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Mechanisms of apoptosis in developing thymocytes as revealed by adenosine deaminase-deficient fetal thymic organ cultures

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Abstract

Adenosine deaminase (ADA) catalyzes the conversion of adenosine and deoxyadenosine to inosine and deoxyinosine, respectively. ADA-deficient individuals suffer from severe combined immunodeficiency and are unable to produce significant numbers of mature T or B lymphocytes. This occurs as a consequence of the accumulation of ADA substrates or their metabolites. dATP is a candidate toxic metabolite because its concentration in RBCs of ADA-deficient patients correlates with the severity of disease. Murine fetal thymic organ culture (FTOC) under ADA-deficient conditions can be used as a model system to investigate the biochemical mechanism responsible for the inhibition of thymopoiesis. In ADA-deficient FTOCs initiated at day 15 of gestation, thymocyte development was arrested at the $CD4^{-}CD8^{-}CD44^{lo}CD25^{+}$ to $CD4^{-}CD8^{-}CD44^{lo}CD25^{-}$ transition. Apoptosis appeared to be involved because the cultures could be rescued by the pan-caspase inhibitor zVADfmk, a *Bcl-2* transgene, or deletion of apoptotic protease activating factor-1. As in ADA-deficient patients, dATP was also elevated in ADA-deficient FTOCs. dATP levels were normalized and thymocyte development was rescued in cultures treated with an inhibitor of adenosine kinase, the enzyme that phosphorylates deoxyadenosine to dAMP. zVADfmk also prevented the accumulation of dATP in ADA-deficient FTOCs, suggesting that deoxyadenosine was derived from thymocytes undergoing apoptosis as a consequence of failing the β selection checkpoint. In contrast, dATP levels remained elevated in ADA-deficient FTOCs with fetal thymuses from *Bcl-2* transgenic mice. These data suggest that thymocyte apoptosis as a consequence of failing developmental checkpoints involves one or more caspases that are not regulated by Bcl-2.

Keywords: Thymocyte development; Adenosine deaminase deficiency; Apoptosis; Fetal thymic organ culture; Bcl-2; Cytochrome *c*

1. Introduction

ADA catalyzes the irreversible deamination of adenosine and deoxyadenosine to inosine and deoxyinosine, respectively (Fig. 1). Mutations in the structural gene for ADA that affect its enzymatic activity cause severe combined immunodeficiency (SCID) in humans [1]. Patients with ADA deficiency are profoundly lymphopenic, especially in the T cell compartment, and suffer from a wide variety of opportunistic infections that eventually become fatal unless enzyme replacement therapy or bone marrow transplantation are undertaken [2,3]. Early studies of ADA deficiency employed T cell lines or adult peripheral blood T cells treated with an ADA inhibitor plus adenosine or deoxyadenosine (reviewed in [2]). Murine FTOC has advantages as a model system to investigate the mechanism by which a lack of ADA leads to a failure of T cell development [4,5]. First, no exogenous substrates are needed to see the consequences of ADA deficiency, as these nucleosides are generated *in situ* during the normal course of thymopoiesis. Second, it is a system in which

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Abbreviations: ADA, adenosine deaminase; AK, adenosine kinase; Apaf-1, apoptotic protease activating factor-1; cNT, cytoplasmic 5′-nucleotidase; dCF, 2′-deoxycoformycin; FTOC, fetal thymic organ culture; MHC, major histocompatibility complex; TCR, T cell receptor; zVADfmk, carbobenzoxy-Val-Ala-Asp-fluoromethyl ketone.

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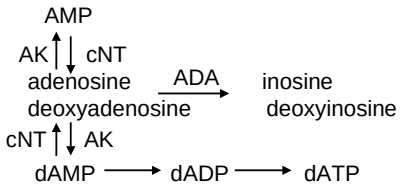

Fig. 1. Metabolism of adenosine and deoxyadenosine. ADA, adenosine deaminase; AK, adenosine kinase; cNT, cytoplasmic 5′-nucleotidase. In normal cells, ADA converts adenosine and deoxyadenosine to inosine and deoxyinosine, respectively. However, when ADA is missing, deoxyadenosine metabolism is altered so that dATP accumulates, especially in developing lymphoid tissue.

differentiation occurs, so the consequences of ADA deficiency upon thymocyte development can be determined. Using FTOC, new insight into the pathogenesis of SCID caused by ADA deficiency has been gained as well as a better understanding of the pathways by which normal thymocytes undergo apoptosis during development.

FTOC is a technique where murine fetal thymic lobes at day 15 of gestation are cultured on cellulose ester filters sitting on gelfoam sponges at the air/media interface for periods of 3–5 days [6]. At the initiation of the cultures, there are 1×10^5 to 2×10^5 cells/lobe and they are virtually all at the $CD4^-CD8^-$ stage of development (see Fig. 2 for the stages of thymocyte differentiation). During the next 5 days, the cells both proliferate and differentiate resulting in a 10–20-fold increase in cell number and a cell surface phenotype that is indistinguishable from that of an adult mouse thymus (i.e. 75–80% of the cells are now at the $CD4^+CD8^+$ double positive stage of development and there are also significant numbers of mature CD4 and CD8 single positive cells). TCR gene rearrangements and selection events take place in a manner similar to that which occurs *in vivo*. There are three important developmental checkpoints during thymocyte development: β selection, positive selection, and negative selection. During β selection, which occurs at the $CD4^-CD8^-CD44^{lo}CD25^+ \rightarrow CD4^-CD8^-CD44^{lo}CD25^-$ transition [7], only those cells that have undergone an in-frame rearrangement at the TCR β locus can continue to develop into αβ T cells [8]. At the positive selection checkpoint, only those cells that have expressed a TCR that can recognize peptide in the context of self-MHC are allowed to survive [9]. Finally, at the negative selection checkpoint, those cells that recognize self-peptides with high affinity are deleted [10]. The positive and negative selection checkpoints occur during the transition from the double positive to single positive stage [11].

2. Thymocyte development is inhibited in ADA-deficient FTOCs

FTOCs performed with thymuses from ADA-deficient fetuses [12] at day 15 of gestation, or from normal fetuses treated with the potent and specific ADA inhibitor, dCF [13], showed a profound inhibition of development past the $CD4^-CD8^-CD44^{lo}CD25^+$ stage [4]. Importantly, exogenous substrates were not required to obtain this effect, so endogenous metabolites could be measured with the goal of assessing the relative contributions of adenosine and deoxyadenosine to the pathogenesis of the disease. The block in development was accompanied by the accumulation of dATP, derived from the ADA substrate deoxyadenosine (Fig. 1). dATP is a prime candidate for the so-called "toxic metabolite" in these cultures as they could be rescued by an inhibitor of adenosine kinase, the enzyme that phosphorylates deoxyadenosine to dAMP [14]. Furthermore, the concentration of dATP in red blood cells of ADA-deficient patients correlates with the severity of their immunodeficiency [2]. The inhibition of differentiation in ADA-deficient FTOCs was not due to a failure of β selection, as TCR β rearrangements and transcription of the T early α locus [15] occurred normally. Rather, the cells appeared to be dying by apoptosis, as the cultures could be rescued by inhibiting apoptotic pathways [4]. FTOCs in which the addition of an ADA inhibitor was delayed for 2 days, so that many cells had already reached the $CD4^+CD8^+$ stage of development, were also dramatically inhibited with respect to both cell yield and differentiation. These cultures could also be rescued by inhibitors of apoptosis (manuscript in preparation).

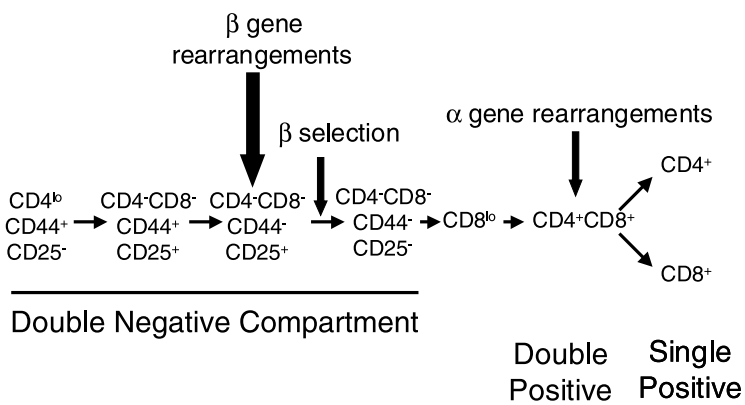

Fig. 2. Murine thymocyte development. The stages in murine thymocyte development are shown.

3. Rescue of ADA-deficient FTOCs by inhibitors of apoptosis

Treatment of ADA-deficient FTOCs with the pan-caspase inhibitor zVADfmk improved both the cell yield and extent of differentiation [4]. This was accompanied by normalization of dATP levels. These results were obtained regardless of whether dCF was added at the time the cultures were initiated or after 2 days to allow the development of $CD4^+CD8^+$ thymocytes. These observations suggested that the action of zVADfmk was not to nullify the toxic effects of dATP, but rather to prevent its accumulation in the first place. Figure 3 shows a schematic diagram of the events occurring in ADA-deficient FTOCs. Apoptosis is a normal event during thymocyte development as the vast majority of developing thymocytes fail to make a T cell receptor that passes the selection criteria described above and die by this route. They are engulfed by macrophages [16] and their intracellular purines are degraded, generating the ADA substrates, adenosine and deoxyadenosine. These are freely diffusible across the plasma membrane and can be taken up by other cells. When ADA is absent, deoxyadenosine is converted to dATP. The large numbers of thymocytes that die by apoptosis [17], coupled with the high capacity of the thymus to phosphorylate deoxyadenosine [18] and low capacity to dephosphorylate dAMP, explain the ability of this organ to accumulate dATP and its high sensitivity to ADA deficiency. Since the rescue of ADA-deficient FTOCs by zVADfmk was accompanied by normalization of dATP levels, we conclude that it acted to prevent (or delay) the death of cells failing developmental checkpoints.

ADA-deficient FTOCs were also rescued by a *Bcl-2* transgene expressed under the control of the proximal *lck* promoter [19]. As with zVADfmk, both the number of cells recovered and their differentiation state improved considerably. However, unlike the zVADfmk-corrected cultures, dATP remained elevated in ADA-deficient FTOCs with *Bcl-2* transgenic fetal thymuses. These results were obtained with dCF added at culture initiation or after 2 days. These observations suggest that the action of Bcl-2 was different than that of zVADfmk. We conclude that rather than preventing the death of thymocytes failing developmental checkpoints, Bcl-2 acted to prevent apoptosis caused by the accumulation of dATP (Fig. 3).

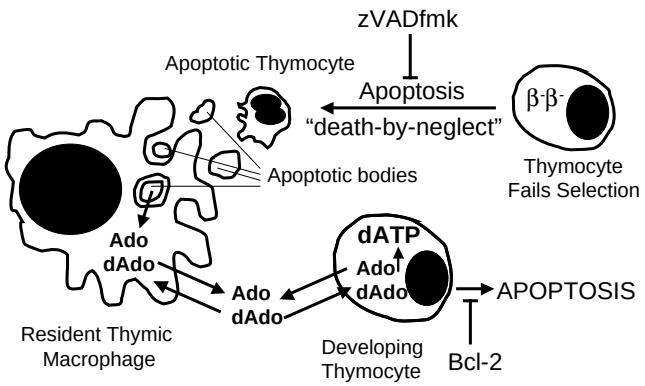

Fig. 3. Adenosine deaminase substrates are generated by thymocytes undergoing apoptosis in the developing thymus. The vast majority of thymocytes fail development checkpoints and die by apoptosis. These are engulfed by macrophages where their intracellular contents are degraded to generate the ADA substrates adenosine and deoxyadenosine. These nucleosides are freely diffusible and can be taken up by other developing thymocytes. If ADA is absent, deoxyadenosine gets converted to dATP and this leads to further apoptosis and a marked decrease in thymic output. The sites of action of zVADfmk and Bcl-2 are indicated.

4. Mechanisms of apoptosis induction

Yang and Cortopassi [20] showed that dATP can cause the release of cytochrome *c* from isolated mitochondria. Thus, we hypothesize that dATP in ADA-deficient FTOCs triggers apoptosis by inducing the release of mitochondrial cytochrome *c* (Fig. 4, Pathway A). Cytochrome *c* can then combine with Apaf-1, procaspase 9, and dATP to form the apoptosome [21]. This will result in cleavage of procaspase 9 into active caspase 9, initiating the apoptotic cascade. Analyses of dATP content of ADA-inhibited FTOCs revealed that dATP accumulated to 4–5% of the level of ATP. ATP concentrations are believed to be in the low millimolar range, so we estimate that dATP concentrations should be about 40–100 μM. This is well within the range that would be expected to cause cytochrome *c* release based on Yang and Cortopassi's *in vitro* experiments. Bcl-2 can prevent the release of cytochrome *c* from mitochondria [22], so it is not surprising that FTOCs performed with fetal thymuses from *Bcl-2* transgenic mice were resistant to the consequences of ADA-inhibition. The observation that deletion of Apaf-1 also rescued our cultures is consistent with dATP-induced mitochondrial cytochrome *c* release as the mechanism causing apoptosis in ADA-deficient FTOCs.

Although our results are consistent with the above mechanism and it is very appealing because dATP has long been hypothesized to be the ''toxic metabolite'' in ADA deficiency, other mechanisms are still possible. It is not surprising that dATP was elevated in ADA-deficient FTOCs for the reasons stated above. However, it is possible that dATP acted indirectly, or merely correlated with some other metabolic change that caused developing thymocytes to undergo apoptosis. We considered the possibility that p53 might be involved (Fig. 4, Pathway B), as Benveniste and Cohen showed that thymocytes from $p53^{-/-}$ mice were resistant to apoptosis induced by a combination of dCF and deoxyadenosine [23]. p53 can cause apoptosis by inducing transcription of the pro-apoptotic Bcl-2 family members Bax, PUMA, and Noxa [24–27]. However, FTOCs with p53 knock-out mice were susceptible to the consequences of ADA inhibition (unpublished observation). Of course, it is still possible that pro-apoptotic Bcl-2

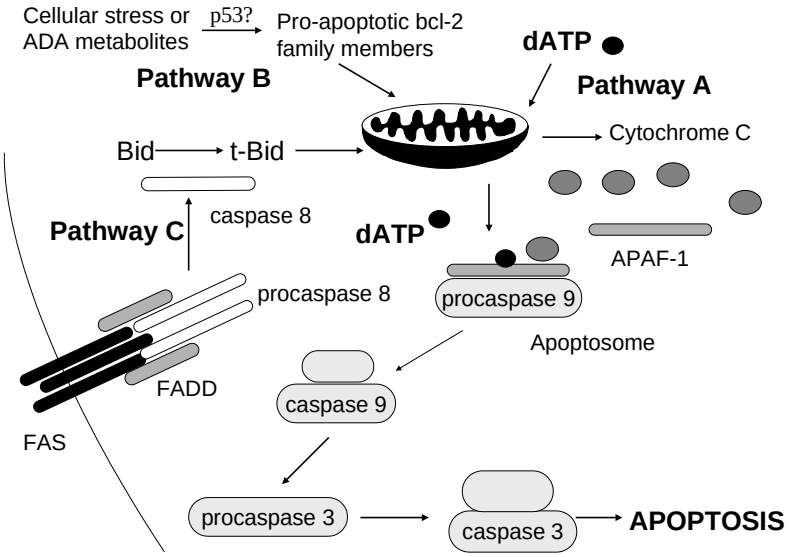

Fig. 4. Postulated mechanisms by which a lack of ADA can cause apoptosis in the thymus. Pathway A: accumulated dATP causes release of cytochrome *c* from mitochondria, leading to formation of the apoptosome and initiation of the apoptotic cascade. Pathway B: abnormal levels of purine metabolites cause the induction of pro-apoptotic Bcl-2 family members, leading to release of cytochrome *c* from mitochondria. Pathway C: abnormal levels of purine metabolites cause induction of death receptor signaling, leading to caspase 8 activation and cleavage of Bid. Truncated Bid then binds to pro-apoptotic Bcl-2 family members leading to release of cytochrome *c* from mitochondria.

family members are involved, but through a p53-independent mechanism. We similarly eliminated death receptor signaling via Fas (Fig. 4, Pathway C), as FTOCs with *lpr* mice remained sensitive to the consequences of ADA deficiency (unpublished observation). Nevertheless, some other death receptor pathway might be involved, as a caspase 8 inhibitor partially rescued ADA-deficient FTOCs. Distinguishing between these alternatives is the goal of ongoing experiments.

5. Conclusions

In summary, both early and late stages of thymocyte development are inhibited by a lack of ADA. Our data suggest that thymopoiesis is inhibited in ADA-deficient murine FTOCs because large numbers of developing thymocytes undergo apoptosis caused by dATP-induced mitochondrial cytochrome *c* release. Furthermore, dATP, produced by phosphorylation of the ADA substrate deoxyadenosine, appears to be derived from thymocytes failing developmental checkpoints. Although the pro-apoptotic Bcl-2 family member Bim has recently been shown to be necessary for negative selection [28], little is known about the mechanism of ''death by neglect'' that occurs when thymocytes fail the β selection and positive selection checkpoints. Our results suggest that one or more caspases are involved and that death by this route cannot be prevented by over expression of Bcl-2. ADA-deficient murine FTOC provides a novel experimental system for further exploration of the regulation of this important apoptotic pathway.

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