Cytotoxic T Cells Overcome BCR-ABL-mediated Resistance to Apoptosis

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Abstract

Chronic myeloid leukemia is a disease marked by expanded clonal hematopoiesis; it is incurable by chemotherapy or radiation but is cured in a majority of patients receiving bone marrow transplantation from nonidentical sibling donors, an outcome generally attributed to a T cell-mediated graft-versus-leukemia effect. In this report, we examine the effect of the p210BCR-ABL fusion protein of the BCR-ABL oncogene, the molecular hallmark of chronic myelogenous leukemia, on the sensitivity of mouse cell lines to apoptosis induced by chemotherapy, radiation, or activated cytotoxic T lymphocytes (CTLs). We find that, although cells expressing p210BCR-ABL by gene transfer are more resistant than their normal counterparts to apoptosis induced by chemotherapy or radiation, they are equally susceptible to apoptosis induced by alloreactive CTLs. These results show that CTLs overcome BCR-ABL-mediated resistance to apoptosis and, therefore, provide a biological correlation for the success of allogeneic bone marrow transplantation in chronic myelogenous leukemia.

Introduction

CML is a hematological malignancy characterized by an initial chronic phase of expanded clonal hematopoiesis with continued differentiation into mature granulocytes; this phase invariably progresses to blast crisis, a terminal stage resembling acute leukemia. The cytogenetic hallmark of CML, the Philadelphia chromosome (1), results from the BCR-ABL gene rearrangement, which encodes a Mr 210,000 fusion protein (p210BCR-ABL) with cytoplasmic tyrosine kinase activity. The BCR-ABL rearrangement likely initiates CML, because mice acquire a disease resembling chronic leukemia when transplanted with bone marrow cells that are infected with a retrovirus encoding BCR-ABL (2). A crucial effect of BCR-ABL expression in CML is the prolongation of hematopoietic cell survival by inhibition of apoptosis, the physiological form of autonomous cell death. We and others have shown that p210BCR-ABL confers upon hematopoietic cells the ability to survive treatments, such as growth factor deprivation or genotoxic chemotherapy, that induce apoptosis in normal cells (3, 4). These findings probably account for the inability of standard chemotherapy to cure patients with CML.

Nonetheless, CML is curable by myeloablative therapy followed by bone marrow transplantation. Relapse of CML occurs in <10% of recipients of marrow from a nonidentical sibling donor, but the relapse rate increases to 50% when the marrow is depleted of T cells or is from an identical twin donor (5). These findings strongly suggest that allogeneic donor T cells exert an immunological antitumor effect in CML. Because CTLs, the major effectors of antitumor immunity, are capable of inducing both apoptosis and osmotic lysis in their targets (6), we were interested to test the effect of p210BCR-ABL expression in targets of CTL-mediated killing. Interestingly, we find that, although p210BCR-ABL protects cells from chemotherapy-induced apoptosis, it fails to protect cells from apoptosis induced by activated CTLs. These findings provide a biological explanation for the relative efficacies of various treatments of CML and provide a rationale for the use of immunotherapy in cancers that are resistant to chemotherapy.

Materials and Methods

Cell Lines. Studies were performed on two IL-3-dependent mouse cell lines, Ba-F3 (early B-lineage; Ref. 7) and FDC-P1 (myeloid; Ref. 8). The cell lines, induced to constitutively express P210BCR-ABL by retroviral infection with a full-length BCR-ABL cDNA sequence, were provided by R. A. van Etten and D. Baltimore (Ba-F3p210, Ref. 9) and S. Cory (FDC-P1p210, Ref. 10). The cell lines were maintained in complete (containing vitamins, amino acids, and lipids) serum-free medium (Serum-Free and Protein-Free Hybridoma Medium; Sigma Chemical Co., St. Louis, MO) in 5% CO2 at 37°C with 100 units recombinant mouse IL-3 (or 10% conditioned medium of the WEHI-3 cell line as a source of IL-3) added to the parental cell lines.

Induction and Assays of Activated CTLs. Spleen cells (4 x 106) from C57BL/10 mice (B10; H-2b) were cultured with 2 x 107 irradiated (3000 cGy) BALB/c (H-2b) or C3H/HeJ (H-2h) spleen stimulators in 2 ml EHAA medium (Biofluids, Rockville, MD) containing 10% FCS, 5 x 10-5 M 2-mercaptoethanol, glutamine, and antibiotics (complete medium). After 5 days of culture, viable CTLs were isolated by centrifugation over Ficoll (Pharmacia, Uppsala, Sweden), counted, and tested for induction of DNA fragmentation or osmotic lysis of H-2b or H-2h-labeled targets, respectively. DNA fragmentation was assessed at various effector:target (E:T) ratios by the JAM test (11). Osmotic lysis was assessed by plating CTL at various E:T ratios with 104 labeled targets in a standard chromium release assay (11).

Analysis of Cellular Viability following Exposure to Activated CTLs or Cytotoxic Agents. Ba-F3 or Ba-F3p210 cells were treated with graded concentrations of VP-16 (0–80 μg/ml for 30 min), were irradiated (0–2000 cGy), or were cultured for 2 h with B10 CTL generated against BALB/c stimulators as described above. The viability of cells exposed to VP-16, irradiation, or CTLs was determined by methylcellulose clonogenic assays as described previously (4).

DNA Fragmentation Assays. FDC-P1 or FDC-P1p210 cells were incubated in medium alone or with activated CTLs for 2 h. Total genomic DNA was then isolated after SDS lysis and proteinase K digestion as described (12). Oligonucleosomal DNA fragments were separated by agarose gel (2%) electrophoresis and visualized by staining with ethidium bromide. One hundred base-size markers (GIBCO-BRL, Gaithersburg, MD) were also separated by electrophoresis as controls.

In Situ Nick-end Labelling of Apoptotic Nuclei. FDC-P1p210 cells were incubated with activated CTLs for 2 h and then fixed in cold 4% buffered formaldehyde (pH 7.4), followed by cold 70% ethanol. The fixed cells were then incubated with 20 μg/ml proteinase K (Sigma), washed in cold PBS, and immersed in 100 μl reaction buffer [0.2 M potassium cacodylate, 25 mM Tris-HCl (pH 6.6), 0.25 mg/ml BSA, and 2.5 mM cobalt chloride] supplemented with terminal deoxynucleotidyl transferase (0.3 U/ml) and biotinylated dUTP. Each experiment was performed with a negative control (without biotinylated dUTP) and a positive control [10-μl pretreatment with 1 μg/ml DNase (Sigma), dissolved in reaction buffer]. Following incubation at 37°C for 30 min, the cells were washed in PBS and incubated with 100 μl FITC-avidin solution [4X saline-sodium citrate buffer (Sigma), 2.5 μg/ml fluorescein isothiocyanate (FITC), 0.1% Triton X-100, and 5% w/v nonfat dry milk] at room temperature for 30 min in the dark. The cells were washed in PBS with 0.1% Triton X-100
The same CTLs were also tested for their ability to induce osmotic lysis by irradiation (Fig. 1a, •) and VP-16 (Fig. 1a, —). Sensitivity of generated CTLs against BALB/c stimulator cells (b–d). Viability of the cells exposed to these agents was then assayed by methylcellulose clonogenic assays (a and b), osmotic lysis of the targets was assessed by a standard chromium release assay (c), and DNA fragmentation was determined by measuring the release of 3H-labeled DNA fragments from the target cell (d). Osmotic lysis and DNA fragmentation of syngeneic B10 targets are also shown (c and d, •).

In recent studies, McGahon et al. (3) and our laboratory (4) have shown that the P210BCR-ABL fusion protein is responsible for the resistance of CML cells and BCR-ABL-expressing mouse cell lines to the induction of apoptosis by chemotherapy or serum or growth factor deprivation. Because activated CTLs kill cells by inducing both apoptosis and necrosis (by osmotic lysis) in their targets (6), we examined whether the P210BCR-ABL fusion protein endows cells with resistance to chemotherapy and radiation without affecting their susceptibility to CTL-induced osmotic lysis. To do this, we compared the Ba-F3 pre-B cell line (derived from a BALB/c mouse) to the same line expressing P210BCR-ABL by gene transfer (Ba-F3P210) for viability following exposure to irradiation, VP-16, or alloreactive CTLs (generated by a 5-day culture of B10 splenocytes with irradiated BALB/c spleen stimulators). Ba-F3P210 cells were more resistant than parental Ba-F3 cells (Fig. 1a, •) to killing by irradiation (Fig. 1a, ——) and VP-16 (Fig. 1a, —). Sensitivity of Ba-F3P210 to chemotherapy and radiation is restored by addition of an antisense, but not a nonsense, oligonucleotide to BCR-ABL, indicating that P210BCR-ABL is directly responsible for the cellular resistance to DNA damage.4 In contrast, Ba-F3 and Ba-F3P210 were equally susceptible to being killed by activated, alloreactive CTLs (Fig. 1b). The same CTLs were also tested for their ability to induce osmotic lysis and DNA fragmentation of both cell lines after 4 h of coculture in chromium and thymidine release assays, respectively. As is shown, Ba-F3 and Ba-F3P210 were equally sensitive to osmotic lysis (Fig. 1c) and DNA fragmentation (Fig. 1d), indicating that the P210BCR-ABL fusion protein offers no protection against the lethal effects of activated, alloreactive CTLs.

T cells induce apoptosis either by ligation of the Fas antigen on the target cell surface or through the action of the constituents of CD8+ T cell lytic granules that are injected into the target cell cytoplasm. Apoptosis induced by granzyme B, a constituent of the lytic granules of CD8+ CTLs, does not require target cell protein synthesis and occurs within 1 h of CTL-target contact (13). In contrast, other forms of triggered apoptosis do not commence until after several hours after the death-inducing stimulus, reflecting the need for synthesis of macromolecules by the cell to die. Therefore, we investigated the mechanism of apoptosis by determining the kinetics of CTL-mediated killing of the FDC-P1 myeloid cell line (also from BALB/c; H-2b) transduced or not with a retroviral vector containing the full-length BCR-ABL cDNA. Splenocytes from C57Bl/10 (B10) mice were cultured for 5 days with irradiated BALB/c or C3H (H-2k) spleen stimulators and then tested for their ability to induce DNA fragmentation.

**Results and Discussion**

In recent studies, McGahon et al. (3) and our laboratory (4) have shown that the P210BCR-ABL fusion protein is responsible for the resistance of CML cells and BCR-ABL-expressing mouse cell lines to the induction of apoptosis by chemotherapy or serum or growth factor deprivation. Because activated CTLs kill cells by inducing both apoptosis and necrosis (by osmotic lysis) in their targets (6), we examined whether the P210BCR-ABL fusion protein endows cells with resistance to chemotherapy and radiation without affecting their susceptibility to CTL-induced osmotic lysis. To do this, we compared the Ba-F3 pre-B cell line (derived from a BALB/c mouse) to the same line expressing P210BCR-ABL by gene transfer (Ba-F3P210) for viability following exposure to irradiation, VP-16, or alloreactive CTLs (generated by a 5-day culture of B10 splenocytes with irradiated BALB/c spleen stimulators). Ba-F3P210 cells (Fig. 1a, •) were more resistant than parental Ba-F3 cells (Fig. 1a, •) to killing by irradiation (Fig. 1a, ——) and VP-16 (Fig. 1a, —). Sensitivity of Ba-F3P210 to chemotherapy and radiation is restored by addition of an antisense, but not a nonsense, oligonucleotide to BCR-ABL, indicating that P210BCR-ABL is directly responsible for the cellular resistance to DNA damage.4 In contrast, Ba-F3 and Ba-F3P210 were equally susceptible to being killed by activated, alloreactive CTLs (Fig. 1b). The same CTLs were also tested for their ability to induce osmotic lysis.

CTL-induced apoptosis in BCR-ABL-expressing cells

Fig. 4. CTLs induce rapid nuclear disintegration of BCR-ABL-expressing cells. FDC-P1P210 cells were exposed for 2 h to a 15-fold excess of B10 CTLs generated against BALB/c (a and c) or C3H (b and d) stimulators. All cells were then fixed and stained by a nick-end labeling technique to identify disintegrating, apoptotic nuclei (yellow) or intact nuclei (red). Low power (a and b) and high power (c and d) views of in situ-labeled cells are shown. The diameter of a CTL is roughly one-half that of a viable FDC-P1P210 cell (as in d).

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...tation in B10, BALB/c, C3H, FDC-P1, or FDC-P1P210 targets (Fig. 2). CTLs generated against BALB/c stimulators induced significant DNA fragmentation in BALB/c (Fig. 2, a), FDC-P1 (Fig. 2, b) and FDC-P1P210 targets (Fig. 2, c) within 1 h of coculture (Fig. 2a). In contrast, CTLs generated against C3H stimulators induced DNA fragmentation of C3H targets (Fig. 2a, d) but not BALB/c, FDC-P1, or FDC-P1P210 targets (Fig. 2b). By 2 h, DNA fragmentation of specific targets of CTLs proceeded essentially to completion (Fig. 2, c and d). The DNA extracted from FDC-P1 and FDC-P1P210 cells exposed to BALB/c-specific CTLs for 2 h was cleaved into the oligonucleosomal fragments characteristic of apoptosis (Fig. 3, Lanes 4 and 5, respectively). By contrast, DNA extracted from FDC-P1 and FDC-P1P210, incubated in medium alone (Fig. 3, Lanes 2 and 3, respectively), or exposed to C3H-specific CTLs (Fig. 3, Lanes 6 and 7, respectively) was not cleaved. FDC-P1P210 cells were also exposed to BALB/c- and C3H-specific CTLs at an E:T ratio of 15 for 2 h, and the cell mixture was then stained by a nick-end labeling technique to identify viable (Fig. 4, red) versus apoptotic (Fig. 4, yellow) nuclei. Low and high power views of FDC-P1P210 exposed to BALB/c-specific CTLs (Fig. 4, a and c) reveal extensive apoptosis, whereas the same targets exposed to C3H-specific CTL show little evidence of nuclear disintegration (Fig. 4, b and d). Therefore, CTL-induced apoptosis of BCR-ABL-expressing cells is both rapid and immunologically specific.

Homeostasis of cell number in hematopoietic tissue occurs because cell production by mitosis equals physiological cell loss by apoptosis. Physiological cell death is induced by a sequence of evolutionarily conserved molecular events, and it is now clear that hematological neoplasms may arise from genetic lesions, such as BCR-ABL (3, 4) or the bcl-2 oncogene of follicular lymphoma (14), that result in a decreased rate of physiological cell death. Using the P210BCR-ABL protein as a model inhibitor of apoptosis induced by drugs or radiation, we have shown that CTLs overcome this resistance to cell death, thereby providing a biological correlation to the effectiveness of allogeneic bone marrow transplantation or donor buffy coat transfusion in the treatment of CML. The precise mechanism by which CTLs overcome this resistance to apoptosis remains to be determined; however, for two reasons we believe that CTLs induce apoptosis by intersecting the death pathway at a very late step: (a) unlike other forms of triggered apoptosis, CTL-induced apoptosis is extremely rapid, commencing within minutes of CTL-target contact, and does not require any metabolic activity by the target cell; and (b) both the BCR-ABL and bcl-2 oncogenes block multiple pathways of apoptosis induction, including those induced by DNA damage, serum or growth factor deprivation, or steroid treatment; yet neither oncogene can protect cells from apoptosis induced by activated CTLs (3, 4, 15, 16). Because of these properties of activated CTLs, T-cell-mediated immunotherapy may be a generally effective strategy for the treatment of tumors harboring oncogenes that cause resistance to chemotherapy and radiation.

References

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