Prevention of Thymic Lymphoma Development in Atm−/− Mice by Dexamethasone

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Abstract

We have reported (M. Yan et al., FASEB J., 15: 1132–1138, 2001) that spontaneous DNA synthesis is markedly increased in the thymocytes from the atrophied thymi of young Atm−/− mice. We, therefore, set out to determine whether this elevated DNA synthesis is responsible for the development of thymic lymphomas in Atm−/− mice. We show here that in Atm−/− mice: (a) DNA synthesis occurs, especially in the immature CD4− CD8− (dominant negative) and CD8+ thymocyte populations; (b) the relative percentage of dominant negative cells increases significantly during postnatal development, with a sharp peak at 4 weeks of age; and (c) dexamethasone suppresses DNA synthesis in these thymocytes and prevents thymic lymphoma development. These observations suggest that ataxia telangiectasia mutated (ATM) down-regulates the proliferation of thymocytes, allowing T-cell development and differentiation. The results also show that dexamethasone, like ATM, checks DNA synthesis in developing thymocytes. Finally, the data document for the first time that dexamethasone prevents or slows thymic lymphoma development in Atm−/− mice.

Introduction

ATM, a pleiotropic protein kinase, is activated by double-strand DNA breaks that occur during meiosis, mitosis, and recombination, or as a result of ionizing radiation (1, 2). On activation, ATM rapidly activates many factors that check cell cycling at multiple checkpoints (3–5). Mutations in the ATM gene result in multisystem disorders, two of which are immunodeficiency and thymic lymphoma (6, 7).

Atm-null mice (Atm−/−) recapitulate most of the features of human ataxia telangiectasia phenotypes, including a high frequency of thymic lymphomas and thymic atrophy (8, 9). In Atm−/− mice, V(D)J recombination is also abnormal (9, 10), suggesting that thymic lymphoma development in Atm−/− mice may be triggered by events that follow V(D)J recombination. Notably, the Rag-1 gene product must be present for V(D)J recombination to occur, and thymic lymphomas do not develop in Rag-1−/− Atm−/− dual-null mice (11).

We have shown previously that the rate of spontaneous DNA synthesis in freshly isolated thymocytes is markedly accelerated in Atm−/− mice, as compared with thymocytes from Atm+/+ littermates (12). This increase in DNA synthesis is not inhibited by FK506, which suggests that it is calcineurin pathway-independent. However, it can be inhibited by the glucocorticoid Dx and by thiols. These results imply that individual Atm−/− thymocytes have two possible fates: (a) death, by cells that fail to pass developmental checkpoints requiring cell cycle arrest (most thymocytes), or (b) survival, by cells that continue to proliferate and to undergo abnormal differentiation after passing developmental checkpoint that normally require cell cycle arrest (a few thymocytes). We show here that four populations of thymocytes in Atm−/− mice, i.e., CD4− CD8− DN, CD4+ CD8− DP, CD4+ and CD8+ SP cells, all exhibit increased DNA synthesis. The distribution of these four populations of thymocytes is also abnormal, in that the percentage of DN cells in Atm−/− thymi increases sharply at 4 weeks of age, and the percentage of CD4+ cells is consistently low. Increased DNA synthesis by all four populations is suppressed to a variable extent by in vitro and in vivo administration of Dx, but this is particularly pronounced in the DN population. These findings suggest that the failure of checkpoint controls in immature Atm−/− thymocytes during development is responsible for the subsequent development of thymic lymphomas in Atm−/− animals. To assess this possibility, we treated Atm−/− mice with Dx and found that Dx completely prevents thymic lymphoma development in these animals.

Materials and Methods

Mice. The Atm−/− mice used here were created by Barlow et al. (8). Heterozygous Atm+/− mice of this line were purchased from the Jackson Laboratory (Bar Harbor, ME), mated, and kept in the Animal Center at M. D. Anderson Cancer Center’s Science-Park Research Division. In some experiments, mice were given i.p. injections with different doses of Dx (Sigma, St. Louis, MO) or with NS as controls. Animal care was in accordance with institutional guidelines.

Flow Cytometry Analysis and Cell Sorting. Thymocytes were isolated as described previously (13). Single-cell suspensions were prepared in HBSS buffer and stained with PE anti-CD4 and FITC anti-CD8 antibodies (PharMingen, San Diego, CA). Analyses were performed with a Coulter EPICS Elite flow cytometer (Beckman Coulter). In some experiments, DN, DP, CD4+, and CD8+ populations of thymocytes were sorted to a purity of >99% and used for measurement of [3H]thymidine incorporation into DNA.

[3H]Thymidine Incorporation into DNA. Single-cell suspensions or sorted populations of thymocytes were incubated in 96-well plastic tissue culture dishes at 37°C in the presence of 0.5 or 1 μCi of [3H]thymidine (Amersham Pharmacia Biotech, Piscataway, NJ) per well. Four to 6 h later, the cells were harvested and [3H]thymidine incorporation into DNA was measured as previously (12) in a scintillation counter (Packard, Meriden, CT). Results were expressed as mean counts per min (cpm) ± SD in triplicate cultures.

Cell Cycle Analysis. Dual-color surface antigen staining of thymocytes was combined with DNA staining with 7-AAD as described previously, with some modifications (14). Briefly, 1 × 10^7 thymocytes were incubated with CD4-PE and CD8-FITC antibodies for 30 min at 4°C. The cells were washed with PBS and the pellet resuspended in 0.875 ml of cold PBS. Cold 2% paraformaldehyde solution (0.125 ml) was then added to fix the cells, and the samples were incubated at 4°C for 1 h prior to resuspension in 1 ml of 0.2% Tween 20 in PBS for permeabilization. DNA of fixed and permeabilized double-labeled thymocytes was stained by incubating the cells in 1 ml of
Atm translation. Thymocytes isolated from four populations (purity, Atm–/–) DNA synthesis during development. To identify the thymocyte populations. Thymocytes were cultured with or without Dx (10⁻⁹ M at 37°C for 2 h). Cell cycle analysis was done by flow cytometry within 2 h. 7-AAD stained with 7-AAD. Cell cycle analysis was performed by FACS. CD4-PE and anti-CD8-FITC antibodies. The DNA of the sorted cells was then stained with 7-AAD solution (25 µg/ml in PBS with 0.1% of sodium azide) for at least 30 min at 37°C. Cell cycle analysis was done by flow cytometry within 2 h.

DNA Fragmentation Assay. Freshly isolated Atm+/+ and Atm–/– thymocytes were cultured with or without Dx (10⁻⁹ M) for 8 h. DNA was prepared and 10 µg were electrophoresed through a 1% agarose gel.

Results

DNA Synthesis Is Unchecked at All Stages of Thymic Development in Atm–/– Mice. Atm–/– thymi are small and contain fewer thymocytes than do thymi of age-matched Atm+/+ animals. However, thymocytes freshly isolated from the atrophic thymi of Atm–/– mice synthesize DNA at a faster rate than do cells of normal thymi, as indicated by their rate of incorporation of [³H]thymidine into DNA (12). This suggests that thymocytes lacking ATM are unable to check DNA synthesis during development. To identify the thymocyte populations responsible for the increased DNA synthesis, freshly isolated Atm–/– thymocytes were sorted by FACS into DN, DP, CD4+, and CD8+ cells, and DNA synthesis in all four populations was measured. For each subset, the rate of thymidine uptake was increased, but this was particularly evident for DN, CD8+, and DP cells (Fig. 1A).

The reasons for increased DNA synthesis in CD8+ thymocytes are unclear. During T-cell development, CD8 is transiently expressed (for <24 h) on the surfaces of DN/DP intermediate cells. These cells are called ISP cells (15, 16). CD8 is subsequently expressed as a permanent surface marker on mature CD8+ thymocytes. Because DNA synthesis in Atm–/– CD8+ thymocytes is significantly elevated relative to Atm+/+ CD8+ thymocytes (Fig. 1A), it seems possible that this population represents cycling ISP cells. To confirm this, cell cycle analysis was performed for the gated CD4+ and CD8+ cells. Fig. 1B shows that the percentages of cells in S phase in the Atm–/– CD8+ thymocyte population is significantly higher than that in Atm+/+ CD8+ thymocytes, whereas the same is not true for Atm–/– CD4+ cells.

Dx Inhibits DNA Synthesis and Induces Apoptosis in Immature Thymocytes. To identify thymocyte populations sensitive to Dx, sorted cells from Atm–/– mice were exposed to 10⁻⁸ M Dx in culture. Results presented in Fig. 2A show that Dx suppresses spontaneous DNA synthesis in all four populations of Atm–/– thymocytes, but that this effect is especially pronounced in the DN and CD8+ ISP populations. This observation implies that immature thymocytes are more sensitive to Dx than mature thymocytes are.

It has been reported that Atm–/– thymocytes undergo increased spontaneous apoptosis in culture relative to normal thymocytes from age-matched Atm+/+ controls (17). Similarly, our results show that both spontaneous and Dx-induced apoptosis are increased in Atm–/– thymocytes (Fig. 2B). The numbers of thymocytes in Dx-treated Atm–/– mice are also reduced relative to untreated Atm–/– mice (Table 1). These results suggest that Dx not only suppresses DNA synthesis in thymocytes but also causes death in these cells.

Distribution of Thymocyte Populations during Development in Atm–/– Mice. To compare the distributions of thymocyte progenitor subsets over time during postnatal development in Atm–/– and Atm+/+ mice, the percentages of the four thymocyte subsets were determined for mice of the two lines at 1, 2, 4, and 8 weeks of age. As shown in Fig. 3A and plotted as a function of time in Fig. 3B, the percentages of DN thymocytes in Atm–/– mice are consistently higher than those of Atm+/+ controls at all time points, but with a

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**Table 1.** The effects of Dx on body weight, thymus weight, and thymocyte number in Atm+/+ and Atm–/– mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Body weight (g)</th>
<th>Thymus weight (mg)</th>
<th>Thymocyte no. (×10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atm+/+ Co</td>
<td>7</td>
<td>13.5 ± 1.1</td>
<td>50.2 ± 7.6</td>
<td>51.7 ± 22.1</td>
</tr>
<tr>
<td>Atm+/+ Dx</td>
<td>7</td>
<td>13.6 ± 1.3</td>
<td>33.3 ± 9.1b</td>
<td>29.9 ± 11.1b</td>
</tr>
<tr>
<td>Atm–/– Co</td>
<td>6</td>
<td>10.4 ± 0.7</td>
<td>19.8 ± 8.4</td>
<td>3.4 ± 1.6</td>
</tr>
<tr>
<td>Atm–/– Dx</td>
<td>6</td>
<td>9.9 ± 1.6</td>
<td>13.5 ± 6.0</td>
<td>2.7 ± 1.0</td>
</tr>
</tbody>
</table>

a Co, control group with NS.
b P < 0.01 versus control groups.

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sharp peak at the age of 4 weeks, followed by a return to “normal” at 8 weeks. By contrast, percentages of CD4⁺ cells in Atm⁻/⁻ mice are consistently lower than in Atm⁺/+ controls. Interestingly, the percentage of CD8⁺ thymocytes also shows a sharp peak in both Atm⁺/+ and Atm⁻/⁻ mice at 4 weeks of age. Together, these observations suggest that the development of thymocytes in Atm⁻/⁻ mice is slowed but otherwise proceeds normally, and that events occurring at 4 weeks (with the accumulation of DN and CD8⁺ ISP cells at this time point) may be critical for the subsequent development of T-cell lymphomas in Atm⁻/⁻ mice. We have used this information to design experiments to test the effects of Dx on thymic lymphoma development (see below).

**Dx Down-Regulates DNA Synthesis in Atm⁻/⁻ Thymocytes in Vivo.** To determine whether Dx inhibits thymocyte proliferation in Atm⁻/⁻ animals, we treated 2-week-old Atm⁻/⁻ mice with Dx i.p. (with 10, 5, 5, 5, 5, 2.5, and 1.25 mg/kg/day) for 2 weeks and then cultured the thymocytes immediately after isolation. As shown in Fig. 4, spontaneous DNA synthesis in thymocytes is inhibited both in Dx-treated Atm⁺/+ and in Dx-treated Atm⁻/⁻ mice. Thymus weights and thymocyte numbers are significantly reduced in Dx-treated Atm⁺/+ mice, but this effect is less pronounced in Dx-treated versus untreated Atm⁻/⁻ controls (Table 1). In both Atm⁺/+ and Atm⁻/⁻ mice of this age (4 weeks), no significant differences in body weight are evident between Dx-treated and control groups (Table 1). These results show that Dx suppresses thymocyte DNA synthesis *in vivo* and decreases thymus cellularity.

**Fig. 3.** Distribution of thymocyte populations during postnatal development in Atm⁻/⁻ mice. A, percentages of DN, DP, CD4⁺, and CD8⁺ thymocytes during postnatal development. Freshly isolated thymocytes were analyzed by FACS with anti-CD4-PE and anti-CD8-FITC antibodies. B, percentages of DN, DP, CD4⁺) and CD8⁺ thymocytes were plotted as a function of time.

**Fig. 4.** Effects of Dx on spontaneous *in vivo* DNA synthesis in thymocytes. Two-week-old Atm⁺/+ and Atm⁻/⁻ mice were treated with Dx or NS, respectively, for 2 weeks. For Dx-treated mice, 10, 5, 5, 5, 5, and 2.5 mg/kg of Dx was administered i.p. in the first 6-day course. Two days later, a second 6-day course began. For control mice, identical volumes of NS were given at the same times. Immediately after the treatment, the mice were killed and the thymocytes isolated and assayed for spontaneous DNA synthesis as described above. ***P < 0.001 for the Dx group versus the NS group by *t* test.
**Dx Administration Prevents Thymic Lymphoma Development.**

Our data showing that Dx suppresses spontaneous DNA synthesis both in vitro and in vivo suggest that glucocorticoids, like ATM, may check DNA synthesis in developing T cells, thereby preventing thymic lymphoma development in Atm−/− mice. To test this idea, eight 30-day-old Atm−/− mice were given 10 mg/kg Dx i.p. daily for 10 days, and then were treated every other day with the same dose for 15 repetitions, followed by 5 mg/kg for another 15 repetitions. The control group contained 4 mice that received the same volume of NS. The doses of Dx used were calculated according to clinical data from control group contained 4 mice that received the same volume of NS. 15 repetitions, followed by 5 mg/kg for another 15 repetitions. The control group contained 4 mice that received the same volume of NS. The doses of Dx used were calculated according to clinical data from Dx use in humans (18).

As shown in Fig. 5A, three of the four control mice died of thymic lymphomas, and one died of an unknown cause, at 2.2–4.8 months of age. By contrast, two of eight Dx-treated mice died of intestinal obstruction at 4.1 and 5 months of age, but without tumors; four died of thymic lymphoma at 6.4–7.6 months of age, and two were still alive and apparently healthy at 8 months of age (P = 0.0022, comparing Dx-treated mice with NS-treated control mice). When the surviving animals were killed and examined for thymic lymphomas or other tumors, none were found.

In the next set of experiments, Dx treatment was started at the age of 15 days in Atm−/− mice. The course of treatment was for 7 days, during which 10, 5, 5, 5, 2.5, and 1.25 mg/kg Dx was administered i.p. This 7-day treatment was repeated on alternate weeks for 2 months, and then was repeated at monthly intervals for an additional 2 months. In this experiment, five control mice died of thymic lymphomas within 4.2 months, whereas all of the 5 Dx-treated mice were alive and healthy at 10 months, without signs of tumor development (Fig. 5B). These observations indicate that Dx, at appropriate dosages and time of treatment, completely prevents thymic lymphoma development in Atm−/− mice.

**Discussion**

In Atm−/− mice, the thymus, spleen, and testes are atrophic at birth, and the animals develop a wasting syndrome as they age (8, 9). The data presented here suggest that ATM-deficiency leads to a severe depletion of thymocytes, accompanied by survival of a few cells that continue to differentiate slowly in the thymus despite marked dysregulation of their DNA synthesis. In time, some of these abnormal proliferating thymocytes give rise to fatal thymic lymphomas.

During normal intrathymic T-cell development, ~3% of DP cells are selected to become mature CD4− or CD8+ SP thymocytes after TCR-β and -α rearrangements. The other 97% of DP cells undergo programmed cell death in the thymus (19). The seeding rate of prothymocytes from bone marrow stem cells is about 5 × 10⁵ cells/day (20). In irradiated mice that have received injections of purified prothymocytes from congenic donors, the transferred cells expand ~50-fold in number in 8 days. This means that immature thymocytes expand their numbers significantly by timely proliferation (21). Many molecules, such as pre-TCR (22), LAT (linker for activation of T cells; Ref. 23), c-Myb (24), SLP-76 (SH2 domain-containing leukocyte protein), and its adaptor GADS (25, 26), are known to be required for immature thymocyte proliferation. Absence of any of the above regulatory genes (in knockout models) results in T-cell development blocked at the DN stage.

Proliferating immature thymocytes normally stop cycling before developing to the next stage. Our data, showing elevated spontaneous DNA synthesis in Atm−/− thymocytes, suggest that ATM is required to check DNA synthesis at defined stages of postnatal thymocyte development. In the absence of ATM kinase, developing immature thymocytes either undergo apoptosis, leading to thymic atrophy, or continue making excess DNA, with thymic lymphomas as the end result. We show here that DN, DP, and immature CD8+ cells in Atm−/− mice exhibit unchecked DNA synthesis, and that this DNA synthesis can be curtailed in vitro and in vivo by Dx. The Dx-sensitive steps in thymocyte development appear to be critical for the development of thymic lymphomas in these animals.

Glucocorticoids are regulators of T-cell growth and differentiation. Dx has been used successfully as a therapeutic agent in the treatment of leukemias and lymphomas (18). We show here for the first time that Dx can completely prevent lymphoma formation in mice lacking the tumor suppressor gene Atm. Our data also show that, for optimal effect, Dx must be available during early thymic postnatal development, rather than later. Only four of eight Atm−/− mice treated at 4 weeks of age with Dx survived without thymic lymphomas up to 6 months of age, whereas all five Atm−/− mice treated at 2 weeks of age lived for 10 months without developing thymic lymphomas.

The mechanisms by which Dx checks DNA synthesis, and prevents thymic lymphoma development in Atm−/− mice, are not clear. Glucocorticoids mediate their biological effects by regulating transcription of many regulatory genes (27). It is well known that glucocorticoids induce programmed cell death in immature thymocytes (28). In this study, we show that Dx increases apoptosis of thymocytes in Atm−/− and Atm−/− mice alike, suggesting that apoptosis is one of the mechanisms by which Dx prevents thymic lymphoma development in Atm−/− animals. However, it seems unlikely that Dx suppression of DNA synthesis in thymocytes is caused only by its ability to induce programmed cell death, because DNA synthesis in

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**Fig. 5.** Dx prevents thymic lymphoma development. A, Dx treatment was begun in 4-week-old mice by the protocols as described in “Materials and Methods.”  *, mouse that died without thymic lymphoma, **, two mice without thymic lymphomas remaining alive at 8 months of age, P = 0.0022 for the Dx-treated group versus the NS group, by the Kaplan-Meier survival/log-rank significance test (SPSS Version 10, SPSS, Inc., Chicago, IL). B, Dx treatment begun in 2-week-old mice by the protocols as described in “Materials and Methods.” ***, five mice without thymic lymphomas remaining alive at 10 months of age.
Deficiency in lymphoma. Dx, like functional ATM kinase, checks thymocyte proliferation, thereby preventing thymic lymphomas. Unlike ATM, Dx cannot prevent immuno-deficiency in Atm−/− mice, because it also induces thymocyte apoptosis.

Freshly isolated viable thymocytes from Dx-treated mice (either Atm−/− or Atm+/+) is also decreased significantly as compared with that in thymocytes from untreated controls. It has been reported that glucocorticoid treatment results in rapid depletion of glutathione in T cells, thereby down-regulating their thiol redox potential and up-regulating apoptotic pathways (29). We suggest that the proapoptotic/anti-proliferative effects of Dx in thymocytes of Atm−/− mice may also be mediated by changes in internal thiol redox status induced by depletion of reduced glutathione (GSH).

In summary, in Atm-deficient mice, most thymocytes fail to survive or complete intrathymic development. In the few cells that do survive, postnatal development is slow, as indicated by the accumulation of immature DN cells. These surviving cells continue to proliferate, and some of these cells ultimately give rise to thymic lymphomas. We suggest that Dx could suppress the elevated spontaneous DNA synthesis, like ATM, and/or it could activate apoptotic pathways that eliminate transformed cells, thereby preventing thymic lymphoma development in Atm−/− mice (Fig. 6).

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