Growth Inhibition and Apoptosis Due to Restoration of E2A Activity in T Cell Acute Lymphoblastic Leukemia Cells

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Summary

Two models have been proposed for the molecular mechanism by which the Tal1 oncogene causes T cell acute lymphoblastic leukemia (T-ALL). The activation model suggests that Tal1 as heterodimers with the E2A transcription factor activates the expression of oncogenes. The inhibition model postulates that Tal1 interferes with the tumor-suppressing function of E2A. In the Jurkat T cell line, originally derived from a patient with T-ALL, Tal1 is complexed with E2A proteins and the transcriptional activity of E2A is very low. When E2A activity was restored by expressing an E2A–Tal1 fusion protein, E-T/2, the Jurkat cells underwent growth arrest and subsequently apoptosis, thus supporting the inhibition model and suggesting that E2A loss may contribute to leukemic progression.

Key words: Tal1 • E2A • apoptosis • growth inhibition • leukemogenesis

The most common genetic alteration found in human T cell acute lymphoblastic leukemia (T-ALL)[1] involves the Tal1 oncogene. Through chromosomal translocation, interstitial deletion, or other unidentified mechanisms, Tal1 is overexpressed in as much as 60% of pediatric T-ALL cases (1–5). Tal1 is normally not expressed in T cells; rather, expression of Tal1 can be detected in the developing hematopoietic system (6, 7). Accordingly, disruption of the Tal1 gene results in mice completely lacking all hematopoietic cells, and consequently these mice die between embryonic day 8.5 and embryonic day 10.5 of anemia (8). Thus, in addition to its involvement in T-ALL, Tal1 plays a crucial role in determining the blood cell lineage (8, 9). Recent data suggest that Tal1 regulates the development of the vascular system as well (10).

Tal1 belongs to the basic helix-loop-helix (bHLH) family of transcription factors involved in cell determination, differentiation, and growth (11, 12). As dimers, these proteins bind to a DNA sequence termed E box (CANNTG) that is located in the enhancers and promoters of genes regulated by bHLH proteins. This family of transcriptional regulators has been divided into two classes based on their dimerization potential and expression patterns (13). Class 1 bHLH members (also called E proteins), consisting of HEB, E2-2, and the alternatively spliced products of the E2A gene, E12 and E47, are expressed ubiquitously and can homodimerize efficiently. Class 2 proteins have a tissue-specific pattern of expression and are incapable of forming homodimers. Instead, they bind to DNA as heterodimers with the class 1 proteins. As a class 2 bHLH protein, Tal1 cannot homodimerize, but it can readily form heterodimers with the class 1 E proteins and bind to an E box (CAGATG) (14). Endogenous heterodimers of Tal1 and E47 have been identified in both leukemic T cells and erythroid cells (15, 16).

The oncogenic potential of Tal1 has been demonstrated in transgenic mice (17, 18). By expressing Tal1 in T cells using the proximal promoter of the lck (lymphoid cell kinase) gene, two separate groups have reported that the resulting transgenic mice develop T cell lymphomas and die with a median survival of 210 and 350 d, respectively (17, 18). Although these experiments show that Tal1 is indeed an oncogene, the mechanism of Tal1-mediated leukemogenesis remains to be determined. Specifically, is Tal1 acting as a transcriptional activator and turning on growth and tumor promoting genes? Or is the oncogenic potential of Tal1 due to its ability to inhibit the E proteins, which are themselves known to suppress cell growth?

Tal1–E47 heterodimers are much less potent in activating an E box reporter construct than E47 homodimers. Thus, in the presence of Tal1, transactivation by E47 homodimers is actually diminished (19–21). This inhibition appears to be due to the incompatibility of their respective

[1]*Abbreviations used in this paper:* bHLH, basic helix-loop-helix; BrdU, bromodeoxyuridine; EMSA, electrophoretic mobility shift assay; GFP, green fluorescent protein; PI, propidium iodide; T-ALL, T cell acute lymphoblastic leukemia; zVAD, zVAD-fluoromethylketone.

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activation domains, since a heterodimer between E47 and a fusion protein consisting of the NH_2-terminal activation domains of E47 and the bHLH domain of Tal1 is as potent as E47 homodimers in transcriptional activation (21). Furthermore, because disruption of the E2A gene renders mice susceptible to T cell lymphomas, E2A gene products are thought to act as tumor suppressors in mice (22, 23). It is possible that E2A products play a role in controlling the proliferation and/or survival of T cells, and loss of this activity eventually leads to deregulated growth and tumor formation. Therefore, oncogenesis mediated by Tal1 may be through inhibition of the growth-regulating activity of E2A. If suppression of E2A activity is important for Tal1-transformed leukemic T cells to proliferate and survive, then restoration of E2A function in these cells should lead to growth inhibition and decreased cell survival. Here we show that restoration of E2A activity in human leukemic T cells results in a profound inhibition of growth and an increase in apoptosis. These data support the hypothesis that Tal1-mediated leukemogenesis is through the inhibition of E2A activity.

Materials and Methods

Expression of E-T/2 in Jurkat Cells. To generate the retroviral construct expressing the E-T/2 protein, a BamH1-Asp718 fragment containing the coding sequence of E-T/2 (21) was first inserted into the green fluorescent protein (GFP)-N1 vector (Clonetech). The resulting plasmid was digested with EcoRI, and the insert was subcloned into the MIGR1 vector (24). The Phoenix packaging cell line was grown in DMEM containing 10% FCS. At 70% confluence, the cells were transfected with 20 μg of DNA, in the presence of 25 μM chloroquine using the $CaPO_4$ precipitation method as previously described (25). The DNA precipitates were removed 10 h later. 24 h after transfection, the media was replaced with RPMI plus 10% FCS. Viral supernatants were collected 24 h later, and loose cells were removed by centrifugation. The supernatants were either used immediately or were frozen on dry ice and stored at −80°C.

Log phase Jurkat/E cells (26), S194, and PD31 were infected with viral stocks at a density of 3×10^5/ml in the presence of 4 μg/ml polybrene for 24 h. The cells were then cultured for 3 d in RPMI containing 10% FCS (PD31 were also supplemented with 50 μM β-ME) and sorted using a Coulter Epics Elite sorter. GFP-positive cells were collected in RPMI containing 20% FCS. In some cases, the cells were sorted again to remove any contaminating cells (usually representing 5% of the sorted cells). The sorted cells were incubated overnight before any analyses were carried out.

Western Blot and Electrophoretic Mobility Shift Assays. For Western blot analyses, infected Jurkat/E cells, S194, and PD31 were lysed in SDS sample buffer at a concentration of 3×10^4 cells/μl. Equal amounts were loaded on a 12% SDS polyacrylamide gel. Proteins were transferred onto a nitrocellulose membrane overnight in transfer buffer (25 mM Tris, 190 mM glycine, and 20% methanol) at 150 mA. The membrane was blocked for at least 2 h in 5% nonfat milk in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) at room temperature. Polyclonal antibodies against E47 (Santa Cruz Biotechnology) were added to the blocking solution at a dilution of 1:1,000 and incubated for 2 h at room temperature. Immunoreactive proteins were detected by ECL (Amersham).

For electrophoretic mobility shift assays (EMSAs), nuclear extracts were prepared as previously described (27). Nuclear extracts were incubated with preimmune sera, anti-E47, or anti-Tal1 antibodies (28) for 10 min at room temperature. The binding reactions using these nuclear extracts were then carried out at room temperature for 20 min in a buffer containing 20 mM Tris, pH 7.5, 60 mM NaCl, 5% glycerol, 1 mM dithiothreitol, 1 mM EDTA, 0.125 mg/ml poly dI-dC, and the ^{32}P end-labeled probe (10^4 cpm). The binding complexes were resolved on a 5% polyacrylamide gel in 0.5× TBE. The sequence of the oligonucleotide probe is as follows: 5′ GTGGACGCCAACCTCAACAGATGGTCGGCTCACGGCATAG 3′ (top strand).

Analyses for Growth and Apoptosis. For growth curve analysis, the sorted cells were resuspended in RPMI containing 1, 5, and 15% FCS at a density of 10^5 or 5×10^4/ml. The cells were stained with trypan blue to exclude dead cells and counted daily in a hemacytometer. For the clonogenicity assay, infected cells were plated onto 96-well plates at a density of two cells per well in RPMI containing 10% FCS. A total of 500 wells were plated for each construct. Jurkat/E colonies were counted 51 d later, and S194 and PD31 colonies were counted 14 d later.

For propidium iodide (PI) staining, $\sim 2 \times 10^4$ cells were spun down and resuspended in 25 μl of PBS with 1% FCS. While vortexing, 0.2 ml of ice cold ethanol was added, and the cells were allowed to dehydrate overnight at 4°C. Cells were collected by centrifugation and resuspended in 250 μl of PBS containing 1% FCS, 0.1 mg/ml RNase A, and 50 μg/ml PI, followed by incubation at 37°C for 2 h. A total of 10,000 events were recorded using FACScan® and analyzed by using the Modfit LT2.0 program (Verity Software House Inc.).

For bromodeoxyuridine (BrdU) and PI double staining, infected Jurkat/E cells were sorted and labeled with 2 μM BrdU for 30 min 3 d later. Cells were fixed and stained with antibodies against BrdU conjugated to fluorescein (Boehringer Mannheim) and PI as described by Lissy et al. (29).

Results and Discussion

Restoration of E2A Activity in Jurkat T-ALL Cells. We chose the human Jurkat T cell line as a system to study the mechanism of Tal1-mediated oncogenesis. Jurkat is a physiologically relevant cell line in that it was originally derived from a patient with T-ALL (30) and it expresses the Tal1 protein (15). As shown in Fig. 1 C, an EMSA using a nuclear extract from Jurkat cells reveals both the E47 homodimeric and Tal1–E47 heterodimeric complexes (lane 1). Preincubation of the nuclear extract with Tal1 antibodies specifically disrupts the Tal1–E47 heterodimer (lane 2), whereas addition of E47 antiserum abolishes both complexes (lane 3). It is interesting to note that the majority of E47 exists as heterodimers with Tal1 in Jurkat cells. Since Tal1–E47 heterodimers have been shown to be transcriptionally inactive (19–21), this result implies that only a small portion of E47 proteins in Jurkat cells is functional in transcriptional activation. Indeed, very little endogenous E47 transcriptional activity is present, since transfection into Jurkat cells of an E box reporter plasmid results in small amounts of expression of the reporter as compared with other cell lines (data not shown).

Because most of E47 in Jurkat cells is complexed with Tal1 as transcriptionally inactive heterodimers, we sought

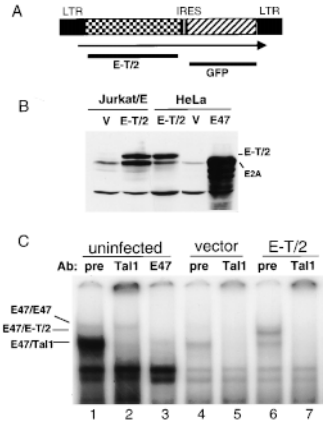

Figure 1. Expression of E-T/2 in Jurkat/E cells. (A) The E-T/2 retroviral construct. The checkered and hatched boxes represent the coding sequences of E-T/2 and GFP, respectively. The transcript of the retroviral construct as depicted by a thin line with an arrowhead encodes both E-T/2 and GFP proteins, which are shown as thick lines. (B) Western blot using anti-E47 antibodies. Total cell extracts were prepared from Jurkat/E cells infected with the indicated retrovirus, or from HeLa cells transiently transfected with the indicated construct cloned in the pcDNA3 vector. The relevant bands are marked on the side. (C) EMSA. Nuclear extracts were prepared from Jurkat/E cells uninfected or infected with vector or E-T/2 retrovirus. The extracts were preincubated with the indicated antibodies before the binding reactions. The specific binding complexes are marked on the side.

to restore E47 activity by expressing exogenous E47, thus increasing the formation of E47 homodimers. If the inhibition of E47 by Tal1 is important for the proliferation and/or survival of Jurkat cells, restoration of E47 activity should then lead to a reduction in growth and/or decreased survival. To test this hypothesis, an E47/GFP expression plasmid was constructed that enabled GFP to be translated from an internal ribosomal entry site located 3′ to the coding sequence of E47. Therefore, any cell that expressed GFP was assumed to also express E47. By sorting transfected cells for GFP expression, it was possible to purify the transfected cells away from the majority of untransfected cells. Cells transfected with the plasmid expressing E47 and GFP or the vector expressing GFP alone, as a control, were sorted for GFP fluorescence 24 h after transfection. The growth rates of the transfected cells were compared 2 d later. Control cells multiplied 3.5-fold on the average, whereas cells expressing E47 did not expand at all. This experiment demonstrates that restoration of E47 activity leads to growth inhibition in Jurkat cells.

Although this transient transfection experiment shows the growth suppressive effect of E47, it is difficult to rule out that the growth inhibition is due to the exceedingly high levels of E47 expression. Therefore, to restore E47 activity in Jurkat cells to endogenous levels, E-T/2, an E47-Tal1 fusion protein, was used. This protein contains the NH_2 terminus of E47, including the known activation domains, fused to the bHLH domain and COOH terminus of Tal1 (21). Hence, E-T/2 cannot form homodimers to bind to DNA and activate transcription on its own. However, when E-T/2 is cotransfected with E47 into HeLa cells, the resulting heterodimers can activate transcription as potently as E47 homodimers (21). More importantly, when transfected alone into Jurkat cells, E-T/2 can increase the expression of a reporter construct by 13-fold. Because the transcriptional activity of E-T/2 is limited by the amount of endogenous E47, this 13-fold activation probably reflects the physiological level of E47 activity.

E-T/2 was delivered into Jurkat cells using a retroviral vector. Due to the extremely low infection efficiency with an amphotropic virus, a Jurkat cell line expressing the receptor for ecotropic retroviruses (Jurkat/E) was used (26). The E-T/2 expressing construct (Fig. 1 A) or the MIGR1 parental vector as a control was transfected into the Phoenix-E packaging cell line to produce ecotropic viruses with high titers. Similar to the plasmids used in the transfection experiment, these retroviral constructs contain the coding sequence for GFP translated from an internal ribosomal entry site. Thus, it was likely that cells that expressed GFP also expressed E-T/2. 4 d after infection, infected cells were purified by sorting for GFP fluorescence. The brightest 5% of the cells were collected and allowed to recover for 24 h before the analyses of growth properties. Expression of E-T/2 in the infected cells was confirmed by Western blotting with antibodies against E47. As shown in Fig. 1 B, whole cell lysates from infected cells 7 d after infection showed expression of the protein. E-T/2 and E47 proteins produced by transient transfection into HeLa cells were used as positive controls. To detect heterodimers between E-T/2 and endogenous E47, EMSA was performed with nuclear extracts prepared from vector- or E-T/2–infected cells 9 d after infection (Fig. 1 C). In addition to the endogenous E47/Tal1 complex, a new complex appeared in the cells infected with E-T/2 (Fig. 1 C, compare lanes 4 and 6). Based on the fact that this complex migrates similarly to E47 homodimers and can be displaced by anti-Tal1 antibodies (lane 7), we conclude that this complex represents the heterodimer between E-T/2 and endogenous E47.

Growth Inhibition as a Result of E-T/2 Expression in Jurkat Cells. Growth rate analyses were set up using vector- and E-T/2–infected cells. As shown in Fig. 2, cells infected with E-T/2 were severely impaired in their ability to proliferate when cultured in 1, 5, or 15% FCS. This impairment was most pronounced at the lowest serum concentration. By day 5, cells infected with E-T/2 had started to level off in their growth. In contrast, cells infected with the control virus continued to proliferate. By day 7, the number of cells in the E-T/2 infection had actually decreased to a level lower than the initial starting point. Significant inhi-

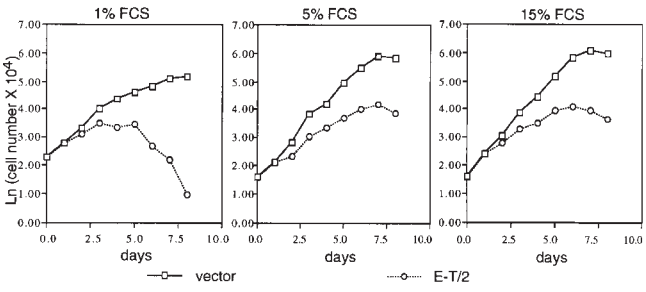

Figure 2. Reduction of the growth rate of Jurkat/E cells by E-T/2. On day 0 (24 h after sorting), the infected cells were plated at densities of 10^5/ml in media containing 1% FCS and 5×10^4/ml in media containing 5 or 15% FCS. Viable cells were counted daily from day 1 to day 8 by using a hemacytometer after trypan blue exclusion of the dead cells. The data is a representative of several experiments.

bition of growth was also observed in cells cultured in the presence of 5 and 15% FCS. The doubling times for E-T/2 cells cultured under these conditions were increased by twofold compared with control cells. To exclude the possibility that the decrease in growth rate of Jurkat/E cells was due to nonspecific toxicities of the E-T/2 protein, negative controls were performed by using two murine B cell lines, S194 (plasmacytoma) and PD31 (Abelson virus-transformed pre-B cell). After these cells were infected with the E-T/2 and MIGR1 viruses, they were sorted for GFP fluorescence and their growth rates were monitored. As shown in Fig. 3, expression of E-T/2 in these two cell lines did not appear to have any effect on their growth rates.

As an alternative measure of growth properties, clonogenicity of the infected cells was determined. Jurkat/E cells infected with the control or E-T/2 virus were purified and seeded in 500 wells at a density of two cells per well in media containing 10% FCS. In two separate experiments, 23 and 38 wells from the E-T/2–infected cells were found to contain proliferating cells, in contrast to 242 and 245 wells for control cells. In comparison, the clonogenicities of E-T/2 infected S194 and PD31 cells were not significantly different from their vector-infected counterparts (131 vs. 260 for S194, and 384 vs. 453 for PD31). These results, together with those from the growth rate analysis, demonstrate that expression of E-T/2 leads to inhibition of cell growth specifically in Jurkat cells, whose growth and/or survival may depend on the absence of E2A function. However, S194 and PD31 cells, which are probably transformed through other mechanisms, are insensitive to the expression of E-T/2 protein.

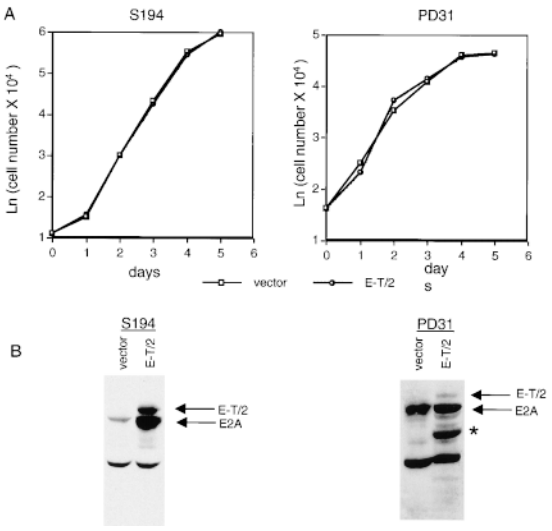

Figure 3. No effect on the growth rate of S194 and PD31 cells by E-T/2. (A) Growth curves. On day 0 (24 h after sorting), S194 and PD31 infected cells were plated at densities of 3×10^5 and 5×10^5 cells/ml, respectively in media containing 5% FCS. Viable cells were counted daily using a hemacytometer after trypan blue exclusion of the dead cells. The data is representative of several experiments. (B) Western blot using anti-E47 antibodies. Total cell extracts were prepared from S194 and PD31 cells infected with the indicated retrovirus. The E-T/2 and endogenous E2A proteins are indicated by the arrows. A degradation product of E-T/2 in PD31 cells is marked by an asterisk.

Apoptosis in Growth-arrested Jurkat Cells. To examine the cell cycle distribution of the infected cells, we stained the cells with PI to determine their DNA content (Fig. 4 A). The E-T/2–infected cells did not show any significant difference in their distribution of G1, S, and G2/M phases compared with the control infected cells. However, the presence of a sub-G1 peak in the E-T/2–infected cells, but not the vector-infected cells, suggested that these cells were undergoing apoptosis. This apoptotic population represented ~15% of the cells. To corroborate these findings, the infected cells were stained with Hoechst 33258 to examine their nuclear morphology (Fig. 4 B). The nuclei of control-infected cells were smooth and round. In contrast, a fraction of the nuclei of E-T/2–infected cells appeared twisted and fragmented, indicative of apoptosis (31).

These results raise the question of whether the growth retardation by E-T/2 is due to a primary apoptotic event or a cell cycle arrest that subsequently results in apoptosis. To address this question, we used the caspase inhibitor, zVAD-fluoromethylketone (zVAD; reference 32), to prevent apoptosis in E-T/2–expressing cells, and asked if the inhibitor could increase the growth rate of these cells. As shown in Fig. 5 A, addition of zVAD increased the growth of neither vector- nor E-T/2–infected cells over a period of 4 d

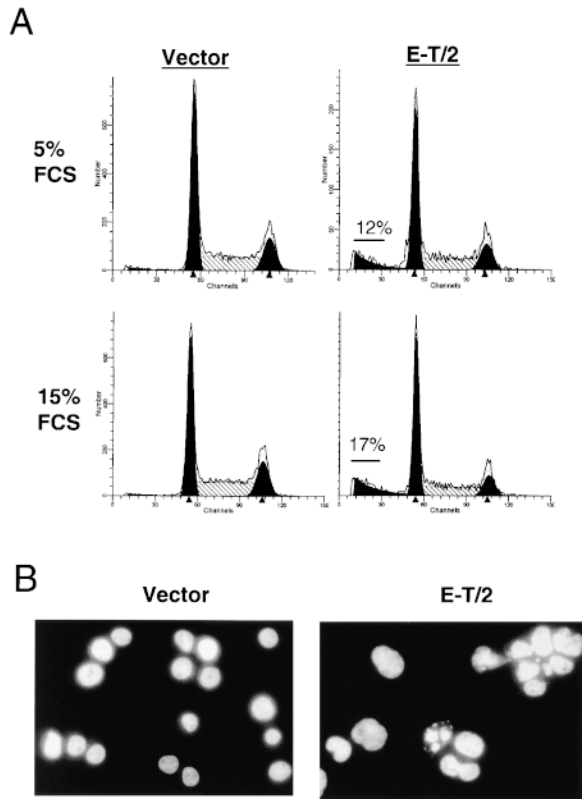

Figure 4. Apoptosis in E-T/2 infected cells. (A) PI staining of sorted Jurkat/E cells, which were cultured in media containing 5 or 15% FCS for 5 d. The sub-G1 peak is marked by a thin line on the top. The percentage of this population of cells is indicated. (B) Hoechst 33258 staining of the sorted Jurkat/E cells infected with the vector or E-T/2 virus and cultured for 5 d in media containing 5% FCS.

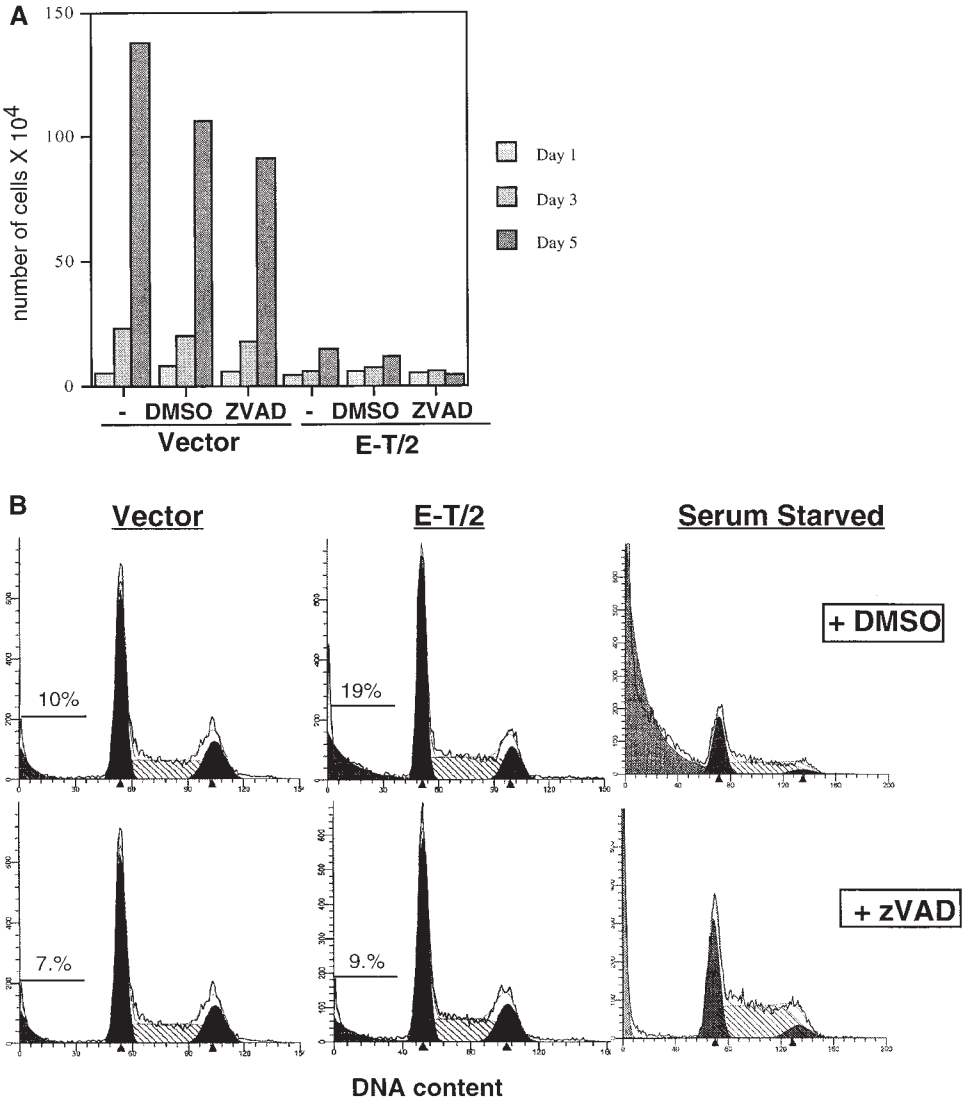

Figure 5. Treatment of infected Jurkat/E cells with the caspase inhibitor, zVAD. (A) The growth of Jurkat/E cells infected with the vector or E-T/2 virus. Cells were sorted for GFP twice and plated in media containing 5% FCS without or with 1:500 vol/vol of DMSO or the same volume of zVAD (20 mM). On the indicated day after plating, cells were counted as previously described. On day 2, an equal volume of fresh medium containing the same concentration of zVAD or DMSO was added. Data shown are representatives of three separate experiments. (B) PI staining. On day 4 after plating, the cells cultured as in A were analyzed. The percentage of apoptotic cells is shown above the thin lines.

(comparing cells treated with the zVAD inhibitor and with the DMSO vehicle). To determine if zVAD can prevent apoptosis in these cells, the vector- and E-T/2 infected cells were stained with PI on day 4. Indeed, the apoptotic population in E-T/2–infected cells was partially reduced by addition of zVAD (Fig. 5 B). As a control, Jurkat/E cells were cultured for 2 d in the absence of FCS to induce apoptosis. In the presence of zVAD, the apoptotic population also decreased dramatically. If the growth retardation in E-T/2–infected cells was due to enhanced apoptosis triggered by caspases, addition of zVAD would have been able to block the process and increase the growth rate. Therefore, our data suggests that the growth inhibition of these cells may not be the direct result of enhanced apoptosis mediated through the caspases, but rather may be due to an intrinsic poor ability of these cells to proliferate.

Inefficient BrdU Incorporation in E-T/2–expressing Cells. To further examine the proliferative properties of E-T/2–expressing cells, we evaluated their ability to incorporate BrdU. 3 d after the infected cells were sorted, they were labeled with BrdU and then stained for both PI and BrdU. As shown in Fig. 6, although the percentage of cells in S phase as determined by PI staining (R1) was similar in vector- and E-T/2–infected cells, those S phase cells expressing E-T/2 appeared deficient in their ability to incorporate BrdU. Within the R1 gate for cells in the S phase, the mean staining for BrdU was 16.0 for vector infected cells, whereas the mean was only 10.8 for E-T/2–infected cells. Furthermore, 60% of vector-infected cells belong to the group with brighter staining for BrdU, whereas only 31% of E-T/2–infected cells have the same staining intensity. Thus, this experiment suggests that one of the reasons for the growth inhibition may be due to the inefficiency of E-T/2–expressing cells to transit through the S phase of the cell cycle. As a result of this aberrant delay in S phase, a fraction of the cells may subsequently undergo apoptosis.

Taken together, our data show that expression of the E-T/2 construct inhibits the proliferation of Jurkat cells, which subsequently leads to apoptosis of the cells. This growth-inhibitory effect of the E-T/2 fusion protein may be due to the restoration of E2A transcriptional activity that is repressed by Tal1 in Jurkat cells. E-T/2, as a heterodimer

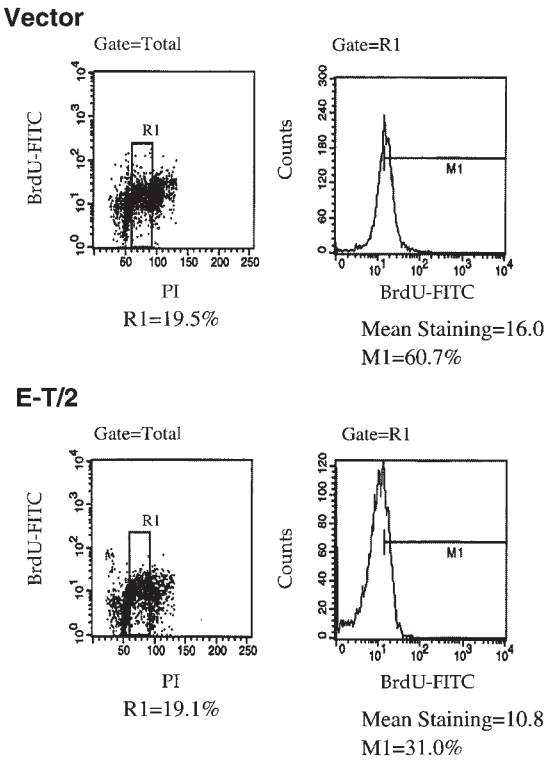

Figure 6. BrdU incorporation of E-T/2 infected Jurkat/E cells. Infected Jurkat/E cells were labeled with BrdU 3 d after sorting and stained with anti-BrdU conjugated to both fluorescein and PI. PI staining was first used to delineate S phase cells (gate = R1). These cells were then quantitated for the intensity of BrdU staining. The data is representative of several experiments.

with endogenous E2A proteins, may be able to activate the expression of E2A-controlled genes, which encode proteins involved in growth suppression. We have previously shown that E2A can stimulate transcription of the gene coding for a cyclin-dependent kinase inhibitor, $p21^{CIP}$, in HeLa, 293T, and NIH3T3 cells (33). However, the p21 protein was not detectable in Jurkat T cells with or without E-T/2 expression (data not shown). Other growth-suppressing genes may be involved. The defect in BrdU incorporation would point to some aspect of DNA synthesis as a possible target, which may be negatively regulated by E2A, perhaps through indirect mechanisms. Differential screening experiments have revealed several transcriptional repressors being upregulated in E-T/2–expressing cells. It remains to be determined if these repressors control the transcription of certain genes crucial for S phase function.

The function of E2A or E-T/2 proteins in suppressing the growth of T cells is consistent with the finding that loss of E2A function leads to the development of T cell lymphoma in either E2A-deficient mice (22, 23) or transgenic mice expressing the Id1 inhibitor of E2A (Kim, D., and X.-H. Sun, manuscript in preparation). Similarly, transgenic mice expressing Tal1 also develop T cell lymphoma, albeit with a longer latency and lower incidence (17, 18). Because Tal1 has been shown to diminish the transcriptional activity of E2A proteins in transfection assays, it is conceivable that Tal1 acts as an inhibitor of E2A in causing T cell lymphoma. This hypothesis is supported by the fact that transgenic mice expressing the truncated version of Tal1 lacking the NH_2-terminal activation domain can still synergize with LMO1 in causing T cell malignancies (34). The same scenario may apply to human T-ALL, where Tal1 may also interfere with the normal function of E2A, i.e., maintaining the balanced growth of T cells. As we have shown using the Jurkat T-ALL cell line, restoration of E2A function by expressing E-T/2 leads to the growth arrest of Tal1-expressing cells. The ability of E-T/2 to inhibit the growth of T-ALL cells may thus be potentially useful for therapeutic purposes. When delivered into bone marrow cells of T-ALL patients through gene therapy approaches, E-T/2 may restore E2A activity to inhibit the growth of leukemic cells.

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