**Deregulated Apoptosis in Ataxia Telangiectasia: Association with Clinical Stigmata and Radiosensitivity**

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**ABSTRACT**

Ataxia telangiectasia (AT) is a recessive genetic disease featuring neurodegeneration, immunodeficiency, chromosomal instability, radiation hypersensitivity, and increased predisposition to cancer. Reduced or delayed induction of the tumor suppressor protein p53 after γ-irradiation was reported. These characteristics may be compatible with an inability to correctly regulate apoptosis. We show here that AT lymphocytes and EBV-transformed lymphoblasts demonstrate a significantly higher level of spontaneous apoptosis, whereas ionizing radiation-induced apoptosis is reduced compared to normal cells. However, neither AT nor normal primary fibroblasts undergo apoptosis after irradiation. Consequently, we conclude that the radiosensitivity of the AT cells is not related to an increased apoptotic response. Finally, we show that SV40-transformed AT fibroblasts undergo γ-irradiated apoptosis, whereas SV40-transformed normal cells do not. This result raises the question of the physiological relevance of the latter cellular model with respect to the AT phenotype.

**INTRODUCTION**

Higher eukaryotes have developed defense mechanisms to protect tissues against genotoxic insults. Growth arrest and cell death constitute predominant cellular responses to treatment with DDA.1 It was speculated that rapid inhibition of cell cycle progression permits more time to repair the induced lesions, and may protect cells against the induction of hereditary genomic alterations. Later, damaged cells die either by apoptosis or necrosis, depending on the extension of the damage. Exposed cells escaping to growth arrest or cell death may harbor genetic changes and have a higher probability of malignant transformation.

It was demonstrated that an important coordinator of these defense mechanisms is the product of the transcription factor and tumor suppressor gene p53. It was clearly established that the p53 protein accumulates in cells exposed to DDA (1, 2), leading to cell cycle arrest at the G1 checkpoint (3) and/or apoptosis (4–6). The cellular genetic background may modulate these p53-mediated effects. For example, primary fibroblasts arrest in G1, but do not undergo apoptosis (7), while in mitogenic-stimulated lymphocytes and EBV-transformed lymphoblasts, both G1 arrest and apoptosis are induced (8). Although the molecular pathway leading to p53 activation following treatments with DDA is unknown, recent work suggested that mutations in the AT gene may play an important role in this activation (9).

AT is an autosomal recessive disease featuring progressive neurodegeneration, immunodeficiency, chromosomal instability, radiation hypersensitivity, and increased predisposition to cancer (10). Cells from AT patients have increased chromosomal and cellular sensitivity to ionizing radiation and radiomimetic drugs (10). Moreover, the inhibition of semiconservative DNA synthesis and other cell cycle modifications such as the G1 and G2 arrest induced by radiation in normal cells are altered in AT cells (11, 12). In these cells, the induction of p53 protein after γ-irradiation is delayed or reduced compared to normal cells (9, 13–15). Since the clinical hallmarks of this syndrome and the alteration of p53 induction may reflect incorrect regulation of the programmed cell death, we previously investigated the apoptotic response in AT and have shown that lymphoblastoid cells from AT patients displayed altered regulation of apoptosis (16). AT lymphoblasts showed augmented spontaneous apoptosis, but reduced frequency of radiation-induced apoptosis as measured 1 day after γ-ray exposure. Since publication of our findings, others have reported that induction of apoptosis is clearly increased in AT fibroblasts transformed with SV40 relative to normal cells (17). To better define the role of apoptosis in the AT phenotype, we compared spontaneous and ionizing radiation-induced apoptosis in primary AT cells (fibroblasts and lymphocytes) with that in their virus transformed counterparts.

**MATERIALS AND METHODS**

**Cell Lines and Apoptosis-inducing Treatments.** Lymphoblast cell lines AH1-H (normal; provided by Dr. W. Thilly, Massachusetts Institute of Technology, Boston, MA), GM3657 (normal), GM3187 and GM3188 (AT heterozygous), GM3189 (AT homozygous) (Human Genetic Mutant Cell Repository, National Institute of General Medical Sciences, Camden, NJ), AT2043 and AT2044 (AT heterozygous), and AT383 (AT homozygous; provided by Dr. M. Swift, New York Medical College, Hawthorne, NY) were grown in suspension in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 12% FCS (Boehringer Mannheim, Mannheim, Germany). Cells were routinely maintained in exponential growth by a daily dilution to 3 × 10^5 cells/ml. Primary fibroblasts GM3658 (normal), AT4BE and AT5B1 (AT homozygous), and the SV40-transformed cell line AT5B1, called AT5BIVA (NIGMS Human Genetic Mutant Cell Repository), were maintained in MEM (GIBCO) supplemented with 10% FCS.

Mononuclear cells were separated from heparinized peripheral blood samples using lymphocyte separation medium, resuspended in RPMI 1640 (GIBCO) plus 12% FCS, and left in a flask for 1 h at 37°C to separate attached macrophages from lymphocytes in the suspension. The latter were resuspended at 5 × 10^5 cells/ml, irradiated, and maintained at 37°C in a CO2 incubator for 24 h before the analysis of apoptosis. Cells were cultured without mitotic stimulation.

For irradiation experiments, cells were exposed to γ-ray from a 60Co source at a dose rate of 15 Gy/min. After exposure, cells were cultivated up to the selected time, after which they were processed as indicated below.

**Detection of Apoptosis.** Flow cytometric analysis of apoptosis in lymphoblasts and lymphocytes was performed as previously described (16, 18), with minor modifications. In brief, cells were resuspended at 10^5/ml in PBS buffer (10 mM sodium phosphate, 136 mM NaCl, and 7 mM KCl) containing 0.1% Triton X-100 (Sigma Chemical, St. Louis, MO) and 100 units/ml RNase A (Sigma) and incubated for 15 min at 37°C for the lymphocytes and 5 min at room temperature for the lymphoblasts. Cells were subsequently stained in propidium iodide at 50 mg/ml and analyzed using a FACScan (Becton Dickinson).

For each sample, 10,000 events were recorded. Photomultiplier sensitivity of the FL2-H parameter was set as to place the peak corresponding to the diploid DNA content (G0/G1 cells) at channel 300 of a linear amplification scale for lymphocytes or at the third log of a logarithmic scale for lymphoblasts.

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4 The abbreviations used are: DDA, DNA-damaging agents; AT, ataxia telangiectasia; SSC-H, side scatter-high; FSC-H, forward side scatter-high.
blasts. In both cases, cells with a hypodiploid DNA content and a granularity (SSC-H) higher than that of G0-G1 cells were regarded as apoptotic (19). The logarithmic amplification scale was used in the case of lymphoblasts to obtain a more complete analysis of the cell population. Cellular debris, apoptotic bodies, as well as chromosomes from broken cells are localized in a region of the dot plot at lower values of FSC-H/SSC-H, with FSC logarithmic and SSC linear. They are easily gated out before analysis. Cell cycle distribution was analyzed with the CellFit software (Becton Dickinson) using the FL2-A parameter.

Apoptosis morphology was determined after staining cells with bis-benzimidide H33342 (Calbiochem, La Jolla, CA) at 0.1 μg/ml in PBS for 15 min at 37°C. Fluorescence observation and image acquisition were performed with an intensified fluorescence digital imaging microscope system. At least 1000 cells were scored for each time point by two independent observers in a blinded fashion.

Apoptosis was also scored using the In Situ Cell Death Detection kit (Boehringer Mannheim) following the procedure indicated by the manufacturer. Results obtained with the three different methods were similar. Only the morphological criteria were utilized to analyze fibroblasts. Adherent cells were trypsinized and mixed to the floating dead cells. All of the population was pelleted by centrifugation and further processed for analysis.

Viability Measurement. At specific time periods following irradiation, viable cell numbers were determined by trypan blue dye exclusion and counting in a hemocytometer. Cells were mixed (1:1) with a 0.4% trypan blue solution (Sigma). Results of all experiments represent the mean of at least three independent observations.

RESULTS

Spontaneous and Induced Apoptosis in EBV-transformed Lymphoblasts. As reported in Table 1, the spontaneous frequency of apoptotic events was significantly higher \( (P < 0.01) \) in EBV-transformed AT lymphoblasts than in normal ones. The progressive accumulation of apoptotic cells in AT heterozygous individuals from family 1 was intermediate between that of normal and homozygous AT patients from this family. In contrast, in family 2, the apoptotic response of cells from the parents is normal. Interestingly, the cell line derived from the AT patient in family 1 showed a higher frequency of spontaneous apoptosis than that from the patient in family 2. This suggests that AT mutation(s) may modulate the genetic control of apoptosis.

Ionizing radiation induces apoptosis, and AT cells are hypersensitive to γ-ray exposure. Moreover, cells from AT heterozygotes are generally slightly more radiosensitive than those from normal individuals, as evaluated using a clonogenic assay (20). It is widely accepted that the degree and extent of apoptosis correlates directly with the cellular sensitivity to genotoxic stress (21). Therefore, it was of interest to compare the γ-ray-induced apoptosis in AT homozygous, AT heterozygous, and normal cells in relation to their respective survival. Table 1 shows the induction of apoptosis at different time points after exposure to 5 Gy γ-rays. The frequency of apoptotic cells was similar among the individuals and was independent of the genetic background. It should be noticed however that the ratio of ionizing radiation induced to spontaneous apoptosis rapidly increased in normal cells from about 4 after 24 h to more than 6 after 72 h of treatment. In contrast, in AT cells, the increase at 24 h was less than twice the spontaneous level, and reached only about three times the spontaneous level at 72 h. Moreover, the increase in apoptosis starts to be visible 4–6 h after irradiation in normal cells, whereas it may be observed only after 10–12 h in AT cells. This strongly suggests that AT cells are defective in the induction of apoptosis after γ-ray exposure. The apoptotic response in the heterozygote from family 1 was intermediate between that of normal and AT cells from this family (Table 1). The heterozygote cells from family 2 behaved like those from normal individuals.

The AT hypersensitivity to γ-irradiation was clearly established using the clonogenic cell survival test (22, 23). Using this test, the γ-ray dose leading to 37% survival was 1.75 Gy and 2.0 Gy for the normal GM3657 and AHH-1 cell lines, respectively, versus 0.75 Gy and 1 Gy for AT GM3189 and AT383 cell lines, respectively. However, the clonogenic cell survival assay evaluates the long-term (2–3 weeks) cytotoxic effect of a treatment. That is why, in our study, to monitor survival in parallel with apoptosis, we established the γ-ray-

### Table 1 Percentage of apoptotic cells in untreated and 5 Gy-irradiated samples from normal, AT heterozygous, and homozygous lymphoblasts

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Normal (AT+/−) cells</th>
<th>AT heterozygotes (AT+/−) cells</th>
<th>AT homozygotes (AT−/−) cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treated</td>
<td>Control</td>
</tr>
<tr>
<td>24</td>
<td>4.39</td>
<td>17.67 (×4.0)</td>
<td>4.63</td>
</tr>
<tr>
<td>48</td>
<td>6.35</td>
<td>39.47 (×6.2)</td>
<td>6.74</td>
</tr>
<tr>
<td>72</td>
<td>9.51</td>
<td>65.07 (×8.8)</td>
<td>9.88</td>
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<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Normal (AT+/−) cells</th>
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<td></td>
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<tr>
<td>24</td>
<td>7.17</td>
<td>16.31 (×2.3)</td>
<td>8.71</td>
</tr>
<tr>
<td>48</td>
<td>8.74</td>
<td>24.14 (×2.8)</td>
<td>13.14</td>
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<tr>
<td>72</td>
<td>13.76</td>
<td>46.01 (×3.3)</td>
<td>15.67</td>
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</table>

Values in parentheses, fold increase of induction of apoptosis over the spontaneous level at the same time.

Family 1.

Family 2.
induced cytotoxicity by determining the number of cells excluding trypan blue. It was found that AT cells are more sensitive than normal and heterozygous cells to induction of cell death by irradiation (Fig. 1). Thus, because the percentage of induced apoptosis in AT lymphoblasts was never higher than that of normal lymphoblasts, our results suggest that apoptosis is not responsible for the hypersensitivity of AT cells to irradiation.

**Spontaneous and Ionizing Radiation-induced Apoptosis in Resting Lymphocytes.** To investigate whether the apoptotic response observed in AT lymphoblasts could be regarded as a general feature of the AT syndrome, we carried out similar experiments on unstimulated lymphocytes from AT patients. Lymphocytes isolated from the peripheral blood of nine normal donors and nine AT AT patients were γ-irradiated and incubated without mitogenic stimulation for 24 h before analysis. Nonirradiated lymphocytes from AT patients (with the exception of patient 14) demonstrated a significantly higher mean frequency of apoptotic cells when compared to normal donors (P < 0.01; Table 2). Moreover, the increase in ionizing radiation-induced apoptosis was significantly lower in AT samples than in normal cells (1.60 versus 9.47; P < 0.01). Thus, the differential response of normal and AT lymphocytes is consistent with that observed in EBV-transformed normal and AT lymphoblasts.

**Apoptosis in Primary Fibroblasts and SV40-transformed Fibroblast Responses.** We compared the apoptotic response in two cellular types, lymphoblasts and primary fibroblasts, derived from the same donors. Indeed, AT4Be fibroblasts (Fig. 2) and GM3189 fibroblasts and GM3657 lymphoblasts are derived from the same normal individual. It can be seen that the frequencies of spontaneous apoptosis in both normal and AT primary fibroblasts (Fig. 2) were clearly lower than those in the corresponding lymphoblastic cell lines (Table 1). Moreover, following γ-ray exposure at the observed times, the normal and AT primary fibroblasts did not undergo apoptosis as compared to the lymphoblastoid cell lines.

We also compared the apoptotic response of primary cultured (AT5Bl) or SV40-transformed AT fibroblasts (AT5BIVA) from the same donor. We show that the spontaneous level of apoptotic cells is not modified by the transformation. The level of γ-ray-induced apoptosis is increased in SV40-transformed AT fibroblasts (4-fold induction) in contrast to their nontransformed counterpart (Fig. 2).

**DISCUSSION**

In this article, we extend our previous observations showing alterations in apoptosis in the AT syndrome. Lymphocytes and EBV-transformed lymphoblasts exhibited an increased level of spontaneous apoptosis. This programmed cell death plays a central role in the control of developmental processes, particularly in epithelium, hematopoietic, and nervous tissues (24). The spontaneously increased apoptosis in the AT syndrome may have an important pathogenetic role in the immunological and neurological deficiencies, which are the major features of the disease. This hypothesis is consistent with the characteristics of the recently cloned AT gene (25). One of the functional domains of the AT gene product is similar to that of phosphatidylinositol-3 kinases, and it was shown that this kinase activity is
required for the prevention of programmed cell death in rat pheochromocytoma cells by nerve growth factors (26).

AT heterozygote lymphoblastic cell lines indicate partial alteration in the apoptotic pathway(s), at least in certain individuals (Table 1; Ref. 16). These data are consistent with other observations demonstrating an intermediate AT phenotype in heterozygotes. Indeed, although phenotypically normal, their risk of developing cancer is higher than that of normal individuals (27). Moreover, at the cellular level, their radiosensitivity is intermediate between that of normal subjects and AT patients (28). Unfortunately, the partial overlap between cellular responses to radiation (DNA synthesis, induced chromosome aberrations, and survival) of normal and AT carriers does not permit the utilization of one of these parameters to directly identify heterozygous individuals (28, 29).

We show here that the increased level of spontaneous apoptosis is restricted to hematopoietic cells, such as lymphoblasts and lymphocytes, which undergo apoptosis under normal physiological conditions, like during differentiation or immunological responses. In this context, dysregulation of apoptosis may affect normal development of the hematopoietic system of AT patients, but may be unaltered in other histocellular types such as fibroblasts.

Our data indicate that the extreme radiosensitivity of the AT cells, which is the principal characteristic of this syndrome at the cellular level, is not related to an increased apoptotic response. This contention is supported by the following findings: (a) ionizing radiation-induced apoptosis is lower and γ-ray-induced cytotoxicity is higher in AT lymphoblasts when compared to normal cells; (b) in primary fibroblasts, which do not undergo apoptosis after treatment with DDA, the difference in radiosensitivity between AT and normal cells is still observed (22); and (c) SV40-transformed AT fibroblasts, which are more resistant to γ-ray cytotoxicity than primary cells (22), undergo apoptosis while primary cells do not.

A deficiency in the radiation-induced apoptosis has been associated with a cellular resistance to γ-irradiation and a lack of p53 activation following this treatment (4–6, 30). Indeed, AT cells display an altered pattern of p53 induction following γ-ray exposure (9, 13, 14), whereas the cells are hypersensitive to such a treatment. All together, these observations indicate that the suboptimal p53 induction in AT cells may contribute to the absence of G1 arrest, to the genetic instability, and to the altered γ-ray induced apoptosis (10, 16), but does not account for the increased radiosensitivity of the syndrome.

An important observation of this work is that the transformation of AT fibroblasts by SV40 results in the activation of the radiation-induced apoptotic pathway, while the corresponding AT primary fibroblasts do not undergo apoptosis. The transformation of normal fibroblasts by SV40 is not associated with an increase of the radiation-induced apoptosis (17). In other words, the overall alterations induced by viral transformation may be extremely different according to the genotype (normal versus AT) of the target cells. It has been previously described that immortalization by SV40 increases radioresistance in both normal and AT cells (22, 31–33). In contrast, while the S-phase delay induced by irradiation remains unchanged after SV40 transformation in normal cells, AT cells lose their radioresistant DNA synthesis and thus behave as normal cells (34). The changes in radiosensitivity were correlated with p53 binding by SV40 T antigen (35). Transformation can also alter the expression pattern of a series of other genes. For example, c-myc expression in the SV40-transformed fibroblasts, AT5BIVA, is 16 times greater than the level present in nontransformed cells, while in normal cells this ratio is about 6 (33). Consequently, the cellular response to DDA in SV40-transformed cells may be affected by viral transformation, and the differences in the apoptotic response seen in cells from different genotypes may be due to the presence of the SV40. This raises the question of the physiological relevance of this cellular model with respect to the AT phenotype. Because normal and AT primary fibroblasts demonstrate a similar low induction of apoptosis, we suggest that the stimulation of apoptosis in SV40-transformed AT fibroblasts is due to molecular modifications induced in these cells by SV40 immortalization. In conclusion, this work presents evidence that primary cells, either fibroblasts or lymphocytes, are equally relevant models for the AT syndrome. Also, EBV-transformed lymphoblasts constitute a reliable system for the determination of the apoptotic response in contrast to SV40-transformed fibroblasts.

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