RAPID COMMUNICATION

BCR-ABL Maintains Resistance of Chronic Myelogenous Leukemia Cells to Apoptotic Cell Death

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Apoptosis is the major form of cell death associated with the action of chemotherapeutic agents on tumor cells, and therefore the expression of genes that interfere with apoptosis can have important consequences for the efficacy of therapeutic approaches. Here we show that K562, a chronic myelogenous leukemia (CML) cell line expressing the BCR-ABL fusion protein, are resistant to the induction of apoptosis by a number of agents and conditions. Anti-sense oligodeoxynucleotides corresponding to the translation start of bcr downregulate bcr-abl protein in these cells and render them susceptible to induction of apoptosis by chemotherapeutic agents or serum deprivation. Expression of a temperature sensitive v-Ab/ protein reverses the effects of the anti-sense oligonucleotides, such that the cells remain resistant to apoptosis at the permissive temperature. These data indicate that bcr-abl acts as an anti-apoptosis gene in CML cells and suggests that the effect is dependent on the abl kinase activity in this chimeric protein. Inhibition of bcr-abl to render CML cells susceptible to apoptosis can be combined with therapeutic drugs and/or treatment capable of inducing apoptosis to provide an effective strategy for elimination of these cells.

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APOPTOSIS IS a form of cell death that occurs largely under physiologic conditions and is characterized by cell shrinkage and internucleosomal DNA cleavage. Previous studies have shown that normal cells and cell lines undergo apoptosis when exposed to a variety of chemotherapeutic agents. A number of genes have been shown to be involved in the control of apoptosis and some of these have been identified as oncogenes. These genes fall into two categories: those that drive apoptosis, such as c-myc and p53, and those that retard or inhibit the process, such as bcl-2 and ras. In relation to c-myc we have shown that cells treated with an antisense oligonucleotide to suppress synthesis of myc are considerably more resistant to apoptosis when compared with their counterparts that express the myc protein. The dual ability of myc to drive proliferation and cell death appears to be determined by the presence or absence of extracellular survival factors. bcl-2-expressing cells, in contrast, show a resistance to apoptosis induced by a variety of agents including a number of chemotherapeutic compounds. The expression of apoptosis suppressing genes like bcl-2 may have important implications for our understanding and treatment of malignant disease. For example, over-expression of these genes may interfere with the normal cell turnover and lead to an abnormal accumulation of cells. This occurs in the case of mice transfected with bcl-2 in which there is an observed lymphoproliferation. In addition, cells expressing apoptosis-suppressing genes may also be resistant to the effects of cytotoxic agents, which would have an effect of drug efficacy.

Chronic myelogenous leukemia (CML) is characterized cytologically by the Philadelphia (Ph) chromosome, which results from a reciprocal translocation between chromosome 9 and chromosome 22. It is also characterized by a large accumulation of mature myeloid cells. The breakpoint of chromosome 22 has been shown to be clustered within a region of 5.8 kb and is referred to as the breakpoint cluster region (bcr). This translocation of the abl gene from chromosome 9 results in the synthesis of a chimeric BCR-ABL protein that expresses elevated levels of ABL tyrosine kinase activity. The bcr-abl gene has been documented as having a significant role to play in the generation of CML and has been shown to induce leukemia in mice transgenic for this fused gene.

The K562 cell line is derived from a CML patient and expresses the BCR-ABL protein. Previous studies from our laboratory have shown that this cell line is particularly resistant to cell death via apoptosis, irrespective to the inducing agent used. This observation led us to speculate whether the aberrant expression of the abl oncogene seen in K562 cells contributed to the observed resistance to apoptosis. Support for such a contention comes from the observation that CML myeloid precursors display normal mitotic indices, normal responses to colony-stimulating factors, and do not proliferate or mature faster than their normal counterparts, yet there is a massive accumulation of cells. Taken together these observations suggest that the myeloid expansion in CML may occur via prolongation of cell survival and that the elevation expression of ABL tyrosine kinase activity may act to suppress apoptosis. These considerations led us to speculate that bcr-abl might function as an anti-apoptotic factor in CML cells.

MATERIALS AND METHODS

Cell culture. K562 and HL-60 cells were maintained in a 5% CO₂ atmosphere at 37°C. Exponentially growing cells were used.

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in all experiments unless otherwise stated. K562 cells were transfected by electroporation with pRP4 (derived from Invitrogen pREP4 [San Diego, CA] by removal of the EBNA-1 gene) or temperature sensitive v-ahl' (DP160, provided by J. W. and University of California San Diego) in pRP4. Stable transfectants were selected for hygromycin (500 μg/mL) resistance, subcloned, and subclones were analyzed for v-ahl expression using reverse transcriptase-polymerase chain reaction. K562 parent cells, cells expressing v-ahl(DP160) or transfected with vector alone (RP4 vector) were used in subsequent experiments.

**Induction of apoptosis.** Cells were seeded at 5 × 10⁵ cells/mL in medium and exposed to the apoptosis inducing stimuli at suitable concentrations. Stock solutions of actinomycin D, camptothecin, etoposide (VP-16), and cycloheximide (all obtained from Sigma) were made up using dimethyl sulfoxide (DMSO) as a solvent. For experimental work such solutions were diluted at least 1:500 before use to ensure a concentration of DMSO less than 0.2%. Appropriate controls were run in all cases. Incubation times were as outlined in the results section and all incubations were performed at 37°C. In addition, serum deprivation was also used as an additional stimulus to induce apoptosis.

**Assessment of cell viability and apoptosis.** Cell viability was assessed by their ability to exclude trypan blue. Cell morphology was evaluated on Leukostat (Fisher Scientific, Orangeburg, NY) stained cytocentrifuge cell preparations. Apoptotic cells were identified morphologically using previously defined criteria. Two other methods were used to measure the levels of apoptosis in K562 cells. These included the use of flow cytometry to quantify the level of forward light scatter in cells undergoing apoptosis. A decrease in forward light scatter has been previously shown by us to be indicative of apoptosis. Finally, an in situ terminal deoxynucleotidyl transferase assay (TDTA) was used to measure nick end-labeling of DNA because K562 and other cells have been shown not to undergo interucleosomal DNA cleavage as they die via apoptosis. Cells 5 × 10⁵/mL were fixed in 1% formaldehyde in PBS (pH 7.4) for 15 minutes on ice. After washing in PBS the cells were resuspended in ice-cold 70% ethanol and transferred to the freezer. The cells were stored in ethanol at −20°C for up to 24 hours before being subjected to the TDTA. After rehydration in

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Fig 1. Effect of oligonucleotide on BCR-ABL expression. K562 cells that had been treated with either antisense bcr-abl or its non-sense counterpart for 48 hours were fixed, permeabilized, and incubated with a sheep anti-ABL antibody. After a washing step an FITC-labeled donkey antsheep antibody was added and fluorescence viewed with a laser scanning microscope. (A) Nomarski and (B) fluorescent images of AS-bcr-abl-treated cells; (C) Nomarski and (D) fluorescent images of NS-bcr-abl-treated cells.
BCR-ABL RESISTANCE TO APOPTOTIC CELL DEATH

Fig 2. Western blot of protein extract from K562 cells treated with either AS-bcr-abl/5-GCCCACCGGGTCCACCAT-3; or two distinct NS-bcr-abl 5'-CGCGCCTCGTCCCAAGCA-3 and 5'-CGCCCTGTTCCCAAGCA-3. The 210-kD BCR-ABL protein was detected using a primary sheep anti-ABL antibody followed a peroxidase-labeled second antibody.

PBS, cells were resuspended in 50 µL of a solution containing: 0.1 mol/L sodium cacodylate (pH 7.0), 0.1 mmol/L dithiothreitol, 0.05 mg/mL bovine albumin, 5 U of terminal deoxynucleotidyl transferase, and 0.5 mmol/L biotin-dUTP. The cells were incubated in this solution at 37°C for 30 minutes, then rinsed in PBS and resuspended in 100 µL of the staining buffer, which contained saline-sodium citrate buffer, 0.1% Triton X-100, and 5% (wt/vol) nonfat dry milk. The cells were incubated in this buffer for 30 minutes at room temperature in the dark. Cell fluorescence was measured using the LYSYS II software on a FACSScan Flow Cytometer (Becton Dickinson, San Jose, CA).

Western immunoblotting. Protein extracts from K562 cells were electrophoresed in polyacrylamide gels, transferred to a nitrocellulose membrane, and probed for expression of BCR-ABL as described previously. Because ABL occurs as a fusion protein with BCR in K562 cells a sheep anti-ABL antibody (Serotech, UK) was used to detect levels of the 210-kD BCR-ABL fusion protein.

RESULTS

In a previous study we showed that the CML cell line K562 was considerably more resistant to the induction of apoptosis than a number of other human hematopoietic cell lines. This resistance did not appear to be related to the type of inducing agent used. With this in mind we examined the potential role of bcr-abl in this resistance by exposing cells to antisense oligonucleotides corresponding to the translation start of the bcr gene (AS-bcr-abl). This approach was suggested by the observations of Szczylk and Skorski who reported that an AS-bcr-abl oligonucleotide can inhibit proliferation of cells from CML patients. As shown in Fig 1, cells pretreated for 48 hours with AS-bcr-abl showed a decrease in expression of the protein, whereas cells treated with a nonsense oligonucleotide control (NS-bcr-abl, with the same base composition as AS-bcr-abl, but in a different order) did not. The Western blot shown in Fig 2 supports this observation by demonstrating that expression of the 210-kD BCR-ABL protein is decreased after treatment with AS-bcr-abl, but two different NS-bcr-abls has no detectable effect.

The effect of decreased BCR-ABL protein expression on susceptibility to apoptosis was then assessed. K562 cells
were pretreated for 48 hours with antisense or nonsense oligodeoxynucleotides and then exposed to conditions capable of inducing apoptosis in other cells. Apoptotic cell death was assessed by morphologic examination of stained preparations. As shown in Fig 3 untreated cells or cells pretreated with nonsense oligonucleotides remained resistant to the induction of apoptosis, whereas those treated with antisense underwent apoptosis upon exposure to actinomycin D, camptothecin, etoposide (VP-16), cycloheximide, or withdrawal of serum. AS-bcr-abl did not enhance apoptosis in the non-bcr-abl-expressing HL-60 cell line treated with cycloheximide, VP-16, or VM-26. This indicates that the effect seen for K562 cells is more than likely mediated through downregulation of the bcr-abl gene rather than some nonspecific effect of the oligonucleotides (Fig 4).

The percent apoptosis is a function of both cell death and proliferation. It was possible that AS-bcr-abl inhibited cell proliferation, leading to a perceived increase in the level of apoptosis without having a direct affect on the process itself. To investigate this possibility we treated cells with a number of agents including AS-bcr-abl that are known to inhibit cell proliferation. Figure 5A shows that AS, VP-16, VM-26, cycloheximide, actinomycin-D, and serum depletion all inhibited cell proliferation. NS-bcr-abl had no detectable effect on proliferation. Figure 5B demonstrates that none of these agents alone had any effect on apoptosis. Taken together these data indicate that AS-bcr-abl probably has a direct effect on the process of apoptosis, rather than an indirect one through inhibition of proliferation.

We observed that while a number of cell lines (eg, HL-60, U937) readily displayed double-stranded DNA fragmentation upon induction of apoptosis, K562 cells did not. In view of this we used two alternative criteria to show that the form of cell death occurring was apoptotic in nature. Cell shrinkage is a hallmark of apoptosis and this change can be readily detected by measuring the light-scattering properties of treated cells. As shown in Fig 6 K562 cells did not display changes in these characteristics upon treatment with AS-bcr-abl or upon serum withdrawal. However, the combination induced apoptosis as evidenced by the sharp decrease in forward light scatter. Another characteristic feature of apoptosis is single-stranded DNA fragmentation. Single-stranded breaks were readily shown using an in situ DNA end-labeling method. Cells treated with AS-bcr-abl or NS-bcr-abl were deprived of serum for 48 hours and examined. As shown in Fig 7, a pronounced increase in DNA strand breaks was observed in the cells undergoing apoptosis in response to antisense treatment and serum withdrawal.

By using AS-bcr-abl we were able to show that downregulation of the expression of BCR-ABL corresponds to an increase in the susceptibility of the treated cells to apoptosis. In the present experiment we sought to show that the kinase activity of abl was central to the resistance seen to apoptosis. To do this we produced stable K562 transfectants with constitutive expression of a temperature-sensitive v-abl (v-abl* or with vector alone (pRP4), and these together with paren-
Fig 5. (A) K562 cells were exposed to either 10 μmol/L AS-bcr-abl, 10 μmol/L NS-bcr-abl, 50 μmol/L VP-16, 50 μmol/L VP-26, 50 μmol/L cycloheximide, 5 μg/mL actinomycin D, or serum depletion and cell numbers measured at 0, 24, 48, and 72 hours. (B) After an identical treatment regimen the level of apoptosis in cultures was assessed morphologically at the indicated time point.

Fig 8. All three cell lines were resistant to the effects of VP-16 without pretreatment or after NS-bcr-abl treatment. When cells were pretreated with AS-bcr-abl and cultured with etoposide, all three lines underwent extensive apoptosis. However, at 32°C the cells expressing v-Abl were resistant to the effect of VP-16 whereas the other lines were not. Similar results were seen for serum deprived cells. v-Abl differs in its translation start from bcr-abl and should therefore be unaffected by antisense oligonucleotides directed to bcr-abl. If AS-bcr-abl mediates its effects only through the targeting and downregulation of BCR-ABL expression, then the presence of v-Abl should reverse its effects. Because this is the result obtained, these conclusions are warranted.
**DISCUSSION**

CML is a disease in which there is a large accumulation of relatively mature myeloid cells. It is characterized cytologically by the presence of the Philadelphia chromosome that results from the translocation of a piece of chromosome 9 to chromosome 22. The net result of the translocation and possibly the root cause of the development of this malignancy is an elevation in the activity of the ABL protein when it is produced as an BCR-ABL chimera. The precise link between this elevation of enzyme activity and the abnormal accumulation of mature cells is yet to be elucidated.

A considerable amount of what we know about the cell biology of this disease comes from studies on cell lines like K562. This particular line was derived from a patient with CML and expresses the characteristic Philadelphia chromosome. Previous experiments from our laboratory showed that K562 cells were particularly resistant to the induction of cell death via apoptosis and suggested a possible link between abnormal expression of c-abl. This in turn led us to speculate that this gene may be a negative regulator of apoptosis and thus contribute not only to the drug resistance seen in these cells, but also possible to the development of CML by abnormally extending the life of Ph-positive cells. Support for this contention comes from two recent studies which showed that v-Abl inhibits apoptosis induced by growth factor withdrawal. While it is likely that the anti-apoptotic effects of Abl involve a signal transduction cascade, the ability of this protein to regulate expression of c-myc may also prove relevant because c-Myc has been implicated in some forms of apoptosis.

To determine whether bcr-abl could function as a suppressor of apoptosis, we used antisense oligodeoxynucleotides corresponding to bcr-abl to switch off production of the BCR-ABL protein. This approach was suggested by the observations of Szczylik and Skorski who reported that an AS-bcr-abl can inhibit proliferation of cells from CML patients. Using laser scan microscopy we showed that pretreating cells with the antisense led to a rapid loss of the BCR-ABL protein form the cytoplasm of the cell (Fig 1). Western immunoblotting confirmed this observation by demonstrating a specific reduction in the level of the 210-kD BCR-ABL fusion protein. When these cells were then exposed to a number of inducers of apoptosis there was a significant increase in their susceptibility to apoptosis irrespective of the inducing agent used (Fig 3). In addition to using a nonsense oligonucleotide as a negative control we also used the HL-60 cell line, which does not express BCR-ABL and showed that AS-bcr-abl did not enhance apoptosis in this system (Fig 4). These data indicate that the bcr-abl gene suppresses apoptosis in a similar manner to that seen for bcl-2, a gene that has been well documented in this regard.

Because the level of apoptosis in a culture is a function of both new cell generation and programmed cell death, it could be argued that the AS-bcr-abl is mediating its effects by simply inhibiting cell production. This in turn would lead to a perceived increase in the number of apoptotic cells.

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![Flow Cytometry Pattern](image-url)
in culture without any change in the overall kinetics of apoptosis. To test the validity of this argument, K562 cells were treated with AS-bcr-abl but not NS-bcr-abl and we showed that cell proliferation ceased without any concurrent increase in the level of apoptosis. A similar effect was seen for VP-16, VM26, cycloheximide, and serum deprivation. No increase in the levels of apoptosis was seen when these nonproliferating cultures were examined. However, when the AS-bcr-abl is used in conjunction with cytotoxic agents there is a marked increase in the level of apoptosis observed. These data show that the effect of the antisense is directly on the process of apoptosis rather than an indirect one acting by inhibiting cell proliferation. Thus, inhibiting expression of the BCR-ABL protein primes the cells for apoptosis induced by a second stimulus.

Internucleosomal DNA cleavage leading to the formation of DNA ladders in agarose gels is a hallmark of apoptosis. However, we could not show this pattern for K562 cells even though other cell lines such as U937, HL-60, MOLT-4 clearly show this type of fragmentation when examined by us. We are not alone in observing this phenomenon and a number of recent reports have demonstrated that apoptosis can occur in the absence of double-stranded breaks but in the presence of single-stranded breaks. Using a DNA nick end-labeling assay we were able to show extensive single-strand DNA breaks. The lack of double-strand breaks in K562 and other cell types is unclear but it is reasonable to assume that either the activity of the endonuclease is inhibited or the enzyme is simply not present in these cells. Support from the former comes from a report by Wyllie who demonstrated that ras-transfected cells lost their endonuclease activity, suggesting that expression of the ras protein somehow interfered with the endonuclease activity. Alternatively, it may be that cell lines which have been in culture for a prolonged period of time have lost the endonuclease. It could be argued that cells which lost such an enzyme in culture would have a survival advantage over cells which expressed the enzyme, because these would be more prone to apoptosis.

AS-bcr-abl is capable of increasing the susceptibility of K562 cells to undergo apoptosis was formally possible that these are unconnected activities of the oligonucleotides. However, in experiments in which we transfected cells with a temperature-sensitive v-abl we were able to clearly demonstrate that the abl gene was a potent suppressor of apoptosis. While it is likely that the anti-apoptotic effects of ABL involve a signal transduction cascade, the ability of this protein to regulate expression of c-myc may also prove relevant, because c-Myc has been implicated in some forms of apoptosis.

The deregulated expression of genes such as bcr-abl and bcl-2 may contribute to an increase in tumor cell number by inhibition of apoptosis, and this can also contribute to resistance to the induction of cell death by therapeutic agents or treatments. This has important implications not only for our understanding of the mechanisms of tumor development, but also for the way we treat such diseases. Therapies aimed at inhibition of the activity of genes that promote apoptosis (eg, myc) may be less desirable than those aimed at the activity of anti-apoptotic genes such as bcr-abl. By re-opening the apoptotic pathway, cells can be rendered susceptible to induction of apoptosis through DNA damage.
or related means. An antisense approach to inhibit expression of apoptosis-suppressing gene in combination with standard chemotherapy may offer a new therapeutic strategy where suppression of apoptosis contributes to the development of the malignancy.

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