Toward a Leukemia Treatment Strategy Based on the Probability of Stem Cell Death: An Essay in Honor of Dr. Emil J Freireich

E. A. McCulloch
The Ontario Cancer Institute, Toronto, MSG 2M9 Canada

Abstract

Dr. Emil J Freireich is a pioneer in the rational treatment of cancer in general and leukemia in particular. This essay in honor suggests that the cell kill concept of chemotherapy of acute myeloblastic leukemia be extended to include two additional ideas. The first concept is that leukemic blasts, like normal hemopoietic cells, are organized in hierarchies, headed by stem cells. In both normal and leukemic hemopoiesis, killing stem cells will destroy the system; furthermore, both normal and leukemic cells respond to regulators. It follows that acute myelogenous leukemia should be considered as a dependent neoplasm. The second concept is that cell/drug interaction should be considered as two phases. The first, or proximal phase, consists of the events that lead up to injury; the second, or distal phase, comprises the responses of the cell that contribute to either progression to apoptosis or recovery. Distal responses are described briefly. Regulated drug sensitivity is presented as an example of how distal responses might be used to improve treatment.

Introduction

Because leukemia is usually a disseminated neoplasm when it is first recognized clinically, systemic therapy is the dominant treatment modality. Dr. Emil J Freireich’s contribution to the development of the chemotherapy of leukemia is major both in theory and practice. The basis of his work is an understanding of cellular mechanisms, tested in clinical research. Each new advance is then based on observations made on patients. Freireich has always stressed that quantitation is the key to useful clinical observations. He measures not only the responses of patient populations but also the struggle between tumor and host in each individual. Thus, he teaches the need for research in supportive care if radical therapy is to be successful in reducing tumor burden. Freireich has earned a place in the small group of clinical scientists who achieved cures in even the most aggressive diseases, such as AML of adults.

The central hypothesis is that chemotherapy is successful because cytotoxic drugs reduce the number of cells with proliferative capacity sufficiently to prevent regrowth and to allow recovery of normal blood-forming cells. This hypothesis provided priority for drug discovery, pharmacology, and pharmacokinetics as the basis of increased cell kill. Research was directed at finding agents with selective toxicity for leukemic cells and for ways to maintain patients during periods of marrow aplasia. Studies of mechanisms of cell kill disclosed many cellular targets, including DNA itself and the cytoplasmic organelles required for mitosis. This permitted the classification of drugs on the basis of their cytotoxic mechanism and the combination of agents with different ways of killing cells. It was soon apparent that combination chemotherapy was superior to single agents used alone. As treatment became more radical, the need for support increased; now patients are not only protected from infection, supported by platelets, but may often be rescued by transplantation of marrow or peripheral blood stem cells.

The central cell kill hypothesis has required some modification as it was developed in clinical research:

(a) Consideration of the numbers of leukemic cells and the drug dose-response curves made it unlikely that doses could be achieved that would be curative if it were necessary to kill every cell. Nonetheless, clinical remissions were obtained regularly. It was then proposed that the lineage models developed for normal hemopoiesis could be applied to leukemic populations. The production of large numbers of normal cells is based on the proliferative activity of a small number of stem cells, able to renew themselves and to give rise to daughters committed to specific lineages. The extensive cytoreduction achieved in treatment with tolerable drug doses could be explained if a small number of leukemic stem cells maintained a large population of terminally dividing or inert cells with leukemic morphology. Then, to obtain remission, only enough drug is needed to kill leukemic stem cells.

(b) The mechanisms by which drugs injured cells were found to explain only part of the outcome of drug-cell interactions. Cells respond to injury by a number of mechanisms that influence the probabilities that the injured cells enter programmed cell death (apoptosis). These latter mechanisms may be considered “distal” in contrast to the “proximal” events that lead from drug/cell contact to initial injury.

This tribute to Dr. Freireich will concentrate on the cellular hierarchy of AML and distal events in AML blasts treated with chemotherapeutic drugs. The two are linked experimentally because the culture techniques that serve to define leukemic lineages are easily adapted for observations of the responses of cells to injury. Cell culture methods developed as assays for leukemic cells can be combined with great advantage with flow

---


2 To whom requests for reprints should be addressed, at Division of Cellular and Molecular Biology, Ontario Cancer Institute, 610 University Avenue, Toronto, MSG 2M9 Canada.

3 The abbreviations used are: AML, acute myeloblastic leukemia; ATRA, all-trans retinoic acid; ROI, reactive oxygen intermediate; GSH, glutathione; GM-CSF, granulocyte-macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; PARP, poly(ADP-ribose) polymerase; ERK, extracellular signal-regulated kinase; SAPK,

stress-activated protein kinase; RB, retinoblastoma; ara-C, 1-β-D-arabinofuranosylcytosine; IL, interleukin.
The regulation of the balance between "birth" and "death" is brought about by regulatory mechanisms that operate on normal hemopoietic cells and their descendants with lineage commitment (6). It is striking that the proliferative capacity in stem cells; it may be considered as the opposite of stem cell self-renewal, the process by which stem cells retain their capacity to maintain the system. Self-renewal may be considered as "birth," its opposite, commitment, as "death," because it is associated with reduced growth potential. The regulation of the balance between "birth" and "death" is fundamental; many accept that this regulation is lax, because clonal expansion from normal stem cells yields populations with very heterogeneous cellular composition (14–17).

If the hierarchical concept of leukemic hemopoiesis is correct, the development of lineages requires that blast stem cells lose proliferative capacity; there must be a "death" probability for leukemic stem cells. Clinical evidence is available showing that the heterogeneity characteristic of AML clones in patients is regenerated when the clones are reduced by therapy and then examined again after re-expansion. However, the pattern in each clone does not "breed-true"; rather clones re-assort themselves across the spectrum of the patient-to-patient variation (18). This is to be expected, if stochastic regulation is operative during expansion of clones from leukemic stem cells, just as it is when normal stem cells give rise to hemopoietic clones.

The role of the "death" probability in regulation is to limit growth. "Death" may play its part by allowing cells to differentiate. It is now appreciated that physiological "death" is also important in regulation; it has its own program that is now widely recognized as apoptosis (19, 20). When it was appreciated that the "death" process in tumor cells after chemotherapy was often apoptosis, events occurring after injury were perceived as potential avenues for therapeutic innovation (21–24). The validity of the idea became immediately apparent with the discovery that ATRA as a single agent could produce remissions in acute promyelocytic leukemia and that the mechanism of remission was increased cell death, recognized morphologically as differentiation (25, 26).

Distal Events

Distal events fall into two classes: (a) injury leads to cellular responses that initiate the processes that lead to apoptosis. Prominent among these are disturbances in cellular redux; and (b) a series of proteins participate in actions that may either increase of diminish the probability of death.

Compromised Intracellular Redux. Often, cell injury, particularly damage to DNA, initiates the generation of highly toxic ROS. Flow cytometry is an effective way of measuring ROS and other molecules that are important in maintaining the cellular redox state. A major contributor is GSH, which protects other molecules that are important in maintaining the cellular redox state. A major contributor is GSH, which protects other molecules that are important in maintaining the cellular redox state. A major contributor is GSH, which protects other molecules that are important in maintaining the cellular redox state.

In this balanced, reduced state, the cell is protected against oxidative damage. A mitochondrial permeability transition may occur. The permeability transition is an active process that involves the opening of pore complexes in the mitochondrial inner membrane, resulting in the loss of the mitochondrial membrane potential and the cessation of mitochondrial ATP generation. Because respiration is feedback inhibited by cellular ATP levels, the permeability transition causes increased electron transport through the respiratory chain (27). This in turn may result in the increased generation of reactive oxygen intermediates, because these are by-products of mitochondrial respiration. When membranes are damaged by ROS (lipid peroxidation), calcium is released from stores, and calcium-dependent enzymes are activated, including the endonucleases required for apoptosis (28–30). In the compromised cellular redux state of injured cells, a number of proteins mediate several different, although interconnected, distal events. A brief description of some of the relevant proteins follows.

The bcl-2 Family. The protein products of the bcl-2 family may either promote mitochondrial membrane transition and apoptosis.
tosis (31, 32) or protect cells against reactive oxygen damage (33). bcl-2, the first of the family to be identified, is homologous to the Caenorhabditis elegans survival gene ced-9 (34) and protects against apoptosis; another family member with similar activity is bcl-xl (35, 36). In normal hemopoiesis, bcl-x but not bcl-2 is expressed in the most primitive precursors (37). A third protective protein is bag-1, which has minimal homology to the bcl-2 family but protects against apoptosis (38). Other weakly protective family members are ininducible: MCL1 responds to phorbol esters; it was isolated from a leukemic cell line and may play a role in differentiation (39). Bfl-1, the human equivalent of the murine GM-CSF-responsive gene Al, was isolated from fetal liver and is expressed predominantly in normal marrow (38). The most prominent member of the opposite, death-promoting class of family members is Bax, discovered by its capacity to dimerize with bcl-2 (40, 41). Bcl-xxs is an alternatively spliced form of Bcl-xl that has death-promoting activity (35, 36). Bad forms heterodimers with bcl-2 and both forms of bcl-xl, but not with bax, although, like bax, it promotes apoptosis (42). Bak is widely distributed, and, like bax, promotes apoptosis (43–45).

A major characteristic of the bcl-2 family is the capacity to form homo- or heterodimers. Both bcl-2 and bcl-xl form heterodimers with bax; it is proposed that the level of bax homodimers determines the extent of its death-promoting ability (41). However, it is of note that bax transgenic mice are viable, suggesting that high levels of bax alone do not produce death (46).

Enzymes. A variety of enzymatic mechanisms are used in repair of damaged DNA (47–50). DNA repair may also require PARP; this DNA-binding nuclear enzyme converts NAD to nicotine, catalyzing the ADP-ribosylaton of nuclear polypeptides. A role for PARP in distal mechanisms is postulated because it is enzymatically cleaved early after exposure of AML cells to DNA, with resulting reduction in catalytic and DNA-binding activity (51). The enzyme responsible for PARP cleavage is YAMA/CPP32b, the mammalian homologue of the C. elegans gene sed-3. This is a member of the interleukin-2b converting enzyme/sed family of serine proteases that have regulatory functions in apoptosis (52, 53).

Other enzymes are components of signaling pathways that allow cells to respond to mitogenic stimuli or injury. The mitogen-activated protein kinase family consists of ERKs; these are serine-threonine kinases that ultimately act in the nucleus on the AP-1 transcription factor (jun/fos dimers; Ref. 54). The usual observed effects are either increased cell growth or differentiation. Analogous and parallel to the ERKs is a cascade of mitogen-activated protein kinases that is activated by many cellular stresses, including chemotherapy (55, 56). This SAPK is the final stage in a cascade that leads to cell death by apoptosis. Thus, cells possess at least two signaling systems, using similar mechanisms, but leading to either survival (ERK) or death (SAPK; Ref. 57).

Negatively Acting Oncogenes. Negatively acting oncogenes (58) play important roles in distal events after injury. Both RB oncoproteins and p53 act in the nucleus to influence the cell cycle. RB binds transcription factors that are required for S phase; posttranscriptional modification of the protein is required to release RB binding and permit the onset of DNA synthesis (59, 60). p53 is a homotetrameric transcription factor; it binds specific sequences in DNA, acting to block cells in G1, by enhancing expression cyclin-dependent kinase inhibitors, such as p21. Depending on the conditions, these mechanisms may mediate either G1 arrest or apoptosis (61–65). The alterations in the cell cycle produced by these oncoproteins may permit time for DNA repair or set the stage for apoptosis.

Distal Mechanisms and the Chemotherapy of AML. Distal mechanisms have been described as discrete protein-based systems; we do not know enough about distal mechanisms to depict their interactions accurately. Yet they all coexist within damaged cells. It seems probable that they form an information network that contributes to the sensitivity or resistance of AML blast cells to chemotherapy.

ara-C remains the principle effective drug in the treatment of AML. ara-C damage challenges the redox system in AML blasts. Using flow cytometry to measure GSH, ROIs, [Ca2+]i, and the mitochondrial permeability transition, a regular sequence of events was observed. The cells responded to injury with an increase in GSH; this increase could not be maintained, and loss of mitochondrial membrane potential was seen, associated with the generation of large amounts of ROIs. Then calcium homeostasis failed, leading to lipid peroxidation and cell death (66, 67). Bcl-2 protects against oxidative damage by maintaining cells in a reducing state with adequate GSH (66) and may also inhibit the mitochondrial permeability transformation (32).

Up-regulation of p53 is usual in cells damaged by irradiation and chemotherapy (68, 69). Cells may emerge from arrest as survivors or may enter apoptosis; each of these fates may be p53 dependant. The amount of the protein present may alter the dose response to either irradiation or drugs (68, 69). Interactions have been reported between p53 and bcl-2 in some systems (70). PARP may participate in response to chemotherapy because it is enzymatically cleaved by YAMA/CPP32b early after exposure of AML cells to DNA, with resulting reduction in catalytic and DNA-binding activity (51). The SAPK signaling pathway may have an integrative function within the distal network.

These few examples show the complexity and potential of the distal mechanisms. The network is protein based, with many examples of regulatory posttranslational modifications. Bray (71) has suggested that the protein-based information is fundamentally similar to the language used by computers. Nonetheless, the outcome of drug/cell interaction is binary: apoptosis or recovery. It may be that distal mechanisms are not prescriptive but rather set the probabilities of life or death for the injured cell.

Exploiting Distal Mechanisms: Regulated Drug Sensitivity

The regulatory networks that are activated after injury provide attractive targets for novel therapeutic interventions. Regulated drug sensitivity provides an example; the phenomenon emerged from studies showing that the sensitivity of ara-C blasts in culture could be modified by regulators. If growth factor-responsive AML blasts were exposed to ara-C in G-CSF, they were more sensitive than if GM-CSF or IL-3 were in the cultures. The effect was seen regardless of which factor was the more powerful mitogen for the cells under test; that is, even if G-CSF gave the major proliferative response, the addition of GM-CSF or IL-3 was protective. Ligands for the steroid intracellular receptor were also able to regulate drug sensitive. Hydrocortisone was seen to be protective when given before drug.
ATRA usually increased sensitivity when administered after ara-C (72). These observations were potentially useful clinically because they might provide a way of overcoming drug resistance. It was necessary to gain some understanding of the mechanism of regulated drug sensitivity before it could be used intelligently in protocol development.

Extensive searches for an influence of regulators on proximal events were unsuccessful (73). The first positive finding was the observation that bcl-2 mRNA was down-regulated in AML blasts after ATRA; growth factors and hydrocortisone were without effect. Nonetheless, other potential connections among ara-C, ATRA, and bcl-2 emerged. ara-C was not then widely considered to act by altering cellular redox; rather, it is incorporated into DNA and acts to terminate chain elongation (74, 75). However, bcl-2 may act to protect cells against changes in cellular redox. A link was made when the radical scavenger N-acetyl-cysteine was found to protect cells against subsequent treatment with ara-C, providing indirect evidence for participation of ROIs in ara-C damage. Flow cytometric measurements of GSH and ROIs readily provided direct evidence of disturbed cellular redox after ara-C (66, 76). A provisional model began to emerge. bcl-2 was seen as a protection against oxidative damage resulting from ara-C incorporation in DNA; ATRA sensitized cells because it reduced bcl-2 function.

Because bcl-2 was central to the proposal, we looked for molecular evidence of its role. bcl-2 cDNA was transfected into two established AML cell lines, OCI/AML-5 and OCI/AML-2. In both cases, the cells were made significantly less ara-C sensitive than controls (77). When transfectants and controls were compared using flow cytometry to measure ROIs and GSH, it was evident that the cells in transfected bcl-2 were more effective in maintaining the reduced state, although ROIs were produced (66).

Nuclear run-on experiments were used to show that ATRA was down-regulating the translation of bcl-2 message; the nuclear run-on data, however, did not provide a complete explanation (76). Experiments using Northern blots provided no evidence for participation of growth factors or hydrocortisone in bcl-2-mediated regulation. If, as suggested, distal mechanisms are a protein-based information network, posttranslational changes may be important functionally. bcl-2 protein was labeled metabolically with [35S]methionine; after labeling, the cells were returned to cold medium, and the decay of bcl-2 was observed. The protein in the control cells was stable, with a half-life of about 20 h. When cells were treated with ATRA before labeling, the protein half-life was reduced by about 50%. In contrast, after hydrocortisone, bcl-2 was significantly more stable. These changes are consistent with the capacity of ATRA to sensitize cells to ara-C and the protective effect of hydrocortisone (78). Regulation by growth factors remained unexplained; neither bcl-2 expression at the Northern level nor the stability of bcl-2 protein was altered when cells grown in G-CSF were compared to others with GM-CSF added to the medium.

In the metabolic labeling experiments, an increase in the amount of bcl-2 protein was noted in cells in G-CSF + GM-CSF compared to cells in G-CSF alone. A different experimental system was required to support a posttranscriptional mechanism for the growth factor effect. The cell line OCI/AML-1 had desirable properties for our purpose. bcl-2 expression is low in these cells, detectable in RNA only by PCR, and the protein gives a weak protein band in Western blots. OCI/AML-1 cells readily show regulated sensitivity to ara-C by growth factors. They are very sensitive to the drug in G-CSF but much less sensitive in G-CSF + GM-CSF (79). Preliminary experiments showed that bcl-2 protein was increased when GM-CSF was added to AML-1 cells growing in G-CSF; the increase was more marked when the cells were cultured in G-CSF with the addition of the GM-CSF/IL-3 fusion protein pIXY (80).

We used kinetic experiments to ask whether the increased amount of bcl-2 protein in cultures with G-CSF + pIXY compared to G-CSF alone could be explained by a change in synthesis. Western blots were used to measure the amount of protein; metabolic labeling provided a measure of synthesis. OCI/AML-1 cells were maintained in G-CSF for 24 h; then pIXY was added to one set of cultures, the other remaining as controls. After a second 24-h period, the cells were washed and recultured in G-CSF alone. At intervals during the 72 h of the experiment, cells were harvested for Western blots. Other cultures were pulsed with [35S]methionine for 60 min and immunoprecipitated, and incorporation of label into bcl-2 protein was determined. The data showed that bcl-2 synthesis had more than doubled by 12 h after the addition of pIXY and fell almost as rapidly when pIXY was withdrawn. The changes in bcl-2 protein were almost as large; the protein level peaked after 24 h and fell more slowly than synthesis. We concluded that growth factors acted after transcription to alter the rate of synthesis of bcl-2 protein. The increased protein synthesis seen in cultures with G-CSF + pIXY compared to G-CSF alone is consistent with the protection afforded against ara-C toxicity by IL-3 and GM-CSF (81).

Taken together, these experiments show that distal mechanisms can be exploited to alter the sensitivity of AML blasts to chemotherapy.

Conclusion

It is proposed that the standard cell-kill model of leukemia chemotherapy be extended to include two additional concepts:

(a) The blast population in AML may be considered as a cellular hierarchy, in which the majority the blasts are maintained by the reproductive activity of a small number of stem cells. This concept has importance for both basic and clinical research. At the basic level, it is required that measurements of clonogenic populations be included within experimental designs to insure that observations on bulk populations represent events in stem cells. At the level of protocol design, the proposal should include a model statement explaining how the proposed treatment would affect the cellular hierarchy.

(b) The second proposed modification is the acceptance of distal mechanisms as distinct entities, with profound influence on the outcome of treatment. At a fundamental level, these mechanisms provide an example of how proteins can form information systems as important as those encoded in nucleic acid. At a clinical level, distal mechanisms provide novel treatment targets; these, of course, will be useful only to the extent that they are understood.

Dr. Freireich will continue to insist that new ideas be incorporated in clinical thinking about leukemia and other malignancies.


Toward a leukemia treatment strategy based on the probability of stem cell death: an essay in honor of Dr. Emil J Freireich.

E A McCulloch


Updated version  Access the most recent version of this article at:  
http://clincancerres.aacrjournals.org/content/3/12/2676

**E-mail alerts**  Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.