Journal of Cellular Biochemistry 60:33–38 (1996)

Regulation of Apoptosis by Oncogenes

Douglas R. Green, Anne McGahon, and Seamus J. Martin

Division of Cellular Immunology, La Jolla Institute for Allergy and Immunology, La Jolla, California 92037

Cancer is usually envisioned as a disease of cellular proliferation. That is, loss of controls on cell proliferation is viewed as being the central, underlying cause of cancer. Recently, however, this idea has undergone a change. With the realization that cell number is a result of the competing effects of cell proliferation and cell death, the latter has taken on a larger significance. Furthermore, like cell proliferation, cell death can be subject to molecular control. Thus, many recent studies suggest that cancer can result from loss of regulation of either cell proliferation or cell death, and here we argue that it is often a combination of the two.

Apoptosis, or active cell death, is thought to be of particular importance in this regard. The significance of apoptosis in the process of oncogenesis is underscored by at least three important observations. The first is that most physical and chemical agents with antitumor activity act to induce apoptosis, as do genes that potentiate their effects [1–3]. The second is that the frequency of apoptotic cells in a tumor correlates very well with outcome. That is, tumors with low apoptotic indices tend to be more agressive than are those displaying higher incidence of apoptotic cell death, and thus apoptosis is almost certainly an important factor in the rate of tumor growth [4]. By contrast, the frequency of necrotic cell death is inversely correlated with outcome (which might, at first glance, be counter-intuitive). The third observation is that a number of oncogenes and anti-oncogenes have been found to regulate the process of apoptosis [2,3,5]. Together, these observations make a strong case for studying apoptosis as a route to understanding oncogenesis.

Thus, the induction of apoptosis should be an important goal of cancer therapy. However, anti-apoptotic activities exist within cells and these work against this goal. Therefore, methods are needed to bypass such effects and thus facilitate the many pro-apoptotic agents that are either currently in use or in development.

Here, we outline our studies that demonstrated that oncogenes can be either pro- or anti-apoptotic, and that the latter can block apoptosis induced by the former. This provides a basis for synergy among oncogene products in transformation. When anti-apoptotic activities are particularly active, the result can be resistance to a variety of forms of therapy. We focus on the anti-apoptotic activity of the Abl kinase and examine the case of apoptosis resistance in a cell line from a chronic myelogenous leukemia (CML) patient. Furthermore, the nature of the resistance to apoptosis induced by Abl is examined. Finally, the prospects for inducing apoptosis as a general approach to cancer therapy is discussed within the context of a theoretical and practical consideration of whether any element of a central "execution" pathway of apoptotic death is likely to be unique to this process.

ONCOGENES AND APOPTOSIS

The realization that cell death, and especially apoptosis, played a critical role in the process of oncogenesis came about in part through a number of observations on the effects of oncogenes (and anti-oncogenes) on cell function. Thus, Bcl-2 [6,7] and Abl [8] promote transformation and interfere with apoptosis, while p53 acts to interfere with oncogenesis and promotes some forms of apoptosis [9–11]. Such observations are certainly consistent with the expected relationships between these activities.

Some oncogenic activities act in a surprisingly different way, however. Myc and Ras are two types of proto-oncogenes that function in normal cells to promote proliferation, and constitutive activity of these gene products is frequently found in a wide variety of tumors. Yet, perhaps paradoxically, both Myc [5,12–14] and Ras [12,15] have been found to promote apoptosis under some conditions.

Received December 29, 1994; accepted October 2, 1995.

Address reprint requests to Dr. Douglas R. Green, Division of Cellular Immunology, La Jolla Institute for Allergy and Immunology, 11149 N. Torrey Pines Road, La Jolla, CA 92037.

Upon reflection, however, the ability of Myc and Ras to promote apoptosis can be seen to be logical. In multicellular organisms without rigid cell walls (such as humans) the unregulated proliferation of even a single cell is potentially lethal to the individual. Any organism with more than a small number of cells should therefore face an almost inevitable cancerous death early in life—a situation that is clearly untenable. Either the organism must greatly restrict its cell proliferation and cell number, as is the case in nematodes, or it is essential that proliferation be very tightly controlled. It appears that animals have solved this problem (probably very early in evolution) by having cell proliferation tightly linked to the mechanism of cell death. In this scenario, a cell that activates its proliferation machinery, in the absence of any external influences, will either die or divide with approximately equal probability.

How could this be generally true, since we know that tissues do grow and that cells do proliferate (and, in fact, that cancers do occur albeit with a relatively low frequency)? The answer lies in the nature of the "external influences." One important physiological influence is the presence of growth factors, which have been demonstrated to block apoptosis under some conditions [16,17]. In the presence of growth factors, Myc [5,13,14] and Ras [15] promote cell proliferation; in the absence of such factors they promote cell death. Thus, growth factors produced by other cells can determine whether or not death or mitosis will occur in a normal cell expressing Myc or Ras. It follows, then, that a cell that constitutively expresses active Myc or Ras (e.g., due to a mutation) will die without the requisite growth factors, and this explains why a single mutation is unlikely to lead to unregulated growth. However, if such a cell were to acquire a second mutation leading to constitutive growth factor production or constitutive signalling from a mutant growth factor receptor the two mutations could synergize to produce transformation. Of course, oncogenesis as a consequence of such mutations has been extensively described. The novel idea here is that such mutations contribute to oncogenesis via the production of constitutive anti-apoptotic signals.

Another important influence on the "decision" of a cell to divide or die is that of other endogenous oncogene signals. As mentioned above, Bcl-2 is a protein with anti-apoptotic activity, and it has been shown to synergize with Myc in transforming cells [18]. We [19] and others [20] have shown that Bcl-2 blocks apoptotic cell death induced by Myc, which provides a mechanism for the co-transformation phenomenon.

Another oncogenic signal that synergizes with that of Myc is provided by the Abl kinase, and this has similarly been shown to have anti-apoptotic activities. The nature of this activity is considered in more detail in the following sections.

PROMOTION OF APOPTOSIS BY INTERRUPTION OF ANTI-APOPTOTIC SIGNALS

CML is associated with a chromosomal translocation that generates a chimeric protein composed of parts of two normal cellular proteins: Bcr and c-Abl. The resulting Bcr-Abl protein has constitutive Abl kinase activity and is oncogenic [21]. This oncogenic activity depends on the function of c-Myc in the cell [22].

We observed that K562, a Bcr-Abl$^+$ cell line derived from a CML patient, is remarkably resistant to the induction of apoptosis by a variety of different stimuli [23]. To determine whether this was a consequence of the expression of Bcr-Abl in these cells, we took advantage of a strategy employing antisense oligodeoxynucleotides to down-regulate Bcr-Abl expression. These short stretches of synthetic single-stranded DNA can directly enter cells, where they bind to mRNA sequences for which they are specific and inhibit expression of the protein. Since antisense oligonucleotides corresponding to Bcr-Abl had previously been shown specifically to inhibit expression of this protein [24], we adopted this approach.

We found (Fig. 1) that antisense oligonucleotides corresponding to Bcr-Abl did, indeed, inhibit expression of Bcr-Abl in K562 cells [23]. By itself this inhibition did not directly affect the viability of these cells, even over several days. However, when we then exposed the cells to agents capable of inducing apoptosis (to which they are normally resistant), the pretreatment with the antisense oligonucleotides was found to have rendered them fully susceptible to the induction of apoptosis. In experiments in which the Abl kinase was reintroduced into these cells, in a form (v-Abl) that would not be affected by the antisense treatment, we found that we could restore resistance to the induction of apoptosis in these cells. Thus, the effects of the Bcr-Abl

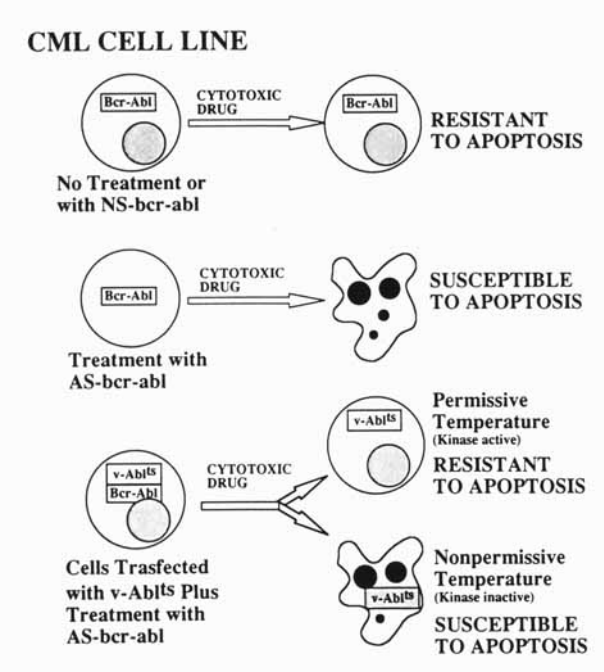

Fig. 1. The Abl kinase regulates susceptibility to apoptosis in a CML cell line. Schematic representation of results published elsewhere McGahon et al. [23]. A CML cell line, K562, was treated with antisense oligonucleotides corresponding to Bcr-Abl (AS-bcr-abl) or with a control oligonucleotide with the same base composition but different order (NS-bcr-abl). Subsequently, cells were treated with cytotoxic drugs (e.g., etoposide) to assess susceptibility to apoptosis. Pretreatment with AS-bcr-abl rendered the cells susceptible. In addition, we examined susceptibility to apoptosis in K562 cells stably transfected with a temperature sensitive v-Abl (v-Abl^{ts}). Antisense treatment with AS-bcr-abl rendered these cells susceptible to induction of apoptosis *only* at the nonpermissive temperature for v-Abl^{ts}. At permissive temperature, the activity of the v-Abl kinase signalled the cells to resist apoptosis. Thus, the susceptibility vs. resistance to apoptosis in this CML cell line is a function of Abl kinase activity.

antisense were specifically mediated by their action on Bcr-Abl expression.

Although such antisense intervention might have potential application in cases such as CML, the therapeutic use of antisense oligonucleotides may continue to be difficult for a variety of reasons. It would perhaps be a better strategy to identify and employ a drug capable of specifically interfering with the anti-apoptotic mechanism induced by the Abl kinase or other anti-apoptotic gene products. To do this, however, it will be necessary to explore in more detail how Abl acts to inhibit apoptosis.

ANTI-APOPTOTIC ACTIVITY OF THE ABL KINASE

To further examine the anti-apoptotic activity of the Abl kinase, we have taken advantage of temperature sensitive mutants of v-Abl (v-Abl^{ts}), which have kinase activity only at the permissive temperature (32°C) and not at 37–39°C. We introduced these into a promyelocytic tumor cell line, HL-60, which does not normally express activated Abl. Thus, unlike K562, HL-60 does not depend on the function of Abl for its transformed properties. Introduction of the v-Abl^{ts} into HL-60 did not alter its rate of proliferation or its morphologic appearance at either temperature.

We then examined the ability of v-Abl to interfere with apoptosis in HL-60 cells. Unlike K562, HL-60 cells undergo apoptosis after exposure to any of a wide variety of agents [1,23]. However, when Abl kinase activity was present, these cells showed a significantly increased resistance to the induction of apoptosis by many agents. This represents a formal demonstration that v-Abl kinase can produce a general anti-apoptotic state, reminiscent of that produced by Bcl-2.

Since Bcl-2 produces a similar state, it was possible that Abl exerted its anti-apoptotic effects through induction of this protein. We found, however, that protein synthesis following activation of the Abl kinase was not necessary for its anti-apoptotic effects, and thus it does not operate via induction of expression of anti-apoptotic genes such as Bcl-2. It was possible, however, that it worked to enhance the activity of Bcl-2. This could potentially occur in at least two ways—Abl could act directly on Bcl-2 or a necessary partner protein to improve its efficacy, or it could act to interfere with an inhibitor of Bcl-2. We found that in normal and in the transfected HL-60 cells, both Bcl-2 and its inhibitor, Bax, are constitutively expressed. Our experiments, however, indicate that neither of these is likely to be involved in the anti-apoptotic effects of the Abl kinase [25]. Our evidence comes from two different sets of observations.

We were fortunate to identify a clone of HL-60 cells expressing v-Abl^{ts} in which no Bcl-2 could be detected at either the protein or RNA level at any temperature, even when a sensitive RNase protection assay was employed. Despite this, v-Abl was capable of inducing an anti-apoptotic state in the absence of Bcl-2 protein.

Our demonstration of an apparent absence of a role for Bax in the effects of the Abl kinase relied on an even more interesting observation. In HL-60 cells expressing v-Abl^{ts}, Bax protein levels were observed to decrease dramatically after culture for two days at permissive tempera-

ture. This loss of Bax would explain the resistance to apoptosis in these cells, if the loss of Bax allows increased function of other Bcl-2 family members (e.g., Bcl-X, which is expressed in these cells as well). Thus, if Bax is generally required for susceptibility to apoptosis in HL-60 cells, its downregulation by v-Abl kinase would produce a state of resistance. However, we also observed that when the v-Abl kinase was inactivated at nonpermissive temperature, the cells rapidly regained their susceptibility to apoptosis. If this were dependent on Bax, then we would have expected a reappearance of the Bax protein upon culture at the higher temperature. Therefore, we cultured HL-60.v-Abl^{ts} cells at permissive temperature (so that Bax disappeared), and then shifted the cells to nonpermissive temperature in the presence of cycloheximide, a protein synthesis inhibitor. While Bax did not reappear under these conditions, the cells nevertheless rapidly reacquired their susceptibility to apoptosis.

These studies indicate that the anti-apoptotic state induced by Abl is independent of that induced by Bcl-2 and the mechanisms of this effect should therefore be studied. Since disruption of Abl activity restores susceptibility to apoptosis, we expect that agents that can interfere with this anti-apoptotic pathway will have significant value in promoting the effects of therapy in cells expressing this kinase. Furthermore, since the kinase activity of Abl is similar to that produced by a wide variety of growth factor receptors, we expect that such agents would have a more general application to a range of tumors that resist apoptosis. This idea (as well as any proposal to promote apoptosis through disruption of Bcl-2) depends on the likelihood that most tumors are capable of undergoing apoptosis once anti-apoptotic activities are disrupted. Clearly, an alternative is that tumors develop defects in the apoptotic machinery itself, and if so, then no attempt to promote apoptosis through interference with anti-apoptotic effects will work. We will end this discussion with a consideration of why this concern is very unlikely to be a problem.

PROSPECTS FOR PROMOTING APOPTOSIS VIA DISRUPTION OF ANTI-APOPTOTIC ACTIVITIES WITHIN TUMOR CELLS

It is commonly believed that the process of apoptosis proceeds via a complex biochemical process with effects in both the cytoplasm and the nucleus of the cell, consistent with many observations. We can envision the process as being the result of any of a number of different triggering pathways, which might lead to only one (or a few) central "execution" pathways responsible for the ultimate death of the cell. If so, it is certainly possible that one or a few mutations in a cell would eliminate the function of the execution pathway, and thus render the cell resistant to apoptosis. In such a case, however, no amount of intervention would induce apoptosis, unless a way could be found to reintroduce the defective part of the pathway or find a way to circumvent the defect. This is a much

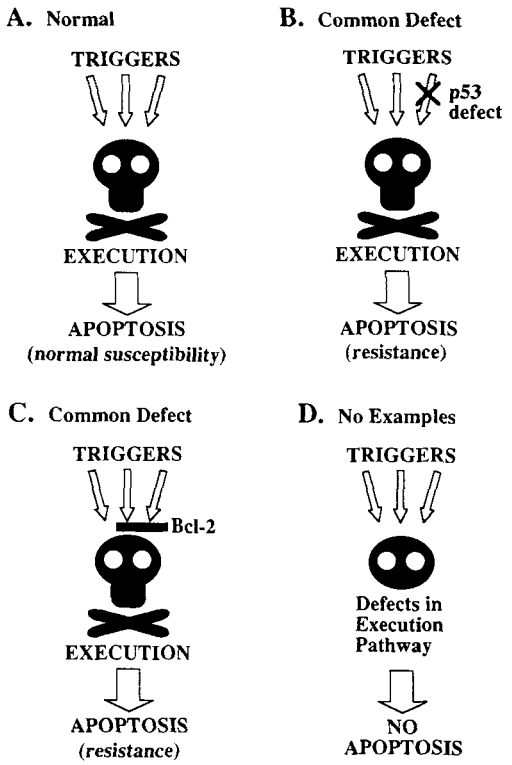

Fig. 2. Effects of defects in apoptosis pathways. The process of apoptosis can be envisioned as a consequence of multiple triggering pathways that lead to induction of a central execution pathway, resulting in cell death (**A**). Cancer cells often display defects in at least one triggering pathway, mediated by p53 (**B**), which results in the cells being resistant to some, but not all, forms of apoptosis. Similarly, Bcl-2 (**C**) can act to block the induction of the execution pathway, thus rendering the cells relatively resistant to several forms of apoptosis. (However, once cells expressing Bcl-2 undergo apoptosis, the process proceeds identically to that of normal cells, and thus we suggest that Bcl-2 acts to inhibit induction of the execution pathway versus action directly on the pathway itself. A similar argument has been made by Gerard Evan [personal communication].) Defects in the execution pathway itself (**D**) should result in cells incapable of undergoing any form of apoptosis. Such cells have not been described.

harder problem than that of removing anti-apoptotic effects that are "overlaid" onto an otherwise fully functional apoptotic pathway, as discussed above. If this reasoning is correct, then our prospects for promoting apoptosis in cancer cells might prove more daunting than we anticipate.

It is not necessarily the case, however, that the execution pathway is composed of molecules that function uniquely in this process. Consider the possibility, instead, that the elements of the execution pathway all have dual roles in both cell death and either cell proliferation or the integrity of the cell itself. In other words, loss of any activity in the central pathway of apoptotic death should cripple the cell.

We base this suggestion on the simple fact that no cell line has yet been described that is incapable of undergoing apoptosis under any condition (Fig. 2). Even cells with potent anti-apoptotic influences, such as Bcl-2 or Abl, that are profoundly resistant to apoptosis, will undergo this form of cell death when challenged with a sufficiently strong stimulus (and when they do, the process is identical to that observed in cells without such resistance). All cells, so far examined, can undergo apoptosis under *some* circumstance.

This "absence of proof" argument may appear rather weak, since it might simply be that we have not examined enough cell lines. However, we suggest that the loss of a central execution pathway, if it did not cripple the cell in any other way, should be such a potent promoter of oncogenesis that such tumor lines should be fairly common. The fact that they are not suggests that such mutants do not grow. This would be the case if all of the elements of the execution pathway had additional essential roles in maintaining the cell cycle or cell integrity (Fig. 3). Without any one of these elements cells might be completely resistant to apoptosis, but they also would be incapable of growth. Examples might include topoisomerases, DNA polymerases, cytoskeletal elements, and/or a variety of other centrally important elements of cell function. The search to find a family of unique "death proteins" that function only in the execution pathway of apoptotic death might be in vain.

But isn't it also possible that there are simply several execution pathways that function in redundant ways, such that a defect in one would not elimate apoptosis? Although this is certainly possible, there are some problems with this idea. First, we might expect that even with a requirement for multiple "hits" (i.e., mutations in several parallel pathways) cases of cells lacking the capacity for apoptosis would emerge, since such a state would be so strongly favored (if it did not affect cell proliferation or integrity). A related problem with the redundancy argument (i.e., the idea that there are multiple parallel pathways that all "execute" apoptosis) is that we should expect members of the population to carry germline defects in some of these pathways, since in this scenario such a defect would not have a phenotype. Such defects would accumulate in the population until, again, "apoptosis-less" states would appear. Complete redundancy, by its very nature, should be difficult to maintain at a population level.

Thus, the absence of cells lacking apoptosis is sufficiently conspicuous to warrant an assertion that such cells will not be found for fundamental reasons (e.g., as proposed in Fig. 3). Perhaps the

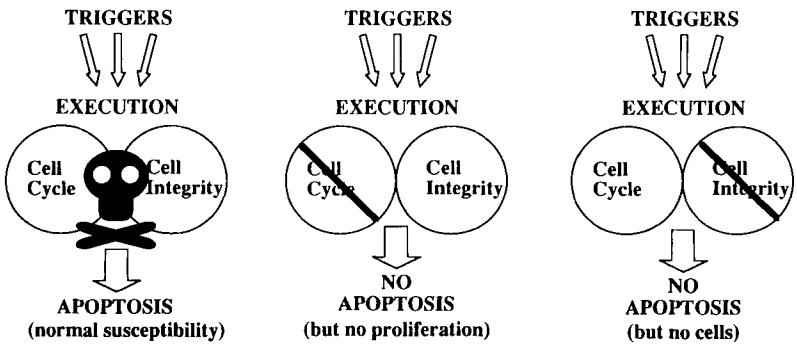

Fig. 3. A possible reason for the apparent absence of cells incapable of undergoing apoptosis. We suggest that all the molecules involved in the execution pathway for apoptosis also have critical roles in either the cell cycle or in maintenance of cell integrity (**left**). Defects in any of these molecules would be expected to produce a state of complete resistance to apoptosis, but cells with this phenotype will not grow, either due to associated defects in cell cycle (**center**) or loss of cellular integrity (**right**).

best support for this line of reasoning comes from an examination of a protein that functions as an important trigger for apoptosis induced by DNA damage and probably other stimuli. This protein, p53, is not an element of the execution pathway per se, since cells lacking p53 can still undergo apoptosis, although some apoptotic stimuli do not function. Nevertheless, mutations in p53 are extremely common in tumors, and germline defects in this protein produce a state of extreme susceptibility to a variety of cancers. Thus, defects in a protein that is even *close* to the execution pathway are readily found and the effects are dramatic. The fact that we have not yet found such a defect in a central pathway (even if there are several, redundant pathways) is important (Fig. 2).

Thus, the prospect of promoting apoptosis through interfering with anti-apoptotic effects is a good one, and research into this problem is being actively pursued. The development of agents that block such anti-apoptotic effects, combined with agents that induce apoptosis (preferably, in a p53-independent manner) holds great promise for a new generation of cancer therapeutics.

ACKNOWLEDGMENTS

The research described in this paper was supported by grant CB-82 from the American Cancer Society. S.J.M. is a Wellcome Trust Traveling Fellow (041080).

REFERENCES

1. Lennon SV, Martin SJ, Cotter TG (1991): Dose-dependent induction of apoptosis in human tumour cell lines by widely diverging stimuli. Cell Prolif 24:203–214.
2. Martin SJ, Green DR (1994): Apoptosis as a goal of cancer therapy. Curr Opinion Oncol 6:616–621.
3. Martin SJ, Green DR (1995): Apoptosis and cancer: The failure of controls on cell death and cell survival. Crit Rev Oncol Hematol 18:137–153.
4. Arends MJ, McGregor AH, Wyllie AH (1994): Apoptosis is inversely related to necrosis and determines net growth in tumors bearing constitutively expressed myc, ras, and HPV oncogenes. J Pathol 144:1045–1057.
5. Bissonnette RP, Shi Y, Mahboubi A, Glynn JM, Green DR (1994): C-myc and apoptosis. In Tomei LD, Cope FO (eds): Apoptosis. II. The Molecular Basis of Cell Death. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp 327–356.
6. Korsmeyer SJ (1992): Bcl-2: A repressor of lymphocyte death. Immunol Today 13:285–288.
7. Reed JC (1994): Bcl-2 and the regulation of programmed cell death. J Cell Biol 124:1–6.
8. McGahon AJ, Cotter TG, Green DR (1994): The abl oncogene family and apoptosis. Cell Death Diff 1:77–83.
9. Yonish-Rouach E, Resnitzky D, Lotem J, Sachs L, Kimchi A, Oren M (1991): Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. Nature 352:345–347.
10. Shaw P, Bovey R, Tardy S, Sahli R, Sordat B, Costa J (1992): Induction of apoptosis by wild-type p53 in a human colon tumor-derived cell line. Proc Natl Acad Sci USA 89:4495–4499.
11. Lowe SW, Schmitt EM, Smith SW, Osborne BA, Jacks T (1993): p53 is required for radiation-induced apoptosis in mouse thymocytes. Nature 362:847–849.
12. Wyllie AH, Rose KA, Morris RG, Steel CM, Forster E, Spandidos DA (1987): Rodent fibroblast tumors expressing human myc and ras genes: Growth, metastasis and endogenous oncogene expression. Br J Cancer 56:251–259.
13. Askew DS, Ashmun RA, Simmons BC, Cleveland JL (1991): Constitutive c-myc expression in an IL-3-dependent myeloid cell line suppresses cell cycle arrest and accelerates apoptosis. Oncogene 6:1915–1922.
14. Evan GI, Wyllie AH, Gilbert CS, Littlewood TD, Land H, Brooks M, Waters CM, Penn LZ, Hancock DC (1992): Induction of apoptosis in fibroblasts by c-myc protein. Cell 69:119–128.
15. Tanaka N, Ishihara M, Kitagawa M, Harada H, Kimura T, Matsuyama T, Lamphier MS, Aizawa S, Mak TW, Taniguchi T (1994): Cellular commitment to oncogene-induced transformation or apoptosis is dependent on the transcription factor IRF-1. Cell 77:829–839.
16. Raff MC (1992): Social controls on cell survival and cell death. Nature 356:397–400.
17. Williams GT, Smith CA, Spooncer E, Dexter TM, Taylor DR (1990): Haemopoietic colony stimulating factors promote cell survival by suppressing apoptosis. Nature 343:76–79.
18. Vaux DL, Cory S, Adams JM (1988): Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. Nature 335:440–442.
19. Bissonnette RP, Echeverri F, Mahboubi A, Green DR (1992): Apoptotic cell death induced by c-myc is inhibited by bcl-2. Nature 359:552–554.
20. Fanidi A, Harrington EA, Evan GI (1992): Cooperative interaction between c-myc and bcl-2 proto-oncogenes. Nature 359:554–556.
21. McWhirter JR, Wang JYJ (1991): Activation of tyrosine kinase and microfilament-binding functions of c-*abl* by *bcr* sequences in *bcr/abl* fusion proteins. Mol Cell Biol 11:1553–1565.
22. Sawyers CL, Callahan W, Witte ON (1992): Dominant negative myc blocks transformation by abl oncogenes. Cell 70:901–910.
23. McGahon A, Bissonnette R, Schmitt M, Cotter KM, Green DR, Cotter TG (1994): BCR-ABL maintains resistance of chronic myelogenous leukaemia cells to apoptotic cell death. Blood 83:1179–1187.
24. Skorski T, Szczylik C, Malaguanera L, Calabretta B (1991): Gene-targeted specific inhibition of Chronic Myeloid Leukemia cell growth by BCR-ABL antisense oligonucleotides. Folia Hist Cytobiol 29:85–90.
25. McGahon A, Martin S, Bissonnette RP, Green DR (1994): Inhibition of apoptosis by the Abl kinase is independent of induction of Bcl-2. (submitted).