Acute- and Chronic-Phase Chronic Myelogenous Leukemia Colony-Forming Units Are Highly Sensitive to the Growth Inhibitory Effects of c-myb Antisense Oligodeoxynucleotides

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We have previously demonstrated that malignant hematopoietic colony-forming units (CFUs) may be purged from normal CFUs by exposure to c-myb antisense oligodeoxynucleotides (oligomers). This novel strategy appeared particularly promising for patients with chronic myelogenous leukemia (CML) in blast crisis, since in some cases complete elimination of bcr-abl-expressing cells was accomplished. We have examined 11 additional patients, including seven in chronic phase, in order to extend these initial observations. We sought in particular to determine if elimination of bcr-abl-expressing clones was a usual event. Exposure of CML cells to c-myb antisense oligomers resulted in inhibition of CFU-granulocyte, macrophage (CFU-GM)-derived colony formation in eight of 11 (73%) cases evaluated. Inhibition was antisense sequence-specific, dose-dependent, ranged between 58% and 93%, and was statistically significant (P < 0.03) in seven of the eight cases. In two cases, CFU-granulocyte, erythroid, monocyte, megakaryocyte (CFU-GEMM)-derived colony formation was also examined and found to be inhibited by the c-myb antisense oligomers in a sequence-specific manner. To determine whether CML CFU had been reduced or eliminated after exposure to the antisense oligomers, we examined cells in the residual colonies for bcr-abl mRNA expression using a reverse transcription–polymerase chain reaction detection technique (RT-PCR). Eight cases were evaluated and in each case where antisense myb inhibited growth, bcr-abl expression as detected by RT-PCR was either greatly decreased or nondetectable. No residual leukemic CFU were demonstrable on replating of treated cells. These results suggest that c-myb antisense oligomers substantially inhibit the growth and survival of CML CFU in both chronic and blast phase of disease. They may therefore prove useful for both ex vivo and in vivo treatment of CML.

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Chronic MYELOGENOUS leukemia (CML) is a hematopoietic stem cell disorder that is characterized by an elevated, left-shifted peripheral blood leukocyte count, a highly predictable clinical course, and a unique cytogenetic marker, the Philadelphia (Ph1) chromosome.1,2 The Ph1 chromosome is found in more than 90% of cases and results from a reciprocal translocation, t(9;22)(q34; q11), which relocates the c-abl protooncogene on chromosome 9 to variable locations within a 5.8-kb span of chromosome 22 known as the breakpoint cluster region (bcr).3,4 The resulting bcr-abl hybrid gene encodes an approximately 210-kD protein with tyrosine kinase activity that is thought to be important in the pathogenesis of this disease.4,5

The treatment of CML is problematic. During the chronic disease phase, elevated white blood cell counts and symptoms due to organ involvement can be controlled with alkylating agents, of which busulfan is the prototypical agent.6 Significant toxicities, including prolonged marrow aplasia and pulmonary damage posttransplant, have resulted in a decline in busulfan use. Hydroxyurea, an S-phase–specific inhibitor of DNA synthesis, is now widely used for primary therapy in these patients. Though less toxic, hydroxyurea requires frequent dose manipulation to effect smooth disease control. In the accelerated and blast phases of disease, intensive chemotherapy can temporarily control disease progression, but virtually all patients rapidly die of the disease once this stage is reached. For this reason, allogeneic bone marrow transplantation is indicated in all eligible chronic-phase patients with a suitable donor.7

Alternative forms of therapy could have a major impact on the management of these patients. One potential approach would be to disrupt the function of a gene(s) that is critical for cell growth and survival. Recent studies from this laboratory have suggested that antisense oligodeoxynucleotides targeted against the c-myb protooncogene might be ideal agents for this purpose.8,9 Studies on a small number of patients also suggested that CML in blast crisis might be a type of leukemia particularly responsive to this form of treatment, since colony formation was inhibited in most cases and bcr-abl expression was diminished in the few cases in which this was examined.10 The potential importance of these findings prompted us to examine additional CML patients in order to extend these initial observations, and to determine in particular if extinction of bcr-abl–expressing clones should be an expected event. The results of these studies demonstrate that c-myb antisense oligomers are highly effective agents for inhibiting the growth of CML progenitor cells, and perhaps for eliminating bcr-abl–expressing cells.

MATERIALS AND METHODS

Patients. Marrow or peripheral blood mononuclear cells (MNC) from nine patients were studied. The studies described were approved by the institutional human studies committees and all patients gave informed consent. Characteristics of the patients are...
The cultures were incubated at 37°C, by the addition of interleukin-3 (IL-3) (20 U/mL) and granulocyte–erythrocyte–monocyte–megakaryocyte (CFU-GEMM) growth was stimulated by anti-HPC1 monoclonal antibody (Becton-Dickinson, Mountainview, CA) and isolation of antibody-labeled cells with immunomagnetic beads (DynaL, Oslo, Norway).

Peripheral blood or bone marrow was also obtained from normal donors and prepared in a similar manner.

antsense oligomers, we examined cells in the residual colonies for bcr-abl mRNA expression using a RT-PCR (Fig 2).

To carry out these studies, RNA was extracted from cells cloned in methylcellulose cultures after exposure to the highest c-myb antisense oligomer dose. The RNA was then reverse-transcribed and resulting cDNA amplified as described above. For each case, mRNA was also extracted from a comparable number of cells derived from untreated control colonies using the same technique. These data provide an index of bcr-abl expression in control (untreated) cells postculture, and, as shown in Fig 2, bcr-abl expression remains high in this cell population. Eight cases were evaluated, and in each case bcr-abl expression as detected by RT-PCR correlated with colony growth in cell culture. In cases that were inhibited by exposure to c-myb antisense oligomers, bcr-abl expression was also greatly decreased or nondetectable with this highly sensitive technique (cases no. 2, 4, 5, 7, and 8). In patient 1, a weak signal was detectable after prolonged exposure of the x-ray film.

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Control Colonies (no oligomer)</th>
<th>myb Sense</th>
<th>myb Antisense</th>
<th>% Decrease (compared with control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>1,148 ± 164</td>
<td>1,180 ± 183</td>
<td>194 ± 8</td>
<td>84%</td>
</tr>
<tr>
<td>10</td>
<td>22 ± 13</td>
<td>21 ± 5</td>
<td>1 ± 1.4</td>
<td>95%</td>
</tr>
</tbody>
</table>

Cells were harvested, exposed to oligomers, and cultured as described in the text. Concentration of oligomer used was 100 μg/mL at time 0 and 50 μg/mL approximately 18 hours later. Colonies were counted on day 15 through an inverted microscope. Values expressed as mean ± SD (patient 8 cultured in quadruplicate, patient 10 in duplicate).

CFUs from patients 3 and 6 were not inhibited by exposure to c-myb antisense oligomers, and cells derived from these cultures manifested easily detectable bcr-abl expression.

These results suggested that bcr-abl-expressing CFU might be substantially or entirely eliminated from a population of blood or marrow mononuclear cells by exposure to the antisense oligodeoxynucleotides. To explore this possibility further, replating experiments were performed on samples from patient 8 (previously frozen) and patient 10 (fresh). We theorized that if CFU belonging to the malignant clone were present at the end of the original 12-day culture period, but not detectable because they were not expressing bcr-abl, they might again express the message upon regrowth in fresh cultures. Accordingly, cells from these patients were exposed to oligomers and then plated into methylcellulose cultures formulated to favor growth of either CFU-GM or CFU-GEMM. As was found with the original specimens, untreated control cells and cells exposed to sense oligomers had RT-PCR detectable bcr-abl transcripts. Those exposed to the c-myb antisense oligomers had none. The duplicate plates of these cultures (control, sense, and antisense) were then solubilized and all cells contained therein were washed, dispersed, and then replated into fresh methylcellulose cultures without reexposing the cells to oligomers. After 14 days, CFU-GM and CFU-GEMM colony cells were again probed for bcr-abl expression. Control and sense-treated cells had RT-PCR-detectable mRNA, but none was found in the antisense-treated colonies (Fig 3A). Cultured cells were also probed for β-actin and, as shown in Fig 3B, β-actin expression was detectable and of approximately equal intensity under all culture conditions. These results suggest that elimination of bcr-abl-expressing cells and CFU was highly efficient and perhaps permanent.

DISCUSSION

The c-myb gene encodes a protein, Myb, which is known to have several distinct functional regions. A phosphorylation-dependent DNA binding domain is found near the amino terminus and is encoded by three imperfect repeats near the 5' end of the message. The midportion of the protein contains a transcriptional activator region, and the carboxy terminus contains a regulatory domain that is deleted from its homologous oncogene, v-myb. The protein is primarily localizable to the nucleus. While structure/function aspects of the protein are reasonably well understood, the biological functions of the Myb protein itself are...
C-MYB ANTISENSE DNA INHIBITS CML CELLS

Fig 2. Detection of bcr-abl mRNA transcripts in colony cells derived from patients no. 1 to 8 CFU. Patient CFU were enriched from marrow or peripheral blood and exposed to oligomers as described in the text and then plated in methycellulose. Total RNA was extracted from colony cells arising in the cultures, reverse-transcribed with a 3' primer specific for the second c-abl exon, and the resulting cDNA amplified by PCR with a primer pair specific for the bcr-abl junction. Amplified DNA was detected by Southern blotting with a 40-nt c-abl probe as detailed in the Methods. In each panel (1 to 8), the left lane (A) represents bcr-abl expression in the untreated control cells, the middle lane (B), sense oligomer-treated cells, and the right lane (C), antisense oligomer-treated cells. Bcr-abl expression is undetectable in patients 1, 2, 4, 5, 7, and 8.

not yet fully defined. However, it seems clear that it is a transcriptionally active nuclear binding protein which controls passage through the G1/S phase of the cell cycle. In addition, a number of laboratories have suggested that the c-myb gene plays a critical role in regulating hematopoietic cell development. This hypothesis has been directly confirmed using antisense oligodeoxynucleotides to abrogate c-myb gene function and more recently using another gene disruption technique, homologous recombination.

The critical importance of c-myb gene function in both normal and malignant human hematopoiesis make it a likely target for therapeutically motivated disruption strategies. However, to be successful, leukemic CFU would have to be more susceptible to inhibition of c-myb gene function than normal CFU. We have found this to be true for a number of cell lines, as well as for primary leukemic CFU. Interestingly, during the course of our previous investigation, we found that cells obtained from CML patients in blast crisis appeared to be particularly sensitive to Myb deprivation. In this regard, we found that clonogenic units from four of five blast crisis cases studied were significantly inhibited by exposure to c-myb antisense oligomers and that inhibition was accompanied by complete loss of bcr-abl-expressing cells.

In the present work we have extended these initial findings. We have now studied an additional 11 patients: four in blast crisis and seven in the chronic disease phase. In agreement with our initial report, CFU-GM colony formation was inhibited in all four blast crisis cases and was accompanied by apparent elimination of bcr-abl-expressing cells. We have also found that CFU from chronic-phase patients are also inhibited by the c-myb antisense oligomers (four of seven cases; 57%) and that such inhibition is also accompanied by elimination of bcr-abl-expressing cells. As might be expected, this phenomenon was not observed in nonresponding cases. Apparent elimination of bcr-abl-expressing cells in chronic-phase disease is particularly important, since it suggests the possibility of a "curative" treatment option other than allogeneic bone marrow transplantation for patients in this phase of their disease. A true cure would of course depend on complete elimination of
bcr-abl—expressing cells. While there is no completely certain way to determine that this in fact has happened, we attempted to address this issue by replating disaggregated colony cells that grew in antisense-treated dishes. In theory, dormant CML cells might well proliferate again in the fresh cultures, but this was not found to occur under conditions favorable to the growth of either CFU-GM or the even less mature CFU-GEMM progenitor (Fig 3).

The mechanism whereby c-myb inhibits leukemic CFU remains incompletely defined. Several tenable hypotheses have been advanced to explain this finding, including the possibility that a greater proportion of CML CFU are in an active cycle, and therefore more dependent on c-myb function than either normal or acute leukemia CFU.11,16 The substantial inhibition of CML CFU-GEMM cloning efficiency by the antisense oligomers (Table 2) is consistent with this hypothesis. A more intriguing possibility is suggested by the marked decrease in bcr-abl expression noted after exposure to the myb antisense oligodeoxynucleotides. Bcr-abl has long been postulated to play an essential role in stimulating the p210 bcr abl oncogene product of the Philadelphia chromosome. Proc Natl Acad Sci USA 84:6558, 1987

References

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