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Retroviral Transfer of Antisense Sequences Results in Reduction of C-Abl and Induction of Apoptosis in Hemopoietic Cells

Key Words
Retroviral gene transfer
Apoptosis
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Growth factor receptors
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Abstract
The c-abl proto-oncogene is ubiquitously expressed during mammalian development. Activated forms of c-Abl proteins are oncogenic and have been shown to suppress apoptosis. The biological role of normal c-Abl protein is unknown. In this study, we have introduced c-abl antisense sequences into various hemopoietic cells by retroviral gene transfer. Introduction and expression of the antisense sequence effectively reduced the amount of c-Abl protein in a number of transduced hemopoietic cells, that consequently underwent apoptosis. When factor-dependent cell lines were examined, we observed that the addition of sufficient amounts of growth factors could suppress apoptosis in myeloid but not in lymphoid lines. The ability of myeloid cells to be rescued by growth factors correlated with upregulation of mRNA level of IL-3 receptor subunits. Our data suggest that c-Abl provides an anti-apoptotic signal during mammalian cell growth, and that myeloid and lymphoid cells are different in their resistance to apoptosis.

Introduction

Apoptosis is the process of regulated cell death under physiological conditions and is characterized by DNA fragmentation, cell shrinkage and disorganized internucleosomal structure. A number of genes are involved in the control of this process. For example, c-myc, p53 and bax are known to induce apoptosis, whereas ras and bcl-2 have been shown to inhibit the process [58]. In the case of c-myc, it can also induce proliferation. This dual function of c-myc appears to be influenced by extracellular microenvironment, represented by the presence or absence of growth factors that maintain cell survival [20]. In the case of p53, it can also induce growth arrest [32, 33]. These various functions have been shown to be regulated by exogenous growth factors as well [4, 61]. The influence of some of these growth factors affecting cell survival appears in turn to regulate the level of bcl-2, which inhibits apoptosis [37, 39, 45].

Oncogenic Abl proteins have been shown to inhibit apoptosis, and the role of their normal counterpart, c-Abl, in this process is unknown. There are several isoforms of c-Abl, and they are located in the cytoplasm as well as in the nucleus [52; for review, see 55]. Biochemical analyses indicate that c-abl plays an important role in cell cycle. The molecule has several functional domains, which include a tyrosine kinase domain, a DNA-binding domain and a nuclear localization signal [55]. C-Abl has been shown to bind to a motif in the hepatitis B enhancer, EP [18], is differentially phosphorylated during the cell-cycle

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by cdk/cdc2 [29, 30], and its tyrosine kinase activity is tightly regulated. Welch and Wang [56] have shown the binding of c-Abl to retinoblastoma (Rb) protein during the G1 phase of the cell cycle, at which time c-Abl is inactive. At the G1/S phase of the cell cycle, when the Rb protein is hyperphosphorylated, c-Abl becomes activated [56]. Overexpression of c-Abl results in a predominance of the protein in the nucleus but some are also present in the cytoplasm [52]. In NIH/3T3 fibroblasts overproducing c-Abl, Sawyers et al. [48] reported a reversible growth arrest. Whether the effects observed in cells overproducing c-Abl are the same as in cells containing a normal level of c-Abl remains unknown.

Additional information has been derived from studies of the two oncogenic forms of Abl: Bcr/Abl and Gag/Abl. Bcr/Abl is generated as a result of the reciprocal translocation between chromosomes 9 and 22, and is found in almost all patients with chronic myelogenous leukemia (CML) and in some patients with acute lymphocytic leukemia (ALL) [41]. It is believed to be one of the initial events for the disease pathogenesis. Retroviral transfer of the bcr/abl oncogene into murine bone marrow cells results in the development in recipient mice of multiple types of hematologic diseases including one resembling CML in humans [16, 19, 28]. It has the ability to convert factor-dependent cells to become factor-independent [15]. When expressed in primary hemopoietic progenitor cells, Bcr/Abl can induce colony formation with or without a supply of exogenous growth factor, albeit in the latter condition the efficiency of colony formation is reduced [21]. In addition, Bcr/Abl has been shown to replace one of the critical functions of growth factors, which is to suppress apoptosis in a number of cell types [34, 36].

Another abl oncogene, the gag/abl or v-abl, is derived from a natural recombination event between the retroviral genome of Moloney murine leukemia virus (MoMuLV) and c-abl proto-oncogene [22, 54]. Despite very significant differences that exist between bcr/abl and gag/abl, such as the absence of the SH3 domain and the presence of a myristylation site in gag/abl [10, 55], there are a lot of biological functional similarities. For example, retroviral transfer of the gag/abl oncogene into hemopoietic stem cells produces a myeloproliferative disease similar to that observed in bcr/abl-induced mice [8, 25]. Expression of gag/abl can convert factor-dependent cells to become factor-independent [12, 46], can induce primary hemopoietic progenitor cells to differentiate without exogenous growth factors [9, 53] and can also inhibit apoptosis [6]. Recently, Renshaw et al. [44] have shown that Bcr/Abl can transform NIH/3T3 fibroblasts; this property was not observed in earlier reports [14]. In contrast, it is well established that Gag/Abl can transform 3T3 fibroblasts [23, 43, 49]. Thus, it appears that the biological effect of Bcr/Abl is very similar to Gag/Abl, except the latter is biologically more potent. The effect of c-Abl protein is not yet known, and its elucidation will be critical for understanding whether different signal transduction pathways may be involved.

We have previously constructed a retrovirus vector, pαA, which carries the anti-abl antisense sequence, and have shown that expression of this antisense sequence could effectively reduce the amount of c-Abl at both the RNA and protein levels [17]. NIH/3T3 fibroblasts cells expressing low level of c-Abl was deregulated in that they entered sooner into S phase of the cell cycle [17]. In addition, we also noticed that a small population of such cells had a DNA content of less than 2n, suggesting that they underwent apoptosis. Since the majority of the transduced cells were dividing, we reasoned that while reduction of c-Abl might have an immediate effect on inducing apoptosis, an alternative mechanism might exist so that the c-Abl requirement could be bypassed and the cells rescued from programmed cell death. To investigate this possibility, we have shown in this study that reduction of c-Abl resulted in the induction of apoptosis in both lymphoid and myeloid cells. However, apoptosis in myeloid but not lymphoid cells could be inhibited by hemopoietic growth factors. This data are consistent with the hypothesis that a generalized alternative pathway to bypass apoptosis by growth factors to their corresponding cell types may exist.

Materials and Methods

Cell Lines and Cell Cultures

A number of hemopoietic cell lines were used in this study. 70Z/3 is a pre-B lymphoid cell line, EL4 is a T-cell line, CTLL-2 is an IL-2 dependent T-cell line, WEHI-3B is an IL-3 producing monomyelocytic leukemic cell line, and FDC-P1 is a growth factor-dependent myeloid cell line. All cell lines were maintained with R10, which is RPMI medium supplemented with 10% fetal bovine serum (FBS), with additional supplements in some cases. For 70Z/3 cells, 50 μM 2-mercaptoethanol was added to R10. For CTLL-2, the medium was supplemented with 50 μM 2-mercaptoethanol and an optimal concentration of 60 U/ml of recombinant IL-2 (R&D systems) except otherwise stated. For FDC-P1, a predetermined optimal amount of WEHI-3B cell conditioned medium (usually 10%) containing IL-3 was also present in the medium.

To perform the colony assays in methylcellulose culture, between 500 and 1,000 virus-infected cells were plated into methylcellulose culture, with or without a source of growth factors. The number of colonies in each ml of culture in a 30-mm dish was enumerated in 7 days or more.

Virus Infection and Co-Cultivation

For retroviral gene transfer, 1 million 70Z/3 or WEHI-3B cells were infected with N2 or αA virus by co-cultivation with near-confluent N2- or αA-virus-producing cells in 100-mm tissue culture dishes containing 10 ml D10 medium (DMEM supplemented with 10% FCS) and 5 μg/ml Polybrene. The time for co-cultivation was 48 h, except those for DNA fragmentation analysis, where a time course of 24-hour infection was carried out.

In some cases, viral supernatant infection was performed. The preparation of the N2 and αA viruses was described previously [17, 59]. Briefly, viral supernatants were harvested from near-confluent virus-producing cells until the cultures became fully confluent. Virus supernatant was harvested every 12–16 h following fresh medium exchange. For infection, one million cells in 1 ml of viral supernatant were incubated for 24 h in a single well of a 24-well plate, with 5 μg/ml Pb, with or without conditioned medium. Heat-inactivation of viral supernatant was performed in some experiments and was done by incubating the viral supernatant at 65 °C for 30 min.

DNA Fragmentation Studies

To perform DNA fragmentation analysis, we extracted genomic DNA following the phenol/chloroform method [47]. One microgram of each sample was loaded into each well of a 2% agarose gel, electrophoresed, and fragment DNA ladder in the gel were stained with ethidium bromide as described [47].

Southern and Northern Analysis on 70Z/3 and WEHI-3B Cells

The extraction of DNA and RNA was performed as described [47]. Twenty micrograms of genomic DNA digested with Kpn I or 30 μg total RNA were loaded into each well of a gel. The samples were electrophoresed, transferred to a nylon membrane filter, and hybridized with a $^{[32]}$P-labeled 2.2-kb BgI II fragment of the v-abl-specific DNA probe.

Immunoprecipitation and Western Blot Analysis on 70Z and WEHI-3B Cells

WEHI-3B cells or 70Z/3 cells were infected with N2 or αA. Forty-eight hours after the onset of infection, the cells were harvested and 2×10^7 viable WEHI-3B cells, 1×10^7 viable 70Z/3 cells and 5×10^6 virus-producing cells were immunoprecipated with 8E9 anti-abl and anti-actin monoclonal antibodies [62]. The immunoprecipitates were resolved on SDS-PAGE gel, transferred to immobilon-P membrane and blotted with 8E9 antibody. The 140-kD c-Abl protein band was visualized on X-ray film by the photoblot chemiluminescent system (Gibco-BRL). As a control, some protein gels were stained for 30 min with 0.05% Coomassie blue in 50% ethanol/10% acetic acid, followed by washing for 4 h in 5% ethanol/7% acetic acid.

Construction and Characterization of αD Retrovirus Vector

A neo^r gene including TK promoter from pMV-7 plasmid [31] was cloned into the *Cla*I site of αA plasmid DNA vector. This vector also contains the anti-abl antisense sequence, which covers the 0.7 kb BgI II/Xho I v-abl fragment in the reverse orientation. The antisense sequence is primarily against the DNA-binding domain located in the c-terminal region of c-abl. GP/E virus packaging cells were transfected by calcium-phosphate DNA co-precipitation [47]. After infection with the αD or N2 virus, FDC-P1 cells (polyclonal) were selected with 1 mg/ml G418 for 1–2 weeks. G418-resistant cells (pooled) were subsequently expanded and RNA were then extracted from these cells, electrophoresed, transferred to filters, and hybridized with one of the probes described in the legend to figure 6. For the αD virus, the titer was 10^4 infectious viral particles/ml, and for the control N2 virus, 10^7/ml. The G probe used to detect viral RNAs was the 0.6-kb BstE II-*Hin*cII fragment of A-MuLV DNA, coding the gag region of A-MuLV. N2 virus also contains the gag region [2, 60]. C probe was the 0.7-kb *Bgl*II fragment of A-MuLV DNA. αC probe is the 2.2-kb fragment of A-MuLV.

Growth Response Studies on FDC-P1 Cells

For growth response studies, N2- or αD-transduced FDC-P1 cells were usually carried in 10% WEHI-3 CM. Prior to the experiments, the cells were washed 3 times with R10 (RPMI+10% FBS). Then, 2×10^5/ml of the washed cells were plated into 24-well plates at various concentrations of WEHI-3CM and the concentration of cells in each well was determined 48 h later. Cell viability and the presence of apoptotic cells were also recorded. Identical conditions were used for cells harvested for RNA analysis (fig. 6b) but at a larger scale. In studies with FDC-P1 cells overexpressing c-Abl, cells were plated onto 96-well plate at a concentration of 2×10^5/ml, or 2×10^4 cells per well, 5 wells for each point. The cell concentration was evaluated 48 h later using a 3-(4,5-dimethylthiazol-2-yl)-2,5-dephenyltetrazolium bromide (MTT) proliferation assay as we previously described [62].

Analysis of IL-3R Expression Level

To detect the IL-3R$\beta_{IL\text{-}3}$, a beta subunit of interleukin-3 receptor, we used the AIC2A cDNA probe from Dr. A Miyajima at DNAX [27], to detect IL-3Rβc, we used the AIC2B cDNA probe [24], to detect IL-3Rα, SUT-1 probe was used [26]. The densitometry analysis was used to determine the relative amount of $\beta_{IL\text{-}3}$ and γ-actin mRNA by means of a phospho-image analyzer. Northern blot analysis was performed as described [47]. Thirty micrograms of total RNA was loaded into each lane. The γ-actin probe was used in our past experiments [17]. In order to evaluate the potential cross-hybridization of ACI2A and ACI2B, we performed Southern blot analysis: FDC-P1 genomic DNA was digested with *Eco*R1 and transferred to nylon filter. Hybridization was then performed at 70 °C. Under these conditions, the density of ACI2B band (15 kb) [24] was limited to about 5% of the ACI2A band (11 kb) density, when filter was hybridized with the ACI2A cDNA. Identical conditions were then used for Northern blot analysis.

Results

Reduction of c-abl Induces Apoptosis in 70Z/3 Lymphoid but Not WEHI-3B Myeloid Cells

We analyzed the biological consequences after retroviral gene transfer of the anti-abl antisense (αA virus) or a control neo gene (N2 virus) into immortalized 70Z/3 lymphoid cells and WEHI-3B myeloid cells (fig. 1a). Transduced cells were plated into semi-solid methylcellulose cultures, and their plating efficiency before and after infection enumerated [17, 59]. No significant changes were observed for WEHI-3B cells, whether or not they were co-cultivated with N2- or αA-virus producing cells

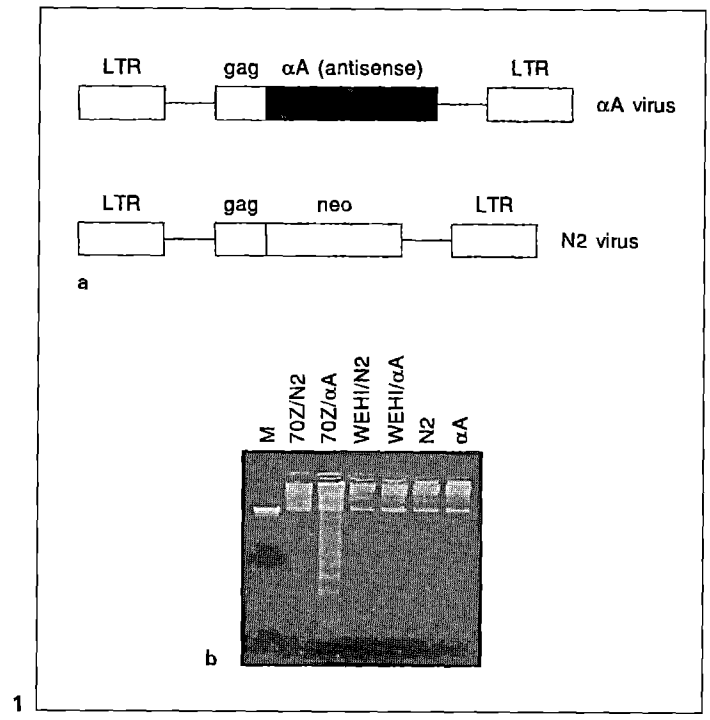

Fig. 1. Infection of 70Z/3 and WEHI-3B cells with αA or the control N2 virus. **a** αA and N2 vectors, as described in the references [17, 59]. **b** DNA fragmentation. The exponentially growing WEHI-3B and 70Z/3 cells were infected with the corresponding virus for 24 h. Genomic DNA were then extracted and analyzed in 2% agarose gel. M = MW markers; N2 = N2-producing cells; αA = αA-producing cells; 70Z/N2 = N2-infected 70Z/3 cells; 70Z/αA = αA-infected 70Z/3 cells; WEHI/N2 = N2-infected WEHI-3B cells; WEHI/αA = αA-infected WEHI-3B cells.

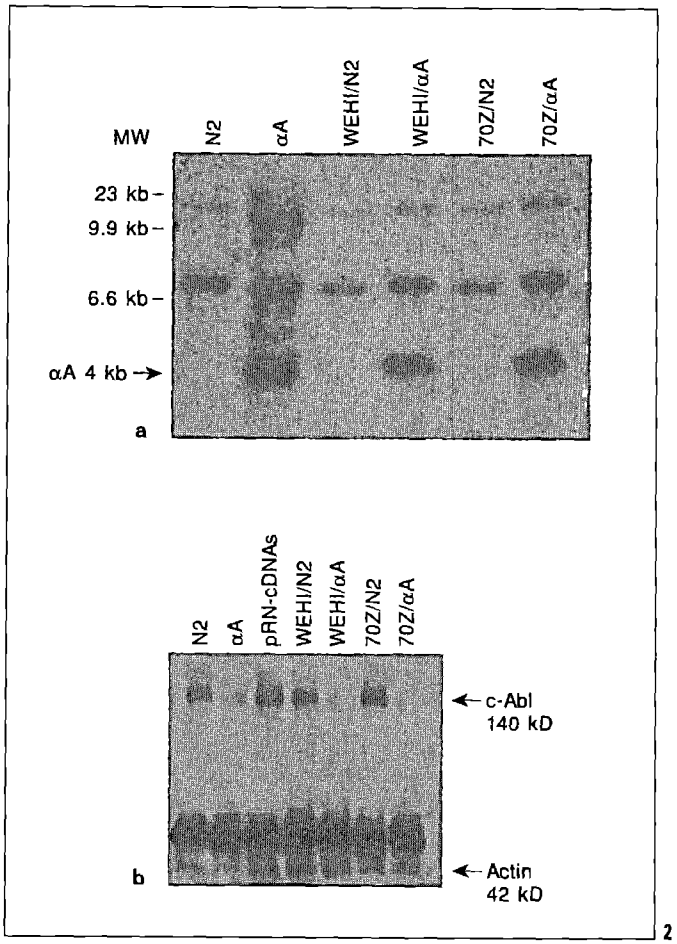

Fig. 2. Retroviral gene transfer and c-abl RNA expression. **a** Southern blot analysis of DNA from infected cells. Genomic DNA extracted from cells infected with N2 and αA viruses for 48 h was digested with Kpn I enzyme, whose recognition site is represented once in each LTR αA provirus. Thus, when the filter was hybridized with a c-abl-specific probe, a 4.0-kb integrated αA proviral DNA should appear if present in the host genome. 70Z/N2-70Z/3 cells infected with N2, 70Z/αA-70Z/3 infected with αA, WEHI/N2-WEHI infected with N2, WEHI/αA-WEHI infected with αA, N2-N2-producing cells, αA-αA-producing cells. **b** Western blot analyses of c-abl protein in N2- and αA-infected cells. Cells were infected under conditions used for Southern and Northern blot analysis, c-abl was immunoprecipitated, transferred to immobilon-P membrane and blotted with 8E9 anti-c-Abl and anti-actin antibodies as described in 'Materials and Methods'. pRN-cDNAs-GP/E cells transduced with a retroviral sublibrary DNA containing about 1,000 different retroviruses were included as controls in the assay.

(table 1). By contrast, a 9-fold reduction was observed when 70Z/3 cells were co-cultivated with αA-virus producing cells compared to N2-virus-producing cells (table 1). There were two likely possibilities: either the 70Z/αA cells were growth arrested or they underwent cell death.

Next, cell viability was examined with time during co-cultivation. While there was no significant difference between N2 and αA producer cells after co-cultivation with WEHI-3B cells, there was a progressive drop in viability with time for αA virus-producing cells co-cultivated with 70Z/3 cells compared to those of N2 (table 2). These data suggested induction of apoptosis in 70Z/αA cells presumably expressing lower amount of c-Abl. To confirm apoptosis, genomic DNA from cells under different conditions were extracted and fractionated in a 2% agarose gel. Indeed, DNA from 70Z/αA, but not 70Z/N2, WEHI/αA or /N2 cells, had fragmentation characteristic of cells undergoing apoptosis [57] (fig. 1b). Morphologically, a large proportion of these cells contained fragmented nuclei – nuclear condensation and disorganization into discrete masses (table 3). These types of cells were not

observed in 70Z/N2, parental 70Z/3, WEHI/αA or /N2 cells under the same conditions.

To confirm that these biological effects were due to successful retroviral transfer and expression of the antisense sequence, we performed Southern and Northen blot analysis. Southern blot analysis was performed on Kpn 1-digested genomic DNA. Using an abl-specific probe, a 4-kb αA proviral DNA fragment was observed in αA-virus producing cells, in WEHI-3B transduced with αA (WEHI/αA) and in 70Z/αA cells (fig. 2a). This band was not present in untransduced cells or N2 virus-producing cells. The two endogenous c-abl sequences, one with a size of 7 kb and the other 18 kb, were evident in all DNA samples. Additional bands were observed in DNA of αA virus producer cells. This was due to the presence of multiple copies of pαA plasmid DNA introduced into the genome of the packaging cells by illegitimate DNA recombination after calcium phosphate transfection [17].

From the signals of the transferred gene based on Southern blot analysis in figure 2, the efficiency of gene transfer is approximately the same for both WEHI-3 and 70Z/3 cells. Because the target cells were infected by co-cultivation with virus-producing cells, it is possible that many virus-producing cells were harvested, giving the false positive signals. To rule out this possibility, we repeated the experiment except this time we used EL4 cells, which are known to produce a replication competent MoMuLV virus and therefore could not be superinfected by αD or N2 virus efficiently. In this case, should we still observe the presence of the antisense DNA in genomic DNA of cells from co-cultivation, the signal would be coming from DNA of virus-producing cells. Our results showed that DNA from EL4 cells co-cultivated with αD or N2 virus-producing cells did not give an antisense signal or neo signal. Furthermore, we transduced FDC-P1 cells in these experiments with αD, which is a retrovirus vector containing both a neo gene and anti-abl antisense sequence against the nuclear localization signal and the DNA-binding domain of c-Abl. The transduced cells were all G418-resistant; nonadherent cells were passaged for 8 weeks or more in the presence of growth factors (see below, fig. 6). Any virus-producing cells which were adherent would have been removed before the analysis. Also, the cells that were maintained in these cultures were factor-dependent hemopoietic cells and not virus-producing fibroblasts because when WEHI-3B CM was removed from the medium, no cells survived after 3 days. Taken together, these data therefore lend strong support that the signal observed in WEHI-3B and 70Z/3 cells co-cultivation was derived from those of transduced cells and not those of virus-producing cells.

Table 1. Methylcellulose colony formation of N2- and αA-infected cells[1]

Cell line	Number of colonies after infection with	
	N2 virus	αA virus
WEHI-3B	499±4	461±4
70Z/3	201±33	23±1

[1] 70Z/3 and WEHI-3B cells infected with αA or N2 virus for 48 h were plated into methylcellulose culture at a density of 500 cells/dish. The number of colonies per dish was recorded 4 days later.

Table 2. Cell viability during infection

Cell line	Virus	Time		
		12 h	24 h	48 h
WEHI-3B	N2	95±2	100	100
	αA	97±2	99±1	100
70Z/3	N2	83±5	83±2	89±4
	αA	76±5	66±2	48±2

Numbers represent the percentage of viable WEHI-3B or 70Z/3 cells during co-cultivation with virus-producing cells. Time is the number of hours after the onset of infection.

Table 3. Percentage of cells with fragmented nuclei 48 h after infection

Cell line	% cells with fragmented nuclei in culture infected with	
	N2 virus	αA virus
WEHI-3B	0	0
70Z/3	12±7	76±4

48 h after infection, the cells were harvested, deposited onto microscope slides and stained with Wright-Giemsa. The numbers of cells with fragmented nuclei as described by Williams et al. [57] were counted. The data are presented as the percentage of these cells with fragmented nuclei over the total numbers of cells evaluated. Triplicate counts were performed for each point.

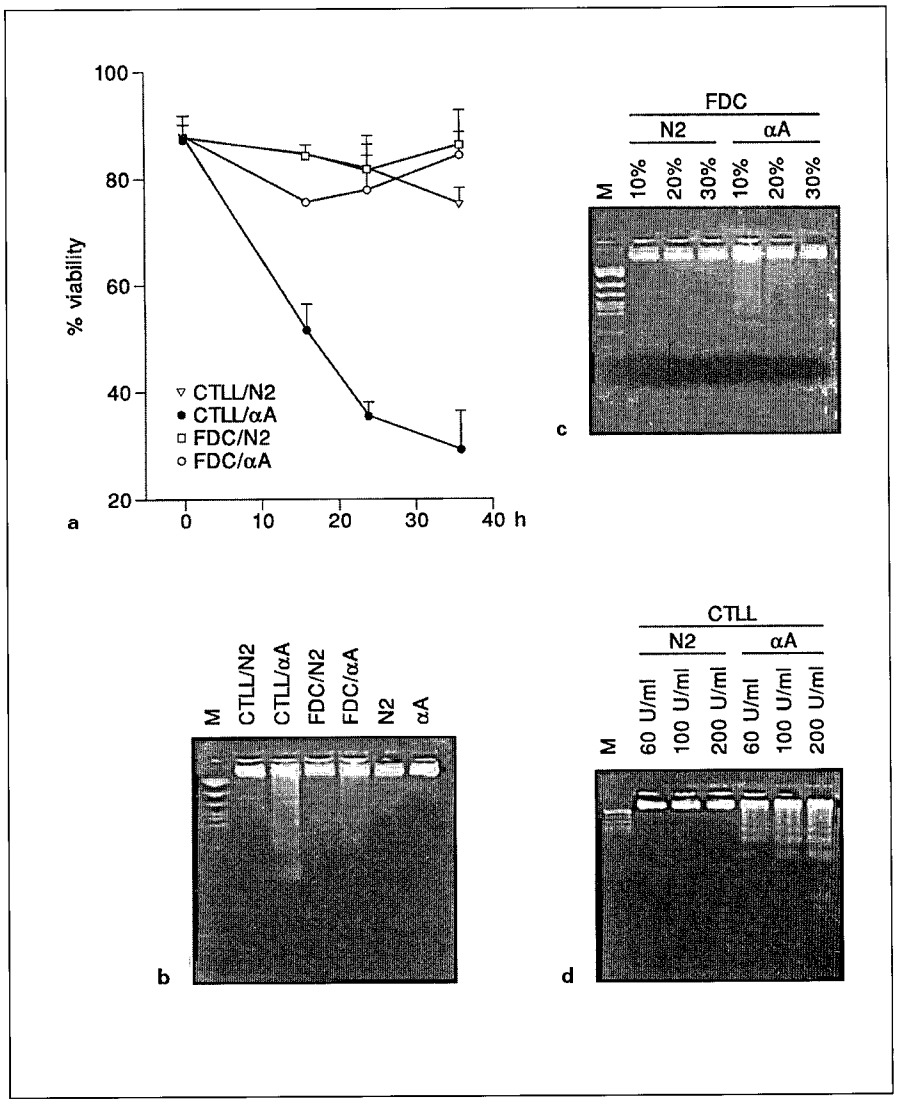

Fig. 3. Infection of factor-dependent lymphoid and myeloid cells and effects of growth factors on DNA fragmentation. **a** Viability of infected cells. Viability was defined as the reduction in the percentage of cells capable of forming colonies in methylcellulose culture. The plating efficiency of 70Z/3 and WEHI-3B cells is 40 and 100%, respectively. This efficiency did not change after virus infection. The cells were co-cultured with virus-producing cells as described in figure 1 for 70Z/3 and WEHI-3B cells. The time was hours after initiation of co-culture. CTLL/N2 = CTLL-2 cells co-cultured with N2-producing cells; CTLL/αA = CTLL-2 cells co-cultured with αA-producing cells in the presence of IL-2; FDC/N2 = FDC cells co-cultured with N2-producing cells; FDC/αA = FDC cells co-cultured with αA-producing cells in the presence of WEHI-3-conditioned medium (WEHI CM). **b** DNA fragmentation analysis. Cells were infected for 24 h in the presence of IL-2 or WEHI CM, genomic DNA was extracted and examined on 2% agarose gel. M = MW markers; N2 = N2-producing cells; αA = αA-producing cells; others are the same as in (**a**). **c** DNA fragmentation from myeloid cells cultured in the presence of increasing amount of growth factors. Genomic DNA from N2- or αA-infected FDC-P1 cells that were co-cultured with virus producing cells for 24 h in the presence of 10–30% of WEHI CM. **d** DNA fragmentation from lymphoid cells cultured in increasing amount of growth factors. CTLL-2 lymphoid cells were cultured in the presence of various concentrations (60–200 U/ml) of IL-2. Genomic DNA from N2- and αA-infected was extracted after 24 h of co-culture with virus-producing cells.

Consisting with our previous results on using the pαA vector [17], Northern blot analysis indicated that the c-abl mRNA, as compared to γ-actin mRNA, was reduced significantly in all types of cells transduced with the vector (data not shown) [17]. As we have shown previously, reduction of c-abl mRNA in αA-transduced cells correlated with the reduction of c-Abl protein [17] (fig. 2b). Coomassie blue staining indicated no significant difference in overal protein levels among all samples. Thus, the difference between WEHI-3B and 70Z/3 cells mirrors the phenotype of c-abl knockout animals, which exhibited lymphopenia but not myelopenia [50, 51]. Reduction of c-Abl in the lymphoid cell line 70Z/3 induced apoptosis, whereas reduction of c-Abl in WEHI-3B myeloid cells did not (fig. 1b). Because WEHI-3B cells produce IL-3 [35], it is possible that the reason these cells were resistant to apoptosis might be related to the ability of the growth factor to rescue cells that are indicated to undergo apoptosis.

Rescuing Apoptotic Myeloid but Not Lymphoid Cells by Hemopoietic Growth Factors

To study the nature of this rescue, we examined whether growth factors could inhibit apoptosis. We again determined the biological effects after the transduction of factor-dependent lymphoid and myeloid cells with the antisense sequence. Transduction of IL-3 dependent FDC-P1 cells with either N2 or αA vector did not result in reduction in viability over time (fig. 3a). On the other hand, transduction of IL-2-dependent CTLL-2 lymphoid cells with αA but not with N2 vector resulted in a significant

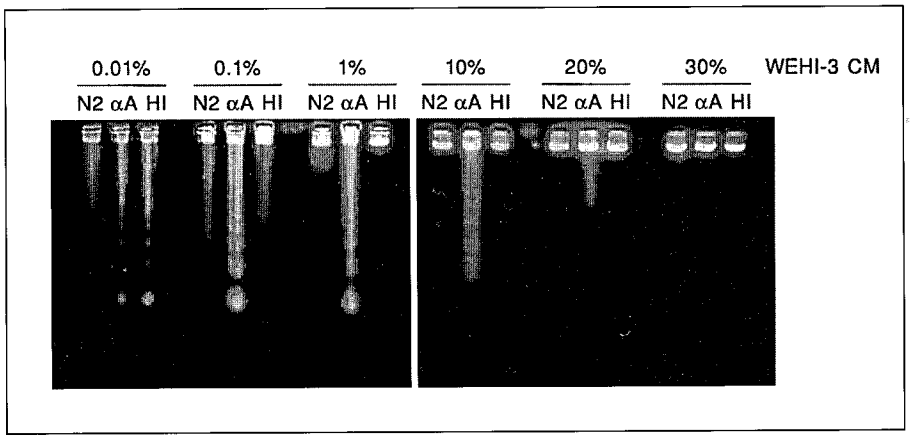

Fig. 4. Infection of FDC-P1 cells with viral supernatants from N2-producing cells, αA-producing cells and heat-inactivated supernatant from αA-producing cells in the presence of various concentrations of cytokines. Cells were infected for 24 h at various concentrations of WEHI-3 CM, genomic DNA was then extracted and analyzed on 2% agarose gel. N2 = FDC-P1 cells infected with N2; αA = FDC-P1 cells infected with αA; HI = FDC-P1 cells infected with heat-inactivated αA. Concentration of WEHI-3 CM is indicated.

loss of viability (fig. 3a). Consistent with the idea that CTLL/αA cells underwent apoptosis, a specific DNA fragmentation ladder was observed in genomic DNA of CTLL/αA cells, but not in CTLL/N2 cells (fig. 3b). However, fragmentation was also observed in DNA of FDC/αA versus the controls FDC/N2 or FDC/αA (heat-inactivated) cells (fig. 3c, 4), albeit weaker than in CTLL-2 cells. Thus, myeloid FDC/αA cells are more prone to apoptosis than myeloid, IL-3-producing WEHI/αA cells. This interesting contrast prompted us to determine whether the ability to rescue cells from apoptosis by growth factors would be the basis for the difference between lymphoid and myeloid cells in terms of the role of c-Abl in cellular development of the two cell types. When various amounts of corresponding growth factors were added during and after retroviral transduction of the cells, a striking difference was observed between the lymphoid and myeloid cells. While FDC/αA cells manifested a small degree of apoptosis in 10% but not in 20 or 30% of IL-3 (WEHI-3CM), CTLL/αA cells underwent apoptosis regardless of the amount of IL-2 supplied to the cultures (fig. 3c, 3d, 4). As FDC-P1 cells proliferated maximally in 10–30% WEHI-3 CM, the disappearance of the apoptotic ladder in DNA of cells maintained at 20–30% CM was not due to a 'dilution' effect – apoptotic cells were not diluted by continuously dividing nonapoptotic FDC-P1 cells. Thus, these data suggest that the reduction of c-Abl in FDC-P1 cells would also induce apoptosis, but the cells could be rescued from apoptosis when given an excess amount of growth factors. Similar data, including those for colony formation as shown in figure 1, were obtained when another IL-3-dependent cell line, 32D, was examined (data not shown). On the other hand, the data also suggest that the reduction of c-Abl in IL-2-dependent CTLL-2 cells stimulated apoptosis, which could not be rescued by an excess amount of IL-2.

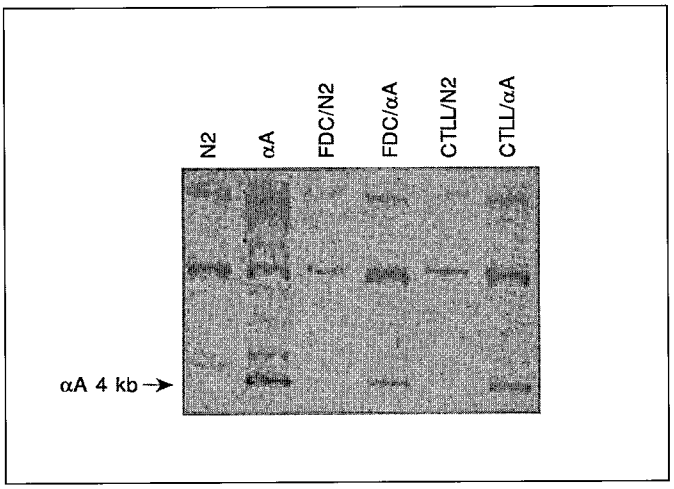

Fig. 5. Southern blot analysis of infected FDC-P1 and CTLL-2 cells. Genomic DNA extracted from cells infected with N2 and αA viruses for 24 h was digested with the *Kpn*I enzyme. The 4.0-kb integrated αA proviral DNA was detected by hybridization with the 2.2 kb *Bgl*II fragment of v-abl DNA. CTLL/N2 = CTLL-2 cells infected with the N2 virus; CTLL/αA = CTLL-2 cells infected with αA virus; FDC/N2 = FDC-P1 cells infected with the N2 virus; FDC/αA = FDC-P1 cells infected with the αA virus. Results were plotted as a percentage of plating efficiency.

To confirm retroviral gene transfer, we did a Southern blot analysis on DNA of cells from the virus-producer cell lines, FDC-P1 and CTLL cells transduced with either N2 control or αA antisense virus. Figure 5 shows that both FDC-P1 and CTLL-2 cells were positively transduced with αA. The hybridization signal of the αA band was approximately the same, suggesting that the efficiency of gene transfer into the two different cell types was the same.

Table 4. Methylcellulose colony formation of αA-infected and N2-infected CTLL-2 and FDC-P1 cells at various concentrations of growth factors

Cell line	Virus	Number of colonies				
		60 U/ml IL-2	100 U/ml IL-2	200 U/ml IL-2		
CTLL-2	N2	76±11	86±10	106±21		
	αA	5±1	10±6	14±1		
		Number of colonies				
		0% WEHI CM	0.01% WEHI CM	0.1% WEHI CM	1% WEHI CM	10% WEHI CM
FDC-P1	N2	0	7±1	28±1	78±1	96±18
	αA	0	1±1	10±1	35±5	120±13

Cells infected with N2 or αA virus for 24 h were plated into methylcellulose culture at a density of 300 cells/dish for FDC-P1 cells and 1,000 cells/dish for CTLL-2 cells, with various amounts of growth factors as indicated. The number of colonies was recorded 14 days later.

Table 5. Viability of FDC-P1 cells stably expressing c-abl antisense sequence

Virus	Viability, %					
	0% WEHI CM	0.01% WEHI CM	0.05% WEHI CM	0.1% WEHI CM	1% WEHI CM	10% WEHI CM
N2	18±2	75±6	88±2	88±5	97±1	98
αD	15±1	44±1	57±5	85±1	98±2	99±1

FDC-P1 cells stably expressing the αD antisense sequence and control cells transduced with N2 virus were passaged for 48 h at various concentrations of WEHI CM. The cells were then harvested and viability was evaluated by trypan blue staining. Three individual samples were counted at each point.

As both FDC-P1 and CTLL-2 cells can proliferate in semisolid methylcellulose culture and give rise to hemopoietic colonies, we reasoned that cells undergoing apoptosis would not be able to develop such colonies. Indeed, table 4 indicates that both cells expressing αA developed a reduced number of colonies. Consistent with the results of DNA gel degradation, colony formation for FDC/αA cells was correlated with the amount of growth factors, but not for CTLL-2 cells (table 4). It is noteworthy that for CTLL-2 cells, the addition of more IL-2 increased the number of colonies slightly. This increase was not significant and could be due to the requirement for CTLL/αA cells to consume the residual amount of IL-2. For FDC/αA cells, the reduction in colony formation was particularly striking at low concentrations of WEHI-3CM (0.01–1%; table 4). Reduction in colony formation for FDC/αA cells was not apparent at 10% WEHI-3 CM (table 4), whereas DNA fragmentation occurred at the same percentage of CM (fig. 3c). This was probably due to an inherent difference of the two assays. In the former assay, the cells were analyzed after 10–14 days in culture; since the plating efficiency of FDC-P1 cells is around 50–70%, it is possible that cells that underwent apoptosis were not colony-forming cells. We conclude that FDC-P1 cells can be rescued by the addition of myeloid growth factors, whereas the lymphoid factor-dependent CTLL-2 cells, like the lymphoid 70Z/3 cells, cannot be rescued by increasing concentration of a lymphoid growth factor.

Increased Expression of Growth Factor Receptors in Cells Expressing a Low Level of c-abl

To investigate further the mechanism(s) accounting for the rescue of myeloid cells from apoptosis, we analyzed a homogeneous population of cells expressing the antisense RNA. To do that, we constructed another retrovirus vector, pαD, which contains a neomycin-resistance gene.

Since growth factors could not rescue lymphoid cells from undergoing apoptosis when expressing antisense abl, we introduced pαD into myeloid FDC-P1 cells. G418 was added to the medium to select for the transduced cells, which were subsequently maintained in the presence of growth factors (fig. 6). Northern blot analysis indicated that the antisense RNA and the neo RNA in the FDC/αD cells were expressed at high level, with a corresponding decline in the c-abl level (fig. 6a).

Because both the antisense and the control N2 vectors have the neo^r gene in the plasmid, we were able to obtain a pure population of vector-transduced, factor-dependent cells. By comparing the growth of FDC/αD cells (FDC-P1 cells expressing anti-c-abl antisense RNA) and control FDC/N2 cells, we noticed that the former cells manifested a higher degree of mortality at concentration of WEHI-3 CM lower than 0.1% (table 5). FDC/αD cells were therefore more sensitive to apoptosis than FDC/N2 cells. Of interest is that FDC/αD cells had the same viability as FDC/N2 cells at 0.1–1% WEHI-3 CM, whereas FDC/αA cells apoptosed even at 1–10% WEHI-3 CM (fig. 3a–c; table 4). Because FDC/αD cells were established by G418 selection, an alternative pathway allowing them to escape from apoptosis may have been triggered, as proposed in the 'Introduction (see also 'Discussion'). On the other hand, FDC/αA cells were not a selected population and were therefore more prone to apoptosis.

One mechanism by which cells respond to increased growth factor requirements is the modulation of growth factor receptors [1]. Therefore, we measured the expression of IL-3 receptors at the RNA level, as IL-3 is the dominant growth factor in the WEHI-3 CM used to rescue apoptotic cells. Washed FDC/αD and FDC/N2 cells were cultured in the presence of 0.1, 1 and 10% WEHI-3 CM, 48 h later, RNA was extracted from cells in these cultures. Northern blot analysis was carried out, and the filter was hybridized with both IL-3Rβ_{IL3} probe and a γ-actin probe. The results indicated that the ratio of the intensity of IL-

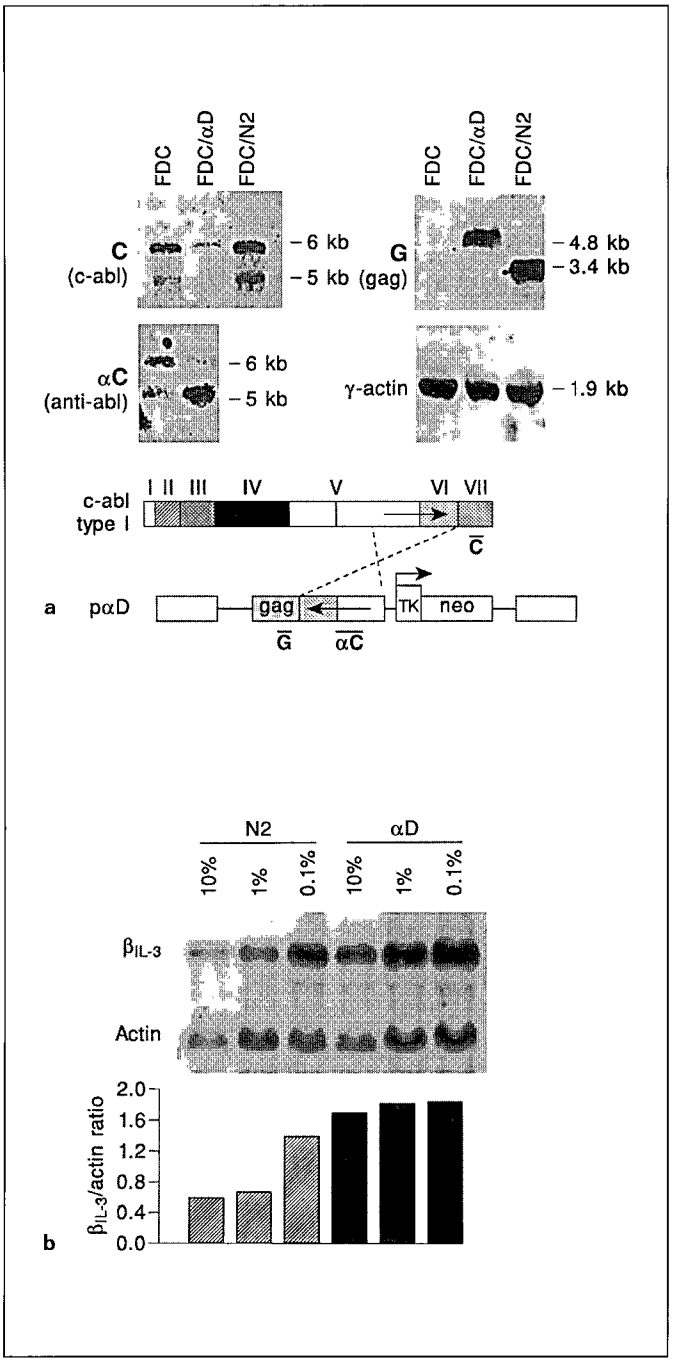

Fig. 6. Northern blot analysis of αD-transduced FDC-P1 myeloid cells. **a** c-abl level of αD-transduced FDC-P1 myeloid cells. Various DNA fragments were used as probes: C probe, which corresponds to C-terminus of c-abl sequence and is absent in pαD, upper left; G probe, which is a gag-specific probe which reveals a 4.8-kb band in FDC/aD cells carrying a proviral genome of anti-c-abl sequences and a TK/neo sequence, and a 3.4-kb band in FDC/N2 cells carrying a proviral gene of a neo gene, upper right; αC probe, which corresponds to a c-abl sequence that is present in both pαD vector and endogenous c-abl sequence, bottom left; and an actin probe, bottom right. Domains of c-abl type I are: I = variable domain; II = SH3; III = SH2; IV = SH1 (tyrosine kinase); V = nuclear translocation signa; VI = DNA-binding domain; VII = actin-binding domain. FDC = Parental FDC-P1 cells; FDC/αD and FDC/N2 = pooled populations of FDC-P1 cells expressing αD or N2 virus, respectively. **b** Expression of IL-3Rβ_{IL-3} subunit in FDC-P1 cells expressing the c-abl antisense sequence. FDC/αD and FDC/N2 cells were carried for 48 h at 0.1, 1 and 10% WEHI-3 CM. Northern blot was hybridized with either a probe specific for IL-3Rβ_{IL-3} subunit or for γ-actin. The subunit/actin ratio was determined by phosphoimage densitometry analysis. FDC/αD and FDC/N2 were selected FDC-P1 cells expressing αD or N2 virus. The concentration of WEHI-3 CM is shown in the figure.

$3R\beta_{IL3}$/actin was increased in FDC/αD cells compared to FDC/N2 cells (fig. 6b). Similar data were obtained with probes of other subunits of IL-3 receptors (IL-$3R\beta_c$ and IL-3Rα), although the increase for either one was not as drastic (data not shown). These data indicate that the IL-3 receptor level in both FDC/αD and FDC/N2 cells could be modulated as a function of available IL-3; however, a reduction of c-abl upregulated the level of IL-3 receptor at all times, representing one mechanism by which cells undergoing apoptosis might be rescued.

Discussion

By retroviral transfer and expression of the anti-abl antisense sequences into various hemopoietic cells, we observed that a reduction of c-abl in both lymphoid and myeloid cells induces apoptosis. However, only myeloid cells expressing a low level of c-abl could be rescued by appropriate hemopoietic growth factors. We have used two different lymphoid and three distinct myeloid cell lines in this studies, and our data are consistent with the idea that there is a difference between lymphoid and lymphoid cells in their relative resistance to c-abl-mediated induction of apoptosis. Recently, our preliminary analysis on IL-3-dependent mast cells derived from the bone marrow and mitogen-stimulated lymphoid cells derived from the spleens suggests that this difference between myeloid and lymphoid cells is also operative on primary cells (data not shown). This difference may be due to the existence of more than one signal transduction pathway for apoptosis. Alternatively, it may be due to the existence of more than one mechanism in rescuing cells from apoptosis.

The majority of fibroblasts and myeloid cells expressing reduced c-Abl do not undergo apoptosis [17; this study], whereas more than 80% of lymphoid cells expressing reduced c-Abl underwent apoptosis (fig. 1, 3, 4). An alternative pathway to bypass apoptosis must exist in myeloid cells and fibroblasts, which is probably not active in lymphoid cells. This alternative pathway should also exist in many other cell types. It is of interest to note that in nature, although c-abl is expressed ubiquitously, its expression is highest in lymphoid cells and testis [38, 42]. Based on this view, one would therefore except that c-Abl may play an important role in lymphopoiesis and spermatogenesis. Indeed, in transgenic c-abl knockout mice, lymphopoiesis is defective and a limited number of mice bred to adulthood appear to have reduced fertility [50, 51]. On the other hand, fibroblasts, myeloid cells or other cell types appear to be relatively normal. Thus this alternative mechanism of inhibition of apoptosis by growth factors may be a more generalized phenomenon.

What is the nature of this alternative pathway to bypass apoptosis as a result of c-Abl reduction? Our study provides a clue indicating that growth factor-mediated pathway is involved. It is interesting to note that at least in embryonic fibroblasts in the aorta of a developing rat, autocrine production of a c-PDGF (cellular platelet-derived growth factor) has been reported to occur in normal cells during development [40]. In our study, we have provided clear evidence that c-Abl can inhibit apoptosis by a supply of myeloid growth factors (fig. 3, 4, 6). Its association with growth factor pathway is further strengthened by the demonstration that reduction of c-Abl level correlates with upregulation of IL-3R (fig. 6b). Therefore, it would be of interest to find out whether a reduction of c-Abl would activate autocrine production of an appropriate growth factor for a corresponding cell type. This would provide one alternative pathway in which the bypass of apoptosis induced by c-Abl reduction can be achieved. This hypothesis becomes even more interesting when the mode of action of the oncogenic Bcr/Abl and v-Abl is considered. Both molecules have been shown to suppress apoptosis, similar to what we showed in this study for c-Abl, and altered patterns of growth factor production by cells transformed by these oncoproteins have been reported [9]. Furthermore, our recent preliminary data showed that expression of Bcr/Abl oncoprotein can induce transformation of fibroblasts, which resulted in creation of an autocrine situation where the transformed fibroblasts were producing an autocrine growth factor [Wong and Wong, to be submitted]. Thus, there may be a generalized alternative mechanism which can rescue cells inclined to undergo apoptosis by means of the production of a growth factor appropriate for the corresponding cell type.

The ability of c-Abl to modulate growth factor receptors provides additional clarification or explanation for some recent findings about the role of c-Abl in growth regulation. We observed that FDC-P1 cells expressing low levels of c-Abl grew faster than the control FDC/N2 cells at high concentrations of WEHI-3 CM, which is due to the elevation of IL-3 receptor numbers (data not shown). Sawyers et al. [48] reported that 3T3 fibroblasts expressing a transdominant negative c-Abl mutant returned to cell cycle faster after serum starvation; these cells also exhibited increased colony formation upon transduction with the oncogene. Similarly, 3T3 fibroblasts expressing the antisense sequences entered into the S phase faster than control cells after serum starvation [17]. It is quite possible that the PDGF receptor numbers might be increased significantly

in the 3T3 cells in these studies after serum starvation and/or expression of the transdominal mutant. Consequently, one would observe fast entry into the cell cycle and an increase in transformability. As apoptosis induced by a reduction of c-Abl can be rescued by hemopoietic growth factors via upregulation of receptors of their corresponding growth factors, induction of apoptosis in myeloid cells as a result of c-Abl reduction can also occur when a supply of growth factors is insufficient. Indeed, by adding anti-abl oligodeoxynucleotides into hemopoietic cultures in vitro, Caracciolo et al. [5] observed a reduction in myeloid colonies in cultures in which an extra amount of hemopoietic growth factors was not added. This result would seem to be contradictory to the normal levels of myeloid cells in knockout animals [50, 51]. Rescue of myeloid cells by growth factors, as demonstrated in figures 3–6 in our studies, would explain this discrepancy.

Considering the significance of the high level of c-Abl in lymphoid cells and in sperm cells, a refractory response of lymphoid cells to be rescued by growth factors may reflect their unique biological functions. They have to be 'educated' to differentiate between self- and non-self, and have to become memory cells, which undergo clonal expansion when the immune system is challenged by foreign particles. It is therefore conceivable that they have more autonomy in regulating their own growth and differentiation, but once apoptosis is induced, as has been observed for T cells in the thymus, no rescue by growth factors may be permissible. Sperm cells are also unique in that they are metabolically active and yet not actively dividing. A high level of c-Abl would certainly permit a significantly extended survival period. Thus, aside from lymphoid cells and sperm cells, rescue of cells from apoptosis by growth factors may be a generalized alternative mechanism.

Oncogenic Bcr-Abl and v-Abl have been shown to suppress apoptosis [6, 34, 36]. The results in this study suggest that c-Abl also plays a role in suppressing apoptosis. Therefore, what is the fundamental difference between the oncoproteins and their normal counterpart? Presently, it is unknown but in our recent review [10] we speculated that this difference might be related to the biological potency of all these molecules, the c-Abl protein being the weakest. Although they have very significant biochemical differences, Bcr/Abl and v-Abl are very similar as measured by a variety of biological functions. The striking consistency is that Bcr/Abl is always weaker in all biological assays used for the test [10]. Therefore, what remains to be tested is whether c-Abl possesses similar biological properties as the oncogenic form. In this case, the sensitivity of the biological assays to be used for the analysis may have to be increased.

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