Positive Selection of Apoptosis-resistant Cells Correlates with Activation of Dominant-Negative STAT5*

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The STAT5 activation has important roles in cell differentiation, cell cycle control, and development. However, the potential implications of STAT5 in the control of apoptosis remain unexplored. To evaluate any possible link between the erythropoietin receptor (EpoR) JAK2/STAT5 transduction pathway and apoptosis, we have investigated apoptosis-resistant cells (ApoR) that arose from positive selection of the erythroid-committed Ba/F3EpoR cells triggered to apoptosis by ectopic expression of the HOX-B8 homeotic gene. We show that JAK2 is normally activated by Epo in both Ba/F3EpoR and ApoR cells. In contrast, both STAT5a and STAT5b isoforms are uniquely activated in a C-truncated form (86 kDa) only in ApoR cells. Analysis of ApoR and Ba/F3EpoR subclones confirmed that the switch to the truncated STAT5 isoform coincides with apoptosis survival and that ApoR do not derive from preexisting cells with a shortened STAT5. In addition, ApoR cells die in the absence of Epo. This indicates that resistance to apoptosis is not because of a general defect in the apoptotic pathway of ApoR cells. Furthermore, we show that the 86-kDa STAT5 protein presents a dominant-negative (DN) character. We hypothesize that the switch to a DN STAT5 may be part of a mechanism that allows ApoR cells to be selectively advantaged during apoptosis. In conclusion, we provide evidence for a functional correlation between a naturally occurring DN STAT5 and a biological response.

Erythropoietin (Epo)1 regulates both the growth and differentiation of immature erythroblasts through its interaction with specific, high affinity cell surface receptor (EpoR). EpoR belongs to the cytokine receptor superfamily (1, 2) and associates with JAK2, a member of the Janus protein tyrosine kinases, through the receptor cytoplasmic, membrane-proximal domain containing the conserved box1 and box2 motifs (3). The activated JAK2 phosphorylates tyrosine residues on the cytoplasmic tail of the receptors that serve as docking sites for the SH2 domain that occur in STAT5 (4, 5), a member of the signal transducer and activator of transcription (STAT) proteins (6, 7). STAT5 dimerizes, translocates to the nucleus and promotes the expression of genes containing specific DNA elements homologous to the γ-interferon activation site (GAS) (8). STAT5 was originally identified in prolactin-mediated responses (9–11) but is activated by several other cytokines (12). In mice, there exist two STAT5 genes that are 96% identical (STAT5a and STAT5b). These encode proteins of 94 and 92 kDa, respectively (11, 13). STAT5a is the principal and also an obligate mediator of mammapoietic and lactogenic signaling (14). Studies on STAT proteins indicate that the transcriptional activation domains reside in the C terminus (15–18). Moreover, C-terminal truncated STAT5 a/b isoforms, generated by protein processing (19, 20), retain high affinity DNA-binding activity and present dominant-negative (DN) character suppressing transcription (16–17). However, the potential physiological significance of DN STAT5 is unknown at present. Another member of the STAT family, STAT1, exists in two forms of 91 and 84 kDa, and it has been demonstrated that they differ in their ability to trans-activate selected genes (21).

Apoptosis, or programmed cell death, is a genetically controlled mechanism for cells to commit suicide in response to specific stimuli such as tumor necrosis factor α (TNF-α) or the fas ligand (22, 23), or it is activated in response to various forms of cell injury or stress (24, 25). The potential role of the JAK/STAT signaling pathway in the induction of apoptosis has been poorly investigated. Recent studies report that activation of STAT1 by gamma interferon (IFN-γ) and epidermal growth factor (EGF) induced apoptosis in HeLa and A431 cells via caspase-1 activation (26), and furthermore, that TNF-α failed to induce apoptosis in STAT1-null cells because of low levels of caspases (27).

In the present study, we have investigated the EpoR JAK2/STAT5 signal transduction pathway in cells that have been positively selected during the HOX-B8-mediated apoptosis of the erythroid-committed Ba/F3EpoR cells (28). HOX-B8 is an homeobox-containing gene, and it is among a variety of transcription factors that have emerged as key components of the regulatory process for lineage-specific development of early hematopoietic cells (29). It has been recently described that HOX-B8 inhibits granulocytic differentiation as evident by rapid apoptosis of the 32DXO-B8 cells treated with G-CSF (30). Furthermore, HOX-B8 exerts a contributory role in leukemogenesis: the integration of an intracisternal A-particle (IAP) genome into the 5′ noncoding region causes the constitutive expression of HOX-B8 and interleukin-3 (IL-3) in the WEHI-3B myelomonocytic leukemia cell line (31).

We show that cells signaling through dominant-negative STAT5 are selectively advantaged during the HOX-B8-mediated apoptosis, suggesting that C-truncated STAT5 isoforms are likely to contribute to a unique biological response in the cells where they are expressed.
EXPERIMENTAL PROCEDURES

Cell Culture and Electroporations—Ba/F3 are murine IL-3-dependent immature progenitor cells (32). Ba/F3EpoR cells have been previously generated by ectopic expression of the EpoR and are irrevocably committed to erythroid differentiation (28). Ba/F3HOX-B8 and Ba/F3EpoRHOX-B8 cells have been generated by transfecting the MPZenNeo/Hox-2.4 vector (33) (now referred to as MPZenNeo/HOX-B8, according to the new nomenclature of HOX genes (34), into Ba/F3EpoR cells, followed by G418 selection in the presence of Epo. Cell subclones were obtained by limiting dilution in the presence of Epo and G418. All cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (HyClone) in 5% CO2 incubators at 37 °C. Recombinant human Epo was purchased from Boehringer Mannheim and was used at 0.2 units/ml. Conditioned medium at 10% from WEHI-3D cells was used as a source of murine IL-3.

Electroporations were performed with an Invitrogen electroporator II (350 volts, 960 microfarads). 10 μg of plasmid DNA were added to 10^7 cells before electroporation. Forty-eight h later, 800 μg/ml G418 (Sigma) was added to the culture medium.

RNA Analysis—RNA protection assays and β-globin riboprobe synthesis were performed as described previously (28). The HOX-B8 riboprobe was generated by subcloning the 300-base pair DNA fragment of the HOX-B8 cDNA into SP72 polylinker (Promega). SP6 polymerase was used to synthesize the radiolabeled antisense riboprobe.

Apoptosis Assays—Cells were harvested by centrifugation, resuspended in the appropriate culture medium at a density of 10^6/ml and allowed to settle on poly-L-lysine (Sigma)-coated glass coverslips for 15 min. The glass coverslips were washed with phosphate-buffered saline, and the cells were fixed with 4% paraformaldehyde for 5 min at room temperature and stained for 1 min with 1 mg/ml DNA-binding fluorochrome Hoechst 33258 dye. DNA fragmentation was analyzed by pulsed field gel electrophoresis as described by Walker et al. (35).

Cellular Extracts and Electrophoretic Mobility Shift Assay—Cells were starved by Epo-depletion for 5 h and subsequently stimulated with 10 units/ml of Epo for 15 min at 37 °C. To obtain whole cell extracts (WCE), cells were resuspended in a high salt buffer (20 mM Hepes, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol supplemented with 1 mM Na3VO4, 50 mM NaF and a standard protease inhibitors mixture) and lysed by three freeze-thaw cycles in liquid N2. Electrophoretic mobility shift assays (EMSA) were performed as described previously (36) using as a probe an oligonucleotide corresponding to the IFN-γ-responsive region (GRR) located within the promoter of FcγRI gene (37).

Antibodies, Immunoprecipitation, and Immunoblotting—Antibodies specific for STAT5a/b mouse monoclonal antibodies raised against amino acids 451–469 were purchased from Transduction Laboratories; two anti-STAT5a/b rabbit polyclonal antibodies, raised against amino acids 5–24 (N-20) and 17–27 (C-17) and anti-STAT1 monoclonal antibody raised against amino-terminal region, were purchased from Santa Cruz Biotechnology. Monoclonal antibodies specific for STAT5a C and STAT5b C' were purchased from R&D Systems. Anti-JAK2 antibodies and anti-phosphotyrosine 4G10 antibodies were purchased from Upstate Biotechnology.

Whole cell extracts from 7 x 10^6 cells were prepared following the same procedure used to prepare WCE for EMSA. A final NaCl concentration of 150 mM was obtained by diluting the extracts with 50 mM Tris, pH 8, and Triton X-100 was then added to make 1% of the final concentration.

Immunoprecipitation and immunoblotting were performed as described previously (38) with some modifications. WCE were immunoprecipitated with a 1:200 dilution of anti-STAT5 (N-20) antibodies overnight at +4 °C. Immunoprecipitates were washed 5 times with washing buffer and once with 1x Tris-buffered saline. In immunoblotting, anti-phosphotyrosine antibodies 4G10 and anti-STAT5 (N-20 and C-17) were used at 1:2000, whereas monoclonal antibodies anti-STAT5 were used at 1:250 dilution.

Luciferase Assays—For luciferase reporter assays, the pGL2 promoter vector (Promega) was used. pGL2 contains the luciferase gene driven by a simian virus 40 basic promoter without an enhancer. The GAS-luc. construct (kindly provided by Bernard Mathey-Prevot, Dana Farber Cancer Institute, Boston, MA) contains four tandem repeat GAS sequences from the murine β-casein promoter (core sequence: ATT-TCTAGGAAATCG) upstream of the luciferase element. Transient transfection experiments were performed as follows. 5 x 10^6 cells/condition were starved by depletion of Epo for 5 h and then transfected by electroporation with 10 μg of test plasmid. After electroporation, cells were replaced in complete medium supplemented with Epo at 1.2 units/ml. Luciferase activity, assayed using the Luciferase Assay System (Promega), was determined after 12 h of stimulation. Each construct was tested four times by independent electroporations with similar results each time.

RESULTS

Overexpression of HOX-B8 in the Progenitor Ba/F3 and in the Erythroid Committed Ba/F3EpoR Cells—The homeobox gene HOX-B8 is not expressed in Ba/F3 (not shown) and Ba/F3EpoR cells (Fig. 1). To understand whether HOX-B8 interferes with erythroid differentiation, we examined the consequences of ectopic expression of HOX-B8 on erythroid-committed Ba/F3EpoR as well as in the progenitor Ba/F3 cells. Three weeks following electroporation, G418-resistant Ba/F3HOX-B8 and Ba/F3EpoRHOX-B8 cells were available. Unexpectedly, keeping the cells in culture in the presence of Epo, a high rate of cell mortality was observed with the Ba/F3EpoRHOX-B8 cells. The same phenomenon was also observed when Ba/F3EpoRHOX-B8 cells were grown in the presence of IL-3. On the contrary, Ba/F3HOX-B8 cells grew normally. To evaluate whether mortality was because of programmed cell death, staining experiments were performed with the Hoechst 33258 fluorescent dye. The morphological features of nuclear chromatin indicative for apoptosis were observed in Ba/F3EpoRHOX-B8 cells (not shown). DNA fragmentation analysis confirmed that apoptosis was indeed the cause of cell death (not shown). However, a small proportion (approximately 3%) of cells survived apoptosis and generated an apoptosis-resistant cell population, named ApoR.

The HOX-B8 expression from the selected populations was determined. As shown in Fig. 1, similar HOX-B8 RNA levels were detected in the Ba/F3HOX-B8, Ba/F3EpoRHOX-B8, and ApoR cells compared with WEHI-3B cells that constitutively express HOX-B8 gene (31). We further determined, by RNase protection experiments, whether the selected ApoR cells main-
tained β-globin gene expression. Interestingly, β-globin transcripts were absent in ApoR, whereas Ba/F3EpoRHOX-B8 and Ba/F3EpoR cells exhibited high β-globin RNA levels (Fig. 1). To verify whether ApoR cells maintained factor-dependence for growth, Epo was removed from the culture medium. Withdrawal of Epo did not allow ApoR cell viability, indicating that the selected ApoR cells were still factor-dependent for growth.

A C-terminal Truncated STAT5 Protein Is Phosphorylated upon Epo Stimulation in Cells Surviving Apoptosis—The fact that apoptosis of the Ba/F3EpoRHOX-B8 cells occurred in the presence of Epo and that the recovered ApoR cells died if Epo was removed from the culture medium, suggested to us that the ApoR cells did not present a general defect in the apoptotic pathway. Therefore, we explored the hypothesis that the EpoR signal transduction pathway, in cells that selectively escaped apoptosis, could have undergone some modification. To study STAT5 activation, we performed EMSA experiments with a radiolabeled GRR probe and WCE from unstimulated or stimulated with Epo, Ba/F3EpoR, and ApoR cells. As shown in Fig. 2, A and B, two bands were detected in both cell types after Epo treatment. To our surprise, however, the two bands from ApoR lysates had higher intensity and migrated slightly faster than the two bands from Ba/F3EpoR lysates. To evaluate whether the retarded bands in ApoR were exclusive to Epo treatment, EMSA experiments were performed with cells stimulated with IL-3 for the same length time. The same pattern bands were observed when ApoR cells were stimulated with IL-3 (not shown).

To determine whether STAT5 is part of the DNA/protein complexes, supershift experiments were performed using antibodies that recognize the C′- and N′-terminal regions, of both STAT5a and b. As shown in Fig. 2A, C′- and N′-terminal antibodies recognized only the DNA-binding complexes in the Ba/F3EpoR cells, but not in the stimulated ApoR cells. The experiment has been repeated with an oligonucleotide sequence specific for β-casein, and the result obtained revealed the same pattern band (not shown). On the contrary, N′-STAT5a/b antibodies, even though at a concentration (dilution 1:5) higher than that of C′-STAT5a/b antibodies (dilution 1:15), completely supershifted the DNA-protein complexes from Ba/F3EpoR cells and decreased the intensity of the bands from ApoR cells (Fig. 2B). Ba/F3EpoR subclones were generated to verify whether the ApoR cells were selected from cells with a truncated STAT5 form pre-existing in the parental Ba/F3EpoR cells. As shown in Fig. 3A, none of the subclones exhibited a truncated STAT5 following Epo stimulation. ApoR subclones, positive for HOX-B8, were also isolated and analyzed for STAT5 activation. As shown in Fig. 3B, top, shows that only the ApoRcl13 presents an activated full-length STAT5. ApoRcl13 was propagated in culture, and within a few days apoptosis was observed (Fig. 3B, middle). Apoptosis-resistant Epo-dependent ApoRcl13 cells (ApoRcl13-R) were obtained, and STAT5 proteins were analyzed by Western blot with anti-STAT5a/b monoclonal antibodies raised against amino acids 451–649. As expected, the shortened STAT5 isoform, comigrating with the one in ApoR cells, was present in the ApoRcl13-R cells (Fig. 3B, bottom). To determine whether HOX-B8 could be the direct cause for the switch to the truncated STAT5 protein, independently of the erythroid program or apoptosis, WCE from a number of Ba/F3HOX-B8 subclones were analyzed. No C-truncated STAT5 was determined in any of the selected Ba/F3HOX-B8, which exhibited HOX-B8 expression levels similar to the one in the ApoR cells (not shown). These data suggest that the specific STAT5 processing observed in ApoR cells, may be related to a particular cellular condition and may not be directly linked to HOX-B8 or its downstream effectors.
The C-truncated STAT5 Protein Present a Dominant-negative Character—To further analyze STAT5 activation, immunoprecipitations with N'-STAT5a/b antibodies followed by immunoblotting with anti p-Tyr antibodies (4G10) were performed on WCE from representative Ba/F3EpoR and ApoR subclones. Interestingly, a unique phosphorylated 86-kDa band was detected in the ApoR cells (STAT5Δ), whereas two phosphorylated 92–94-kDa bands were present in Ba/F3EpoR cells (Fig. 4A). To verify whether in ApoR cells, the phosphorylated 86-kDa protein was truncated at the C'-terminal, the same filter was stripped and reprobed with C'-STAT5a/b antibodies. Immunoprecipitated STAT5 proteins were recognized by these antibodies in the parental cells (Fig. 4B), whereas in the ApoR cells, STAT5 bands were not detected. Finally, the same filter was stripped and reprobed with anti-STAT5 antibodies raised against an internal domain of the protein. These antibodies not only recognized the same 86-kDa protein detected by 4G10 in ApoR cells, but also the two 92–94-kDa proteins in Ba/F3EpoR cells (Fig. 4C). To confirm whether both STAT5a and STAT5b were switched to the truncated form, Western blot experiments were performed with monoclonal antibodies for STAT5a and STAT5b. As expected, because both monoclonal antibodies were recognizing the C terminus of STAT5, no bands were detected in ApoR cells. On the contrary, different bands corresponding to STAT5a and STAT5b were present in the Ba/F3EpoR cells (not shown). This result confirmed that both STAT5a and b were processed in ApoR cells.

Because STAT1 exists in a differently spliced, C-truncated isoform of 84 kDa that is implicated in resistance to apoptosis, we explored the hypothesis of whether STAT1 could be present in its C-truncated form in ApoR cells. Immunoblot experiments with monoclonal antibodies that recognize the amino-terminal region of STAT1, showed that STAT1 was present in its full-length form of 91 kDa in both Ba/F3EpoR and ApoR cells (not shown).

Moreover, to verify whether JAK2 was equally activated in Ba/F3EpoR and ApoR cells, immunoprecipitation with JAK2 antibodies followed by Western blot analysis with anti p-tyr 4G10 antibodies was performed. The results showed a similar phosphorylation level of JAK2 in cells before and after apoptosis (Fig. 5).

To analyze whether STAT5 in the ApoR cells presented dominant-negative properties, functional assays with a GAS-Luc construct were performed. The results, representative of four independent transfections are shown in Fig. 6. The two plasmids PGL2 and GAS-Luc were separately introduced into Ba/F3EpoR and ApoR cells. In the Ba/F3EpoR cells, it was seen that luciferase activity with the GAS-Luc construct was eight times higher than with the PGL2 vector in the same cells. In contrast, in the ApoR cells, there was a negligible increase in GAS-Luc activity even though the basal luciferase activity was one-third of that recorded in the Ba/F3EpoR cells.
DISCUSSION

Our first observation is that HOX-B8 overexpression causes apoptosis in cells that have irreversibly passed the checkpoint for erythroid maturation. But it does not do so in the immature progenitor Ba/F3 cells. The fact that the erythroid phenotype is not permissive for HOX-B8 expression is supported by the finding that cells which survived apoptosis lost β-globin transcripts. However, before apoptosis β-globin transcript is easily detectable in the parental Ba/F3EpoR and in the Ba/F3EpoRHOX-B8 cells. In line with our results, it has been recently shown that ectopic expression of HOX-B8 in hematopoietic progenitor cells negatively affects granulocyte development and positively regulates macrophage development; thus confirming that inappropriate constitutive HOX-B8 expression can alter growth, differentiation, and survival of hematopoietic cells.

Apoptosis of Ba/F3EpoRHOX-B8 cells was detected only after Neo-selection had occurred. A possible explanation for this is that a period of latency is required before the HOX-B8 interacts with cellular targets and thus exerts its functions. We also notice that HOX-B8 overexpression does not abolish the factor-dependence (IL-3 or Epo) in any of the Ba/F3-derivative cells. This supports the finding that derivative cells are not fully transformed by HOX-B8 alone (34). Accordingly, the enforced expression of HOX-B8 in bone marrow cells yields IL-3-dependent, nontumorigenic cell lines, and the progression to a fully malignant state is favored, as in WEHI-3B cells, by somatic mutations conferring autocrine production of IL-3 (34).

Studying STAT5 activation by Epo, we observe by EMSAs bands with higher intensity and faster migration in ApoR cells than in the Ba/F3EpoR cells. This is consistent with the higher affinity for the DNA target sequence of the C-truncated STAT5 isoform, as previously reported (16, 17). Accordingly, antibodies against the C-terminal STAT5 region do not supershift the complex. Thus, activation of the C-truncated STAT5 by Epo is very representative of cells that escaped apoptosis. This is confirmed by the ApoR subclone-13 that undergoes apoptosis. In fact, the derived apoptosis-resistant cells exhibit only the activated C-truncated STAT5 variant, upon Epo stimulation, and retain their Epo-dependence for growth.

Significantly, in the ApoR cells, the phosphorylated 86-kDa STAT5 does not coexist with the phosphorylated full-length 92–94-kDa STAT5a/b proteins, as previously reported for other cell lines (11, 13). Therefore, this is indicative of a complete processing occurring on all the STAT5a/b proteins present in the cells. Furthermore, the 86-kDa size that we report for the phosphorylated STAT5 form in cells that survived apoptosis does not coincide with the 77–80-kDa size previously described for the naturally occurring C-truncated STAT5 isoforms in 32Dc13 or CTLL cells (40). A possible explanation for the different size of the C-truncated STAT5 is that the 86-kDa form originates from a different STAT5 processing that might be related to the maturation state of ApoR cells. In this context, it has been reported that in immature myeloid cells, IL-3 activates the 77–80 kDa STAT5, whereas in mature myeloid cells IL-3 activates the full-length 94–96 kDa STAT5; thus indicating that IL-3 signals through multiple isoforms of STAT5, depending on the differentiation state of target cells (41). Indeed, we speculate that this might be the case for the ApoR cells that might be reverted to an immature state by HOX-B8. Nevertheless, further studies are required to better characterize the maturation stage of the ApoR.

We also show that the C-truncated STAT5 form presents a dominant-negative feature since transient expression of the GAS-luciferase construct in cells that escaped apoptosis have negligible luciferase activity. As mentioned above, we hypothesize that the 86-kDa STAT5 isoform accounts for a selective STAT5 processing, peculiar to ApoR cells, and that the inhibition of transcription by the dominant-negative STAT5 may be restricted to certain genes. Therefore it might be that alternative STAT5 processing discriminates between different functions, for example, as in ApoR cells, allowing proliferation and inhibiting apoptosis-connected functions. However, Epo, like a number of cytokines, activates a variety of signaling pathways such as the tyrosine phosphorylation of SHC and subsequent activation of the ras pathways (42) or the tyrosine phosphorylation of the p85 subunit of phosphatidylinositol 3-kinase (PI3-kinase) (43) and phospholipase C-Y1 (44). Therefore, we cannot exclude the possibility that the DN STAT5 isoform in ApoR cells may not be directly involved in the control of cell proliferation.

In conclusion, our observations that cells presenting a DN STAT5 protein are selectively advantaged during HOX-B8-mediated apoptosis led us to speculate a potential physiological role for C-truncated STAT5 isoforms. Studies are presently under way to determine the antiapoptotic activity exerted by the ApoR-DN STAT5. This could account for the role of Epo in stimulating different responses in ApoR cells, depending on the particular cell-differentiation stage.

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