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Role of *c-jun* in the Inhibition of Erythropoietin Receptor-Mediated Apoptosis

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Human bone marrow cells express both a truncated and full-length form of the erythropoietin receptor (EpoR-T and EpoR-F, respectively). Transfection experiments using the murine interleukin (IL)-3-dependent cell line, Ba/F3, revealed that the cells coexpressing EpoR-F and EpoR-T (Ba/F3-FT) were more likely to undergo programmed cell death (apoptosis) than cells expressing EpoR-F (Ba/F3-FF), even in the presence of erythropoietin (Epo). When Ba/F3-FF cells were stimulated with Epo or IL-3, rapid induction of *c-myc, c-fos, c-jun* and *junB* genes was observed. A similar effect was also seen in IL-3-stimulated Ba/F3-Ft cells. However, in Ba/F3-FT cells expression of the *c-jun* gene was not induced by Epo stimulation. Administration of Epo could prevent apoptosis induced by IL-3 deprivation in Ba/F3-FT cells expressing ectopic c-Jun protein. These results indicate that induction of c-Jun through the Epo signaling pathway has an important role in the inhibition of apoptosis. © 1996 Academic Press, Inc.

Erythropoietin (Epo), a major regulator of mammalian erythropoiesis, promotes the proliferation of erythroid progenitor cells and their subsequent terminal differentiation to circulating erythrocytes (1). cDNAs for the human and murine Epo receptors (EpoRs) have been cloned (2, 3). In a previous study we have isolated a truncated EpoR cDNA from normal human bone marrow cells and found that the truncated EpoR was encoded by an alternatively spliced form of the EpoR mRNA (4, 5). The human EpoR gene consists of eight exons and seven introns (6). Intron 7 contains an inframe stop codon. When intron 7 is not spliced out, the message encodes a truncated EpoR that lacks most of the cytoplasmic region except for 56 amino acid residues encoded by exon 7 and intron 7. The truncated form (EpoR-T) is predominantly expressed in immature erythroid progenitor cells, while the full-length form (EpoR-F) is dominant in relatively mature erythroid progenitor cells. When compared with Ba/F3 cells transfected with the EpoR-F cDNA alone (Ba/F3-FF), cells cotransfected with the EpoR-T and EpoR-F cDNAs (Ba/F3-FT), were more likely to undergo apoptosis at lower concentrations of Epo (4, 5).

Apoptosis or programmed cell death is characterized by chromatin condensation, DNA fragmentation, and cytoplasmic blebbing (7). It appears to be an active process that requires induction of early response genes such as *c-fos* and *c-jun.* These genes have been associated with apoptotic cell death induced by growth factor deprivation in lymphoid cell lines (8). Moreover, it has been reported that apoptotic cell death *in vivo* is preceded by continuous *c-fos* expression (9). It has also been shown that *c-myc* induction activates apoptosis, while *bcl-2* overexpression prevents apoptosis (10). Thus, the expression of these protooncogenes may represent an important early event in the activation or inhibition of apoptosis. In this study we show that c-Jun is required for prevention of apoptosis mediated by the Epo signaling pathway.

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MATERIALS AND METHODS

Cell culture. Ba/F3-FF cells and Ba/F3-FT cells were established from IL-3 dependent murine Ba/F3 cells. Ba/F3-FF cells have full-length homodimers of the EpoR and Ba/F3-FT cells have heterodimers of full-length and truncated types of the EpoR (4, 5). The amount of full-length and truncated EpoR expressed on Ba/F3-FT cells was estimated to be almost equal by the reverse transcriptase polymerase chain reaction (RT-PCR) method(5). Both transfectants were maintained in RPMI-1640 medium supplemented with 10% FCS and IL-3 (1ng/ml).

Isolation of RNA and Northern blot analysis. Ba/F3-FF and Ba/F3-FT cells were maintained in RPMI-1640 medium supplemented with 10% FCS and IL-3 (1 ng/ml). After 7 hr of growth factor deprivation, IL-3 (1 ng/ml) or Epo (1 U/ml) was added to the culture. At the indicated times the cells were harvested and total cellular RNA was prepared by the acid guanidinium thiocyanate-phenol-chloroform method (11). RNA samples (25 μg/lane) were electrophoresed on a 1.0% formaldehyde-agarose gel and transferred to Zeta-Probe blotting membrane (Bio-Rad Laboratories, Richmond, CA). Hybridization was carried out according to the manufacturer's manual. The probes were labeled with a-[^{32}P] dCTP by the random priming method. The same membranes were rehybridized with each probe after stripping of the membrane.

DNA fragmentation assay. The DNA fragmentation analysis was a modification of the method of Sellins and Cohen (12). In brief, 1.5×10^6 cells cultured under various conditions were washed in RPMI-1640 and pelleted in a microfuge tube. The cells were gently resuspended in 400 μL of hypotonic lysing buffer (10 mM Tris, pH 7.5, 1 mM EDTA, 0.2% Triton X-100) and slowly rocked for 30 minutes at 4°C. Nuclei were pelleted by centrifugation at 12,000×g for 15 minutes and the supernatants were transferred to a new microfuge tube. DNA was precipitated from supernatants with 50% isopropanol and 0.5 M NaCl, pelleted at 12,000×g for 15 minutes, dried, and resuspended in loading buffer (3 mM EDTA, 0.4% SDS, 15% glycerol, 0.1% bromophenol blue) with RNAse (100 μg/ml, Sigma). The samples were loaded on a 2% agarose gel, and DNA was visualized with ethidium bromide.

Gene transfer. Ba/F3 cells were transfected with mammalian expression vector (pcDEBΔ) alone or pcDEBD containing *c-jun* cDNA (pcDEBΔ*c-jun*) (13) by a conventional electroporation procedure (250V, 960 μFD). These transfectants were termed Ba/F3-FTV and Ba/F3-FTJ, respectively. We selected five independent Ba/F3-FTJ and three independent Ba/F3-FTV clones resistant to hygromycin B (1 mg/ml).

RESULTS

To clarify the mechanism of the susceptibility of the Ba/F3-FT cells to apoptosis even in the presence of Epo, we compared the Ba/F3-FT and Ba/F3-FF cells in forms of their expression of the *c-fos*, *c-jun*, *junB* and *c-myc* oncogenes after Epo or IL-3 stimulation. These transfectants were passaged in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) and IL-3 (1 ng/ml). After 7 hours of culture without growth factors, the cells were stimulated with IL-3 (1 ng/ml) or Epo (1 U/ml) for various times up to 90 min and then harvested for the isolation of total cellular RNA. When the two transfected cell lines were stimulated with IL-3, rapid induction of *c-myc*, *c-fos*, *c-jun*, and *junB* genes occurred during this observation period and with the same kinetics in both cell lines (Fig.1A). Epo stimulation also induced the rapid expression of *c-myc* and *junB* genes in both Ba/F3-FF and Ba/F3-FT cells (Fig. 1B). The kinetics as similar to the case of

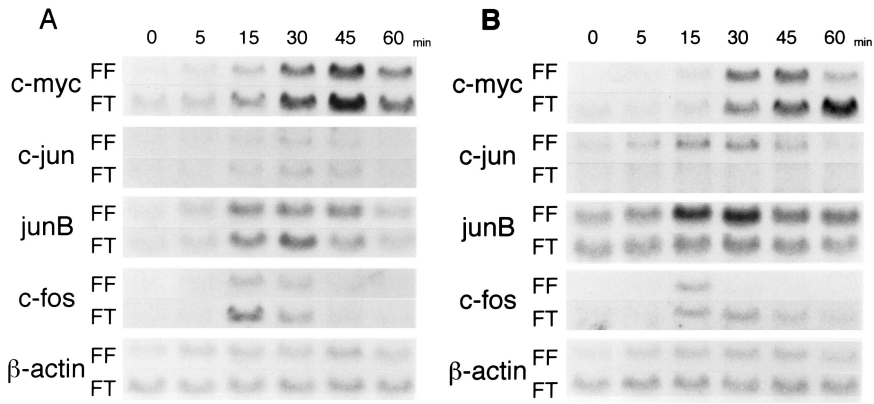

FIG. 1. Expression of *c-myc*, *c-jun*, *junB* and *c-fos* messenger RNA in Ba/F3-FF and Ba/F3-FT cells following stimulation by IL-3 (1 ng/ml; A) or Epo (1 U/ml; B). Bovine β-actin cDNA fragment was used as a quantitative standard.

IL-3 stimulation suggested that early induction of these two genes is a common event in Epo and IL-3 stimulation, but not specific to the Epo or IL-3 signaling pathway. However, Epo-induced *c-jun* expression in Ba/F3-FT cells was completely different from that in Ba/F3-FF cells. The *c-jun* gene expression in Ba/F3-FF cells was rapidly and transiently induced by Epo stimulation (Fig.1B), while no effect was seen in the Ba/F3-FT cells. To exclude the possibility that the lack of *c-jun* gene induction was due to diminished sensitivity to Epo, we added an excess of Epo (10 U/ml) to the Ba/F3-FT cells, and *c-jun* transcripts were still undetectable (data not shown). Since both Ba/F3-FF and Ba/F3-FT cells can proliferate in response to EPO (4, 5), our observations suggested that induction of *c-jun* was not essential for Epo-induced proliferation.

We then isolated DNA from the cells before and after 24 hr of IL-3 deprivation to look for the internucleosomal DNA cleavage characteristic of apoptosis. DNA fragmentation was observed to the same degree in both Ba/F3-FT and Ba/F3-FF cells deprived of IL-3. The addition of Epo immediately after IL-3 deprivation inhibited the formation of the DNA ladder in Ba/F3-FF cells but not in Ba/F3-FT cells (Fig. 2). This finding suggested that the truncated EpoR could not mediate the signal for inhibition of apoptosis as could the full-length EpoR.

These results raised the possibility of *c-jun* involvement in the inhibition of apoptosis in this system. Consequently, an expression vector encoding the c-Jun protein was introduced into the Ba/F3-FT cells (Fig. 3A), and cell death profiles of triple transfectants were analyzed. Epo could not inhibit the formation of DNA fragmentation in control Ba/F3-FT cells transfected with vector alone (Fig. 3B, lane 3). However, DNA fragmentation in the presence of Epo was significantly diminished in Ba/F3-FT cells harboring the c-Jun plasmid (Fig. 3B, lane 6), suggesting that the *c-jun* transfected Ba/F3-FT cells had acquired the capacity to inhibit IL-3 deprivation-induced apoptosis. To assess the effect of Epo on the survival of Ba/F3-FT cells in culture without IL-3, Epo was added to the culture immediately after IL-3 deprivation. Trypan blue staining indicated that *c-jun* cDNA transfected Ba/F3-FT cells remained viable, but the transfectant harboring control vector alone and the parental Ba/F3-FT cells did not (Fig. 3C). There was no significant difference in responsiveness to IL-3 between the transfectants and their parent cells (data not shown). Moreover, ectopic expression of c-Jun did not inhibit apoptosis induced by IL-3 deprivation in Ba/F3-FT cells (Fig. 3B and C), suggesting that ectopic expression of c-Jun did not affect the IL-3 signaling pathway. Taken together, our data indicate that *c-jun* gene induction by Epo is a critical event in the inhibition of apoptosis that is mediated by the Epo signaling pathway.

DISCUSSION

Several early response genes, including *c-myc*, *c-jun* and *c-fos*, are commonly induced by stimulation with various cytokines. Induction of *c-myc* is critical for progression of the cell cycle

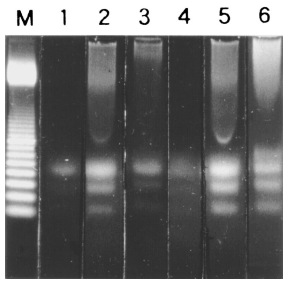

FIG. 2. Gel electrophoretic analysis of low molecular weight DNAs isolated from cytokine-deprived Ba/F3-FF and Ba/F3-FT cells. Ba/F3-FF (lanes 1–3) and Ba/F3-FT (lanes 4–6) cells were maintained in medium supplemented with 10% FCS and IL-3 (1 ng/ml). These cells were harvested before (lanes 1 and 4) or after incubation for 24 hours with 10% FCS alone (lanes 2 and 5) or both 10% FCS and Epo (1 U/ml) (lanes 3 and 6). Each analysis was performed at least twice and similar results were obtained.

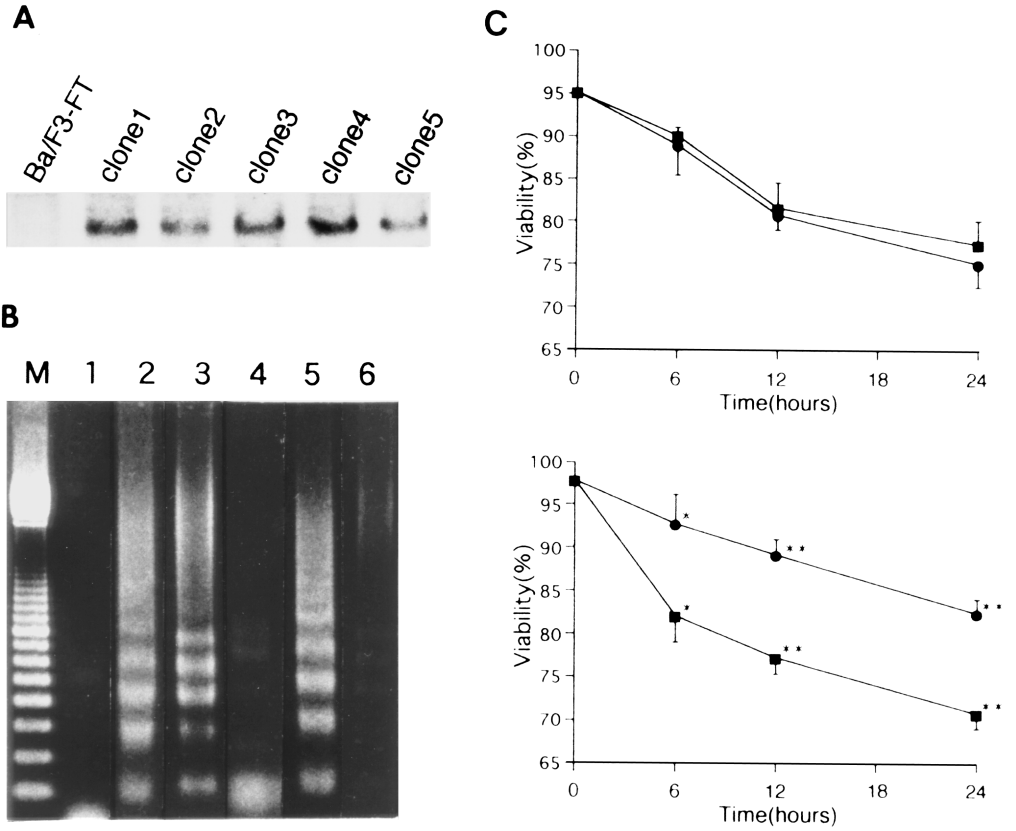

FIG. 3. (A) Constitutive expression of the *c-jun* gene in Ba/F3-FTJ clones. These transfectants were maintained with IL-3 (1 ng/ml) and harvested for isolation of RNA. Total cellular RNA was extracted, and northern blot analysis was performed using a ^{32}P-labeled c-jun cDNA as described in materials and methods. Five independent *c-jun* transfectants are shown as clones 1–5. (B) Gel electrophoretic analysis of low molecular weight DNAs isolated from Ba/F3-FTV and Ba/F3-FTJ cells. We performed DNA fragmentation analysis as described. These transfectants were cultured with IL-3 (1 ng/ml) (lanes 1 and 4), or medium alone (lanes 2 and 5), or Epo (lanes 3 and 6). After incubation for 24 hours, the cells were harvested for extraction of DNA. Representative data from Ba/F3-FTV clone 1 (lanes 1–3) and Ba/F3-FTJ clone 1 (lanes 4–6) are shown. Similar results were obtained from other clones. (C) Kinetics of cell viability after IL-3 deprivation or addition of Epo in Ba/F3-FTV and Ba/F3-FTJ clones. The cells were maintained with RPMI-1640 supplemented with 10% FCS and IL-3 (1 ng/ml). After washing IL-3 from the culture medium, the cells were cultured without (■) or with Epo (1 U/ml) (●), and harvested at the indicated time to determine cell viability by the trypan blue exclusion technique. All experiments were performed in duplicate and similar results were obtained from all clones. Representative data are shown (upper panel, Ba/F3-FTV clone 1; lower panel, Ba/F3-FTJ clone 1). The data are represented as the mean ± standard deviation (n = 3). *p<0.05, **p<0.005.

from the late G1 to S phase in the process of cell proliferation (14). However, it seems that *c-fos* and *c-jun* induction is not always essential to the proliferation, and the biological significance of the induction of these genes is not well understood. In this paper we show that *c-jun* gene induction mediated through the Epo signaling pathway is a key event in the inhibition of apoptosis.

Recently it has been reported that induction of the activated *ras* gene complemented the impaired signaling downstream at the mutant GM-CSF receptor b chain, a receptor defective in the activation of Ras, Raf-1 and mitogen-activated protein (MAP) kinase. This resulted in the prevention of apoptosis (15). Continuous activation of Ras appears to prevent apoptotic cell death induced by nerve growth factor (NGF) deprivation in the NGF-responsive cell line, PC12, and neurons (16, 17). Taken together, these results suggest that Ras activation is involved, at least in part, in the

inhibition of apoptosis. Activated Ras can also activate transcription of the *c-jun, c-myc* and *c-fos* genes through an independent pathway that includes Jun N-terminal kinases (JNKs) and extracellular signal-regulated kinases (ERKs), members of the MAP kinase family (18–21). These findings strongly support our data that *c-jun* is involved in the inhibition of apoptosis.

However, *c-jun* induction is likely to have the opposite effect on apoptosis under some circumstances. Induction of the *c-jun* gene has also been observed in the process of apoptotic cell death caused by growth factor deprivation (8) unpublished data and DNA-damaging agents such as tumor necrosis factor (TNF) or ultraviolet light (22, 23). Moreover, it has been reported that treatment with a *c-jun* antisense oligonucleotide inhibited apoptotic cell death of lymphoid cells induced by growth factor deprivation (8). These observations suggest the importance of *c-jun* gene induction in the promotion of apoptosis. On the contrary, the present findings provide evidence for the importance of *c-jun* gene induction in the anti-apoptotic effect mediated through the Epo signaling pathway. Based on these observations and the present data, we suggest that in spite of a key role of *c-jun* in apoptosis, this molecule may not be a determinant factor of apoptosis. This hypothesis is also supported by our observation that ectopic expression of c-Jun did not directly affect apoptosis induced by IL-3 deprivation. In fact, it is well known that c-Jun acts as a transcription factor. It forms homodimers or heterodimers with a variety of proteins. These proteins include those encoded by *c-jun/c-fos* related genes, many kinds of basic-leucine zipper (bZip) proteins and activating transcription factor (ATF), NF-E2 p45, Maf and related molecules. These protein complexes bind to a consensus DNA sequence termed the activation protein-1 (AP-1) site or related sequences, and function as a positive or negative regulatory factor for a variety of cell promoters (24–28). In addition, the transcriptional activity of the AP-1 complex seems to be modulated by the phosphorylation state of the c-Jun molecule (28). Therefore, interaction of c-Jun with other molecules or itself under appropriate circumstances may induce or repress expression of apoptosis-associated gene(s).

Many apoptosis related genes have been identified. For example, ectopic c-Myc expression induces apoptosis of fibroblasts (29), while Bcl-2 inhibits apoptosis of several cell lines (30, 31). Recently, it has been reported that there are three distinct signaling pathways linked to the IL-2 receptor. These consist of the *c-fos/c-jun, bcl-2, and c-myc* gene induction pathways, and cooperation or crosstalk between these pathways leads to maximum growth (32). Although there were no significant differences in the kinetics of *bcl-2* and *c-myc* gene induction after Epo stimulation of Ba/F3-FF and Ba/F3-FT cells (Fig. 1, data not shown), we cannot exclude the possibility that *bcl-2, c-myc*, and same as yet unidentified gene(s) may be involved in the apoptosis observed in Ba/F3-FT cells. This may explain the finding that ectopic expression of c-Jun could not completely rescue Epo-stimulated Ba/F3-FT cells from IL-3 deprivation-induced apoptosis (Fig. 3B, lane 6).

It is likely that Epo stimulation can initiate both mitogenic and anti-apoptotic pathways. Recent studies have stressed the dominant negative effect of the truncated EpoR (deletion of 221 amino acids in the cytoplasmic region) on proliferation through inactivation of the JAK2 tyrosine kinase (33). However, we propose that the truncated EpoR may also function as a dominant negative regulator of an anti-apoptotic pathway, since our *in vitro* experiments showed that apoptosis occurs even in the presence of Epo in Ba/F3-FT cells.

In conclusion, a dominant negative effect of the truncated EpoR may provide the explanation for the fact that cells expressing the truncated form easily undergo apoptosis even in the presence of Epo.

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