the induction of apoptosis in T cells are mediated by distinct sets of genes.

pathway is likely to be mediated by a distinct set of gene products. We propose that these pathways may converge into a more common pathway that ultimately leads to the death of the cells. Further elucidation of the genes that lie along this common path may lead to a better understanding of the common molecular events that result in apoptosis in thymocytes. Additionally, it is expected that such knowledge may eventually lead to therapeutic strategies that result in the manipulation of cell death.

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