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BCR-ABL and constitutively active erythropoietin receptor (cEpoR) activate distinct mechanisms for growth factor-independence and inhibition of apoptosis in Ba/F3 cell line

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The interleukin-3 dependent murine Ba/F3 cell line has been widely used as an experimental model of cell transformation by BCR–ABL oncogenes as assessed by induction of growth-factor-independence and inhibition of apoptosis *in vitro*. The signaling pathways used by BCR–ABL oncogenes to exert these effects are unknown. To gain insights into this phenomenon, we have introduced the p190- and p210-encoding BCR–ABL oncogenes as well as the constitutively activated oncogenic murine erythropoietin receptor (cEpoR) into Ba/F3 and compared the behavior of individual clones in response to apoptotic stimuli. Both p210 and p190 BCR–ABL vectors induced IL-3-independent growth and the same result was obtained with the cEpo-R vector. Individual clones of Ba/F3 cells expressing BCR–ABL exhibited significant resistance to apoptosis induced by either etoposide, serum deprivation or growth-factor withdrawal. In contrast, Ba/F3 cells expressing the constitutively active cEpoR behaved like parental Ba/F3 cells undergoing apoptosis when similarly treated with etoposide or upon serum deprivation. Bc12 and Bax levels were similar in all BCR–ABL and cEpoR-transfected clones. However, in band-shift assays, nuclear extracts from growth-factor-independent Ba/F3 clones expressing cEpoR had no detectable STAT activity as opposed to the constitutive STAT activation detected in all Ba/F3 clones expressing p210 or p190 BCR–ABL. Our results indicate that although both constitutively activated cEpoR and BCR–ABL oncogenes induce growth-factor independence in Ba/F3 cells, only BCR–ABL is able to protect cells from etoposide and serum-deprivation-induced apoptosis and induce a strong constitutive activation of STAT factors, suggesting a role for these molecules in the anti-apoptotic activity of BCR–ABL.

Keywords: BCR–ABL; apoptosis; EpoR

Introduction

The chimeric fusion gene BCR–ABL is the molecular hallmark of chronic myelogenous leukemia (CML) (Kurzrock *et al*., 1988). The molecular mechanisms of BCR–ABL-induced-cell transformation have been extensively studied during recent years in experimental systems such as the IL-3-dependent murine cell line Ba/F3, to demonstrate the oncogenic potential of BCR–ABL oncogenes (Daley and Baltimore, 1988; McWhirter and Wang, 1993; Kabarowski *et al*., 1994). In several hematopoietic cell lines tested including Ba/F3, BCR–ABL has been shown to induce growth-factor-independence and to confer a strong antiapoptotic effect (Laneuville *et al*., 1994; Kabarowski *et al*., 1994). However, recent data indicate that the obtention of a fully transformed phenotype by BCR–ABL is a complex event requiring multiple activation signals (Puil *et al*., 1994; Salgia *et al*., 1996; Carpino *et al*., 1997; Jain *et al*., 1997) and an appropriate cellular context (Ilaria and Van Etten, 1995; Goga *et al*., 1995). Some domains of BCR–ABL molecule might also have different roles in terms of growth-factor independence inducing ability, proliferation or inhibition of apoptosis (Pendergast *et al*., 1993a; Cortex *et al*., 1995) the latter being probably a major mechanism by which BCR–ABL could exert its transforming effect, perhaps by predisposing cells to a cytogenetic instability (Bedi *et al*., 1994).

The precise mechanisms of the antiapoptotic effects of BCR–ABL oncogenes remain unknown but it has been assumed that they might act, at least in the context of a growth-factor-dependent cell line, by replacing the action of a growth factor to which the target cell is usually responsive (Sirard *et al*., 1994). Although current experimental data indicate that BCR–ABL could activate several growth-factor-induced signaling pathways (Puil *et al*., 1994; Pendergast *et al*., 1993b; Carlesso *et al*., 1996) there is no evidence demonstrating the presence of a common pathway for growth-factor-independence and inhibition of apoptosis. As a matter of fact, even growth factors such as IL-3 and GM-CSF which are the prototypes of antiapoptotic molecules, could use differential signaling for their proliferative and their anti-apoptotic effects (Kinoshita *et al*., 1995).

In this work, we wished to study the relationships between growth-factor-independence and the antiapoptotic activity of BCR–ABL. We have used for this purpose Ba/F3 cell line which we rendered growth-factor-independent by enforced expression of either BCR–ABL oncogenes (p210 and p190) or the constitutively active form of the EpoR harboring an Arginine-Cysteine (R129C) mutation (Watowich *et al*.,

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1992). The latter has been shown to induce a strong proliferative and transforming effect of hematopoietic progenitor cells in mice (Longmore *et al*., 1994). The antiapoptotic potential of both BCR–ABL and cEpoR was therefore assessed in the same cellular context. Our results indicate that although both BCR–ABL and cEpoR render Ba/F3 cells growth-factor-independent, only BCR–ABL is able to inhibit serum-deprivation and etoposide-induced apoptosis. This differential effect was correlated with a differential activation of STAT molecules.

Results

Expression of BCR–ABL and cEpoR in Ba/F3 cells

All Ba/F3 cells transfected with BCR–ABL plasmids and amplified in the presence of WEHI-CM and G418 were found to be growth-factor-independent upon removal of IL-3. Similarly, growth-factor-independence were readily obtained in Ba/F3 cells transfected with cEpoR plasmids. Figure 1 shows a Western blot analysis of individual Ba/F3 clones expressing BCR–ABL p190 (Clones #6, 4 and 3) and BCR–ABL p210 (Clones #6, 5 and 3) constructs. Four clones designated as p210-C5, p210-C6, p190-C3 and p190-C6 were selected for functional analyses.

To demonstrate the presence of cEpoR expression at the surface of Ba/F3 cells transfected with the cEpoR construct, we used a radioactive Epo-binding assay on four individual clones as well as on the polyclonal Ba/F3-cEpoR stock. As can be seen in Figure 2, all clones as well as the polyclonal cEpoR stock bound very efficiently iodinated Epo, with the highest binding being observed on clone cEpoR-C3. As expected, no Epo-binding was detectable on the parental BaF3 cell line. The level of cEpoR binding observed on the polyclonal stock was similar to that observed on clones cEpoR-C1, cEpoR-C5 and cEpoR-C8. These results clearly demonstrated expression of cEpoR at the surface of all Ba/F3 transfectants.

Comparative analysis of growth characteristics of Ba/F3 BCR–ABL and Ba/F3-cEpoR clones

Growth characteristics of BCR–ABL clones (p210-C5, p210-C6, p190-C3 and p210-C6) were compared to that observed in a Ba/F3 clone transfected with a NeoR gene as well as to that seen in two Ba/F3-cEpoR clones (cEpoR-C3 and cEpoR-C5). Figure 3 shows the results of a ^{3}H-thymidine proliferation assay demonstrating growth factor independence of the clones p210-C5 and p210-C6 and that of the clones p190-C3 and p190-C6. Upon stimulation by IL-3, these clones did not exhibit an increased proliferative response. On the other hand, clones cEpoR-C3 and cEpoR-C5 expressing cEpoR at their surface, showed a reduced growth in the absence of IL-3 despite their growth-factor-independence. Their growth could further be enhanced by exogenous IL-3 (10% WEHI-conditioned medium). Similar results were obtained with MTT and daily viability count analyses (data not shown). Altogether, these data demonstrated that the expression of cEpoR at the surface of Ba/F3 cells induced growth-factor-independence similar to the phenomenon observed with the expression of BCR–ABL oncogenes whereas cells transfected with NeoR gene remained strictly growth-factor-dependent (Figure 3). BCR–ABL and cEpoR clones as well as control Ba/F3 cells were then tested for their ability to resist to apoptotic signals.

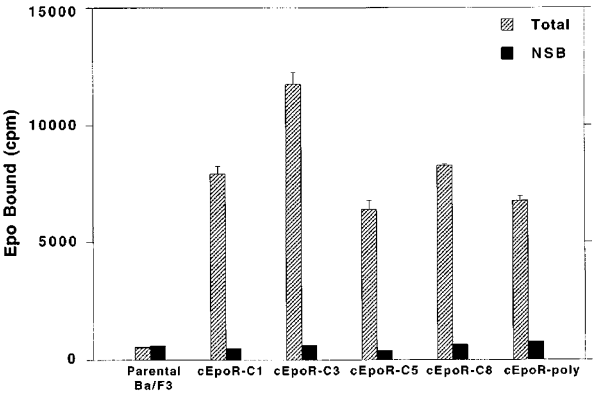

Figure 2 ^{125}I Epo-binding of cEpoR-transfected Ba/F3 cells. 5.10^{6} cells were incubated with 2 nM of 125 ^{125}I-Epo in the absence (total binding) or in the presence of 500 nM unlabeled Epo (NSB: non-specific binding). After 1 h at 37°C, the cells were washed and cell-bound radioactivity was measured. Each point is the mean of three determinations

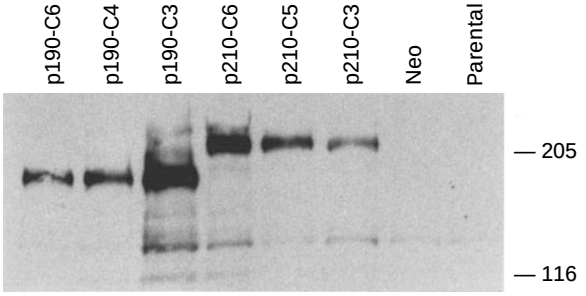

Figure 1 Western blot analysis of Ba/F3 clones expressing BCR–ABL p190 (C6, C4, C3) and BCR–ABL p210 (C6, C5, C3) Neo: Ba/F3 cells transfected with the control MSCV-Neo2.1 plasmid. In addition to the 190 kd and 210 kd bands corresponding to BCR–ABL, a 145 kd ABL gene product is seen in each cell line

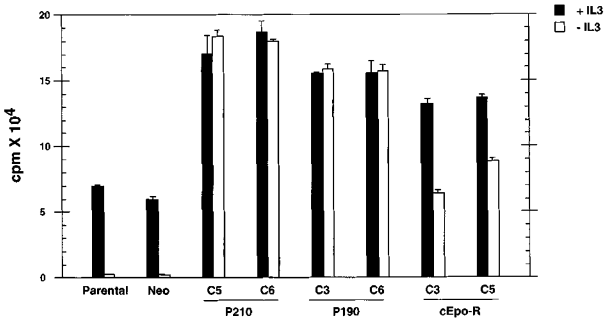

Figure 3 Thymidine incorporation assay using different Ba/F3 transfectants. 10^{4} cells were incubated in triplicate for 48 h in RPMI containing 10% FCS, with or without IL-3 as indicated, before a ^{3}H-thymidine pulse of 18 h

Inhibition of apoptosis by BCR–ABL

Figure 4 shows the results of a representative quantitative propidium iodide assay in all clones expressing BCR–ABL and cEpoR as well as in control NeoR and parental cells to determine the percentage of apoptotic nuclei. The results obtained represent the mean of three separate experiments. At the beginning of the experiments, spontaneous apoptosis rate was low in all clones (<5% apoptotic nuclei). Apoptosis was induced by growth-factor withdrawal, serum deprivation or by the use of etoposide.

As shown in Figure 4b, IL-3 withdrawal for 48 h induced apoptosis in both parental and Ba/F3 NeoR-cells (53% and 63% apoptotic nuclei respectively). On the other hand, upon removal of IL-3, all BCR–ABL-expressing clones (p210-C5, p210-C6, p190-C3 and p190-C6) remained non-apoptotic for 48 h (mean percentage of apoptosis: 8% for Ba/F3-p210 and 7% for Ba/F3-p190 clones). Similarly, cEpoR transfectants cEpoR-C3 and cEpoR-C5 grown without IL-3 in 10% FCS were not apoptotic for over 48 h (11% and 7% of apoptotic nuclei) (Figure 4b). This latter finding suggested that the constitutive activation of cEpoR was sufficient to transmit a mitogenic signal, circumventing the need for the exogenously provided IL-3.

We have therefore compared BCR–ABL and cEpoR transfectants for their behavior with regard to the use of other apoptotic stimuli such as serum deprivation and use of etoposide. As can be seen in Figure 4c; serum deprivation for 48 h induced a significant apoptosis in both parental and control Ba/F3-NeoR cells (45% and 56% of apoptotic nuclei respectively). Conversely, all BCR–ABL clones remained totally resistant to apoptosis induced by serum deprivation in the same period of time (4–8% of apoptotic nuclei in all clones). Interestingly, growth-factor-independent Ba/F3-cEpoR clones were not resistant to serum-deprivation-induced apoptosis, with a mean % of 36–42% of apoptotic nuclei observed after 48 h of serum deprivation (Figure 4c). This discordant behavior between BCR–ABL and cEpoR transfectants was also found when cells were treated with an optimal dose of etoposide known to induce apoptosis in our Ba/F3 cell line. Upon etoposide treatment for 20 h (20 μg/ml) all BCR–ABL clones remained non-apoptotic (9–10% apoptotic nuclei for p210 clones and 8% for p190 clones) whereas parental cells and Ba/F3-NeoR cells underwent a significant degree of apoptosis (41% apoptotic nuclei for parental cells and 51% for NeoR cells). Similarly, both growth-factor-independent cEpoR clones expressing cEpoR at their surface were not protected from the etoposide-induced apoptosis (34% and 36% of apoptotic nuclei in both clones) (Figure 4d) suggesting that growth factor-independence provided by the constitutive activation of cEpoR was not sufficient to transmit an antiapoptotic signal, this effect contrasting with the antiapoptotic effect constantly observed in Ba/F3-BCR–ABL clones. Thus, despite the established oncogenicity of both BCR–ABL and cEpoR, there was a clear dissociation between the antiapoptotic effect of these two genes in the same cellular context.

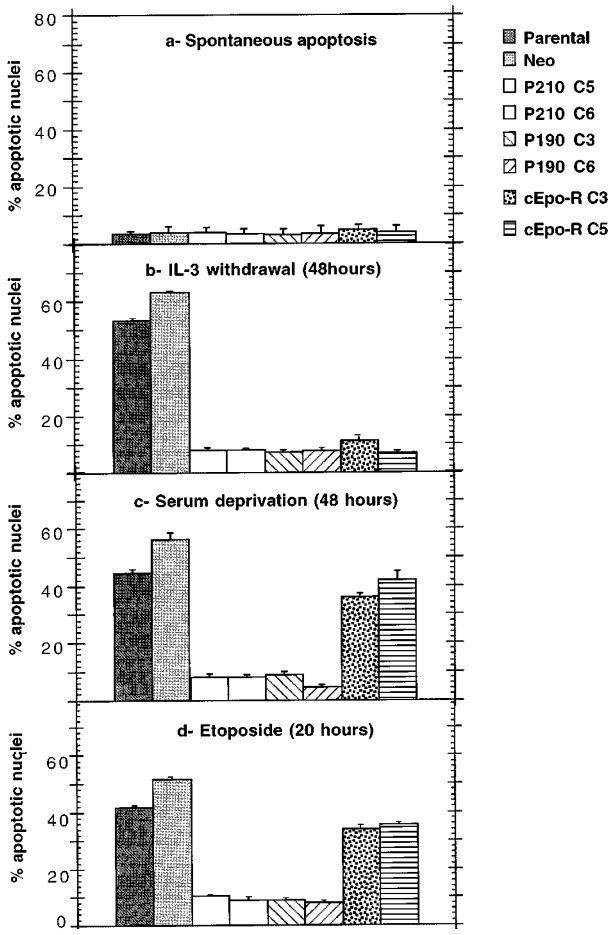

Figure 4 Diagram showing the percentage of apoptotic nuclei observed by flow cytometry in control Ba/F3 cells and in individual Ba/F3 BCR–ABL and cEpoR clones: (**a**) At the beginning of the experiment to determine the rate of spontaneous apoptosis; (**b**) After IL-3 withdrawal for 48 h; (**c**) After serum deprivation for 48 h; (**d**) After incubation with etoposide for 20 h. $n=3$ experiments

Bc12 and Bax expression in BCR–ABL and cEpoR clones

In order to gain insight into the mechanisms of the differential antiapoptotic effect provided by BCR–ABL and cEpoR, we next studied the expression of Bc12 and Bax protein in our clones. Figure 5 shows the result of a Western blot analysis using a Bc12 antibody. As shown in this Figure, we did not find any significant difference of Bc12 protein levels in cells expressing BCR–ABL or cEpoR. However, control Ba/F3 cells (parental or transfected with a NeoR gene) expressed lower levels of Bc12. Similarly, we did not observe any significant differences in the levels of the

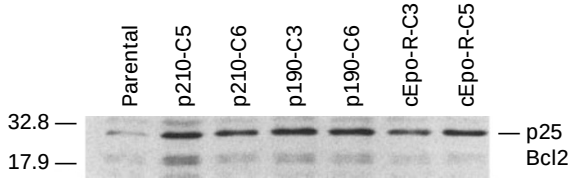

Figure 5 Western blot analysis of BCR–ABL p210, p190 and cEpoR clones to determine Bc12 expression (p25 Bc12). A consistent increase of the Bc12 levels was found in BCR–ABL and in cEpoR clones as opposed to control cells

proapoptotic protein Bax between our BCR–ABL and cEpoR transfectants (Figure 6).

Comparative analysis of STAT activation in BCR–ABL and cEpoR clones

Cytokines such as IL3 and Epo induce activation of STAT factors in hematopoietic cells. This signaling pathway has been shown to be constitutively activated in cells expressing some oncogenes such as v-*src* (Yu *et al*., 1995; Cao *et al*., 1996) and v-ABL (Danial *et al*., 1995) or in lymphocytes infected by HTLV-1 retrovirus (Migone *et al*., 1995), with a strong correlation between cell transformation or transition from a cytokine-dependent to a cytokine-independent growth with STAT activation. We therefore examined the activation of STAT factors in Ba/F3 cells expressing BCR–ABL or cEpoR oncogenes. Nuclear extracts from permanently growing Ba/F3-p210, Ba/F3-p190 and BaF3-cEpoR clones were prepared. The presence of active STAT factors was tested by their ability to bind to three different STAT binding sequences, namely the sis-induced element (SIE) from the c-*fos* promoter, the IFN-γ activated sequence (GAS) from the interferon regulatory factor-1 (IRF-1) promoter and the GAS from the β-casein promoter. Each of these sequences exhibit a different level of affinity for the STATs identified at the present time and often preferentially detects one or two active STATs. As shown in Figure 7, neither the polyclonal stock (lanes 1, 5 and 9) nor the Ba/F3 clones expressing high amount of cEpoR at their surface (lanes 14 and 15) were able to induce STAT factor activation with DNA binding activity for any of the GAS used. As expected, control Ba/F3 cells expressing the neomycin resistance gene were able to respond to IL-3 and IFN-γ stimulation by activation of STAT5 (lanes 3, 7 and 11) and STAT 1 (lanes 4, 8 and 12), respectively, as previously described (Pallard *et al*., 1995). In the same conditions, STAT factors were constitutively activated in cells expressing p190 (lanes 2, 6 and 10) and p210 (lane 16) BCR–ABL oncogenes. Supershift experiments further indicated that BCR–ABL oncogenes preferentially activated STAT5A, STAT5B and STAT6, with a much weaker activation of STAT1 (data not shown).

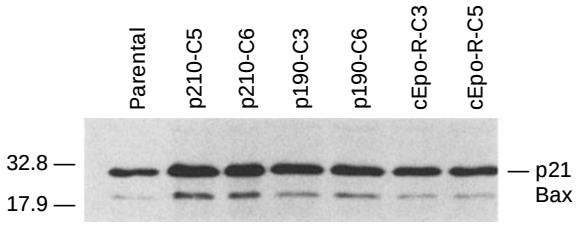

Figure 6 Western blot analysis of BCR–ABL p210, p190 and cEpoR clones to determine the expression of Bax (p21 Bax)

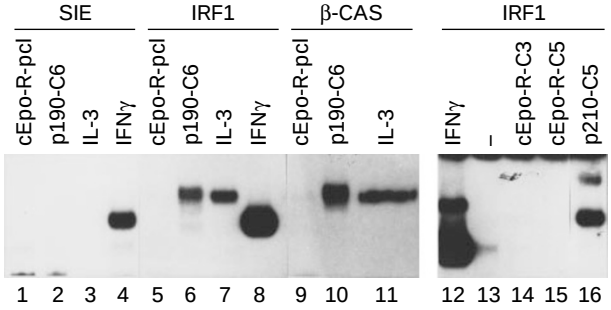

Figure 7 Analysis of the activation of STAT factors. Nuclear extracts from permanently growing Ba/F3 cells expressing BCR–ABL (p190-C6 or p210-C5) or cEpoR (cEpoR-polyclonal stock, cEpoR-C3, cEpoR-C5) oncogenes, or from Ba/F3-NeoR cells stimulated in the presence of IL-3 or IFN-γ were prepared and analysed by EMSA using as a probe, either the SIE (lanes 1–4) or the IRF1-GAS (lanes 5–8 and 12–16) or the β-casein-GAS (lanes 5–8)-containing oligonucleotide. The difference in the mobility of the major complexes activated in the presence of IL-3 and IFN-γ reflects the dominant activation of STAT5 and STAT1 in the presence of these two cytokines, respectively. The left and right panels represent two different EMSAs

Discussion

Inhibition of apoptosis seems to be one of the major effects of BCR–ABL oncogenes (Bedi *et al*., 1994; Kobarowski *et al*., 1994). Although the exact mechanisms of this phenomenon are not clearly understood, it is thought to be a direct consequence of the presence of the BCR–ABL fusion protein inside the cell, rather than being a secondary event. Experimental data in favor of this hypothesis include the reversal of the anti-apoptotic effect upon treatment of the transformed cells with BCR–ABL anti-sense oligonucleotides (McGahon *et al*., 1994) as well as with specific inhibitors of the *ab1* tyrosine kinase activity (Druker *et al*., 1996).

The relationships between growth-factor-independence and the antiapoptotic effect induced by BCR–ABL in hematopoietic cell lines are unclear. Experiments using a BCR–ABL deletion mutant (delta 176–427) in murine 32D cells have shown that the antiapoptotic effect of BCR–ABL could be dissociated from its proliferative effect with activation of Ras and inhibition apoptosis but absence of growth-factor-independence (Cortex *et al*., 1995). On the other hand, growth-factor-independence is probably not an absolute requirement for the antiapoptotic action of BCR–ABL, as v-ABL has been shown to protect the autonomously growing cell line HL-60 from FAS-induced apoptosis (McGahon *et al*., 1995). There is however, growing evidence indicating that BCR–ABL could use the same signaling pathways as some growth-factor-receptors to induce cell transformation. First, BCR–ABL has been shown to lead to constitutive activation of *ras* pathway through binding of GRB-2 to Y177 of the BCR–ABL molecule (Pendergast *et al*., 1993b). A number of cytokines such as IL-3, GM-CSF and Steel factor activate also the *ras* pathway by tyrosine phosphorylation of SHC and its binding to GRB2, leading to an increase of *ras*-GTP, which in turn activates Raf and the MAP-kinase (Sato *et al*., 1993). However, although *ras* activation is sufficient to induce a proliferative response in fibroblastes such as 3T3 cells (Marshall, 1995), IL-3 or Steel-factor-induced *ras* activation in hematopoietic cells can preferentially induce a survival or proliferative response depending on the cytokine used (O'Farrell *et al*., 1996). Similarly, inhibition of *ras* pathway by a dominant-negative *ras* in 32D cells blocks the IL-3-induced proliferation without interfering with the differentiation induced by G-CSF (Okuda *et al*., 1994).

The second evidence of the involvement of the BCR–ABL in the growth-factor-induced signaling pathways has been obtained by experiments demonstrating constitutive activation of STAT molecules in hematopoietic cell lines after v-ABL or BCR–ABL gene transfer (Danial *et al*., 1995; Carlesso *et al*., 1996) or the necessity of an intact phosphatidylinositol 3′ kinase pathway for obtention of a transforming effect (Skorski *et al*., 1995). There is no evidence yet linking disruption of these pathways to the reversal of the anti-apoptotic effect induced by BCR–ABL.

In order to study the relationships between growth-factor-independence and apoptosis, we studied in the same cellular context the effects of two genes rendering Ba/F3 cells factor-independent, i.e., BCR–ABL oncogenes and constitutively activated cEpoR (Watowich *et al*., 1992). The cell lines transfected with p210-BCR–ABL, p190-BCR–ABL and cEpoR were growth-factor-independent and their response to growth-factor-deprivation was essentially identical with evidence of protection from apoptosis whereas Ba/F3 cells transfected with a NeoR construct showed significant degree of apoptosis in response to growth-factor-deprivation. However, there was a significant discrepancy between the Ba/F3-cEpoR cells and Ba/F3 p210 or p190 cells in response to serum deprivation which induced apoptosis in Ba/F3-cEpoR cells whereas Ba/F3-p210 and p190 were resistant (Figure 4). Persistent activation of *ras* pathway in cells expressing BCR–ABL (Puil *et al*., 1994) could probably not explain this difference as there is evidence indicating that hyperexpression of RAS and Rho could predispose cells to serum-deprivation-induced-apoptosis (Esteve *et al*., 1995; Ferrari and Greene, 1994). To determine if Ba/F3-cEpoR cells, as opposed to Ba/F3-p210 and Ba/F3-p190, had truly no resistance to apoptosis despite their growth-factor-independence, we have used etoposide as an apoptotic agent. A polyclonal stock of Ba/F3-cEpoR cells as well as all four growth-factor-independent Ba/F3-cEpoR clones expressing significant amounts of cEpoR at their surface were not able to resist to etoposide-induced apoptosis (Figure 4). Overall, these results suggest strongly that in Ba/F3 cell line antiapoptotic effects and growth-factor-independence can be induced by different signaling events. In order to determine if this discordance could be due to the differential expression of molecules of the Bc12/Bax family, we have performed Western analyses using total cell lysates from our different clones. The results of these experiments indicate that there is no significant difference of Bc12 expression between the apoptosis-resistant BCR–ABL and the apoptosis-sensitive cEpoR-transfectants, although our parental Ba/F3 cells seemed to express lower levels of Bc12 (Figure 5). Similarly, there was no significant difference between the BCR–ABL and cEpoR clones in terms of Bax expression (Figure 6). Currently, the only experimental evidence suggesting a direct relationship between BCR–ABL induced cell-transformation and Bc12-activation is the inhibition of the growth-factor-independent and tumorigenic phenotype of Ba/F3-BCR–ABL transfectants by the use of an enforced expression of an anti-Bc12 expression vector (Sanchez-Garcia and Grutz 1995). Although our experiments confirm the increased expression of Bc12 in cells transformed by BCR–ABL oncogenes, we show here that Ba/F3-cEpoR clones seem to express also a significant amount of Bc12 protein (Figure 5). This finding is interesting as Bc12 has been shown to be increasingly implicated in growth-factor-receptor-signaling pathways such as the IL2-receptor signaling (Miyazaki *et al*., 1995). More recently, experiments with fusion proteins CD16/JAK2 in Ba/F3 cells demonstrated that the activation JAK2 kinase by crosslinking of CD16, induces Bc12 expression with inhibition of apoptosis (Sakai and Kraft, 1997).

In our study, we have also asked whether the differential behavior of BaF3-cEpoR and BCR–ABL transfectants could be due to the different STAT activation induced by these two types of oncogenes. Our results confirm previous findings (Carlesso *et al*., 1996; Ilaria *et al*., 1996) demonstrating a constitutive activation of STAT factors (STAT 1, 5 and 6) by BCR–ABL. In contrast, growth-factor-independent Ba/F3 cEpoR polyclonal cells and two independent clones expressing the highest levels of cEpoR at their surface were not able to induce a DNA binding activity on either SIE, IRF-1 or beta-casein STAT-responsive sequences (Figure 7). Although the absence of STAT 1 activity in the nuclear extracts of cEpoR-transfected cells is not surprising, the absence of detectable STAT5 binding activity is an intriguing finding as STAT5 is clearly involved in Epo-induced signaling (Gouilleux *et al*., 1995; Gobert *et al*., 1996; Sawyer and Penta, 1996). One possibility is the presence, in our cEpoR-transfectants, of a low level of STAT5 activation which is not detectable by the currently used oligonucleotide binding assays. This low level of activation would then be sufficient to lead to a growth-factor-independent phenotype and protection from apoptosis in the absence of growth factor but would not be able to provide signals sufficient to protect cEpoR-transfected cells from etoposide and serum-deprivation-induced-apoptosis.

The precise mechanisms of growth factor independence induced by BCR–ABL in hematopoietic cell lines are unknown. It is still unclear whether this effect has any relevance with regard to human CML, either at diagnosis or during disease progression. As a matter of fact there is no growth factor independence in primary clonogenic CML cells except for some subtle abnormalities such as Epo-independent behavior (Eaves and Eaves, 1987) or Steel factor-induced erythroid differentiation (Issaad and Vainchenker, 1994). How and if STAT activation has any relevance with regard to the antiapoptotic effect of BCR–ABL remains to be determined and awaits experiments with either antisense STAT oligonucleotides or dominant-negative STAT molecules in BCR–ABL expressing hematopoietic cells. Our results in Ba/F3 cell line indicate that growth factor independence and inhibition of apoptosis can be differentially induced by two oncogenes (i.e., BCR–ABL and cEpoR) and are accompanied by a differential activation of STAT molecules in the same cellular context. They also suggest that STAT factor activation might be a major effect underlying the antiapoptotic potential of BCR–ABL. There is recent experimental evidence linking inhibition of STAT factors to apoptosis

(Bright *et al*., 1997; Naka *et al*., 1997). Our results provide therefore a rationale for further study of the involvement of STAT factors in the antiapoptotic action of BCR–ABL oncogenes.

Materials and methods

Cells

Ba/F3 cell line was generously provided by G Krystal (Terry Fox Laboratory) and maintained in RPMI containing 10% FCS and 10% WEHI-CM. For some experiments the recombinant mIL-3 was used at a concentration of 10 ng/ml.

Plasmids

Full-length cDNA for p210 BCR–ABL was provided by Dr RK Humphries (Terry Fox Laboratory, Vancouver, Canada). cDNA for p190 BCR–ABL was obtained from E Canaani (Weizmann Institute of Science, Rehovot, Israel). The pXMcEpoR plasmid containing cEpo-R cDNA subcloned between *Kpn*I and *Eco*RI sites was obtained from Dr Gregory Longmore (Washington University School of Medicine, St Louis, MO).

Construction of retroviral vectors

Murine stem cell vectors (MSCV) developed and generously provided by Dr RG Hawley (Toronto) were used in all gene transfer experiments. These vectors contain modifications of their 5′ and 3′ LTR's and allow high levels of heterologous gene expression in ES cells (Hawley *et al*., 1994). MSCV-p210 vectors were constructed by subcloning the 6.5 kb *Eco*RI fragment from the pK562 plasmid into the *Eco*RI site of the MSCV-Neo2.1 vector in the 5′–3′ orientation. To construct MSCV p190, we have used the pSP65–p190 plasmid which contained the full length cDNA cloned between *Eco*RI and *Hind*III. The *Hind*III site was first transformed into an *Eco*RI site using *Eco*RI linkers (New England Biolabs). The resulting plasmid was digested by *Eco*RI and the 6 kb fragment was subcloned in the 5′–3′ orientation into the dephosphorylated MSCVNeo 2.1 construct. To construct MSCV-cEPOR, the *Kpn*I site of pXMcEPOR was transformed into an *Eco*RI site using *Eco*RI linkers and the resulting 1.6 kb *Eco*RI-*Eco*RI fragment was subcloned into the *Eco*RI site of MSCV Neo 2.1. All plasmids were purified using ultracentrifugation through CsC1 gradients.

Electroporation and cell cloning

Gene transfer into Ba/F3 cells was accomplished using a Cell-ject electroporator (Eurogentec, Brussels, Belgium) 20–30 μg of purified plasmid were electroporated into 5. 10^6 cells using the following protocol: Cells were fed the day prior to electroporation (250 V, 1500 microF). Following a medium change performed 24 h later, cells were expanded for another 24 h before being subjected to a G418 selection (1 mg/ml, Life Technologies, MD) in a semi-solid medium (Methylcellulose, Stem cell Technologies, Inc, Vancouver, Canada) at day 2, either in the presence of G418 (1 mg/ml) plus 10% WEHI-CM or in the absence of both. Cell concentrations ranging from 1000 ml up to 10 000 per ml were used to determine cloning and gene transfer efficiencies. Colonies growing in the presence of G418 (1.0 mg/ml) and 10% WEHI-CM, were individually plucked and amplified. A fraction of each clone was then frozen and cells were tested for growth-factor-independence.

Epo-binding assays

In order to determine if the transfected EpoR construct was effectively expressed at the cell surface of Ba/F3 cells, we have used a radioactive Epo binding assay. Parental Ba/F3 cells, polyclonal cEpoR cells as well as four individual Ba/F3 clones which we rendered growth-factor-independent by transfection of the cEpoR, were incubated for 1 h at 37°C with 2 nM iodinated Epo. Non-specific binding was determined by incubating the cells with 500 μM unlabeled Epo. After this period of incubation, cells were washed three times with ice cold phosphate buffer saline and the radioactivity associated with cells was measured by gamma counting.

Cell proliferation assays

Cell proliferation assays were performed using standard thymidine incorporation technique in 96-well plates. 10^4 cells were cultured for 48 h with and without growth factors at 37°C and then incubated with ^{3}H-TdR (Amersham) for 18 h at a concentration of 1 mCi per well. Thymidine incorporation was determined using a Betaplate counter (LKB Wallac; St Quentin-en-Yvelines, France).

Western blotting

0.4–1 10^6 cells were lysed in Laemmli lysis buffer, loaded on a 7.5% (BCR–ABL) or 12.5% (Bc12/Bax) SDS-polyacrylamide gel, separated by electrophoresis, and electrotransferred onto nitrocellulose filters. Blots were incubated in 5% milk, 10 mM Tris, 137 mM NaCl, 0.1% Tween (blocking solution) for 2 h before adding the antibody (anti-*abl*, Ab-3, Oncogene Science; Polyclonal anti-bc12 and Polyclonal anti-Bax, Santa Cruz Inc Tebu, France) for 18 h at 4°C. Immune complexes were detected by enhanced chemiluminescence detection system (ECL, Amersham Corp. Arlington Heights, IL) using horse radish peroxydase coupled antibodies (anti-mouse IgG1 and for BCR–ABL an anti-rabbit-IgG for Bc12 and Bax) (Amersham).

Electrophoretic mobility shift assay (EMSA)

Permanently growing Ba/F3 cells expressing BCR–ABL or cEpoR were washed in serum-free medium and nuclear extracts were prepared and incubated with 10–15 fmol of a ^{32}P-labeled oligonucleotide as previously described (Pallard *et al*., 1995). As a control, Ba/F3-NeoR cells were deprived of cytokine-containing medium for 18 h and incubated in the presence of recombinant IL-3 (10 ng/ml) or IFN-γ (1000 U/ml) for 10 min. After washing, nuclear extracts were prepared from the control and transfectant clones. Each oligonucleotide probe (SIEm67, 5′-CAT TTCCCGTAAATC-3′; IRF1-GAS 5′-GATCCATTTC CCCGAAATGA-3′; β-Casein-GAS 5′-AGATTCTAG-GAATTCAAATC-3′) was end-labeled using T4 polynucleotide kinase to a specific activity of 8000 c.p.m./fmol (Pallard *et al*., 1995).

Induction of apoptosis

To induce apoptotic death in different cell lines, we have used stimuli including serum deprivation, growth factor deprivation (IL-3 for Ba/F3 and Epo for Ba/F3 transfected with the wild-type EpoR) or etoposide. To determine the optimal concentration of etoposide in parental Ba/F3 cell line, preliminary experiments were performed using etoposide doses ranging from 5 μg/ml to 30 μg/ml. The concentration of 20 μg/ml was found to induce apoptotic death in >95% of cells as determined by the quantitative FACS analysis. Therefore, this concentration was used in

all experiments. To induce apoptosis by growth factor or serum deprivation, cells were washed twice in PBS and were grown in RPMI supplemented with Glutamax (Gibco BRL-Life Technologies, Paisley, Scotland) with or without addition of 10% WEHI-CM for 48 h and then tested for apoptosis. To determine if the apoptotic effect was not due to accumulation of free radicals in the RPMI medium rather than to the serum deprivation, each experiment was repeated in RPMI medium reconstituted prior to the experiment from powder according to the manufacturer's recommendatinos (Gibco BRL-Life Technologies, Paisley, Scotland).

Quantitative FACS-based assay for apoptosis

This assay was performed as described by Nicoletti *et al*., 1991. Briefly, 2.5. 10^6 Ba/F3 cells treated with either etoposide (20 microgrammes per ml) or grown in the absence of growth factors or serum, were centrifuged and washed once in PBS. They were suspended in a 1.5 ml solution containing Triton-X 100 0.1%, NaCitrate 0.1%, and propidium iodide 50 microgrammes/ml (Sigma) in polypropylene tubes at 4°C overnight. The samples were analysed the following day using a FACScan flow cytometer (Becton and Dickinson, Mountain View, CA, USA). Apoptotic nuclei appeared to be easily distinguishable and quantifiable in this assay due to high low side scatter signal generated by the condensed nuclear chromatin.

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