Disrupted Signaling in a Mutant J 2E Cell Line That Shows Enhanced Viability, but Does Not Proliferate or Differentiate, with Erythropoietin*

Peta A. Tilbrook†, Thomas Bittorf‡†, Samantha J. Busfield, David Chappell, and S. Peter Klinken**

From the Departments of Biochemistry, University of Western Australia and Royal Perth Hospital, Perth, Western Australia 6001, Australia and the Department of Medical Biochemistry, University of Rostock, 18057 Rostock, Federal Republic of Germany

The immature erythroid J 2E cell line proliferates and terminally differentiates following erythropoietin stimulation. In contrast, the mutant J 2E-NR clone does not respond to erythropoietin by either proliferating or differentiating. Here we show that erythropoietin can act as a viability factor for both the J 2E and J 2E-NR lines, indicating that erythropoietin-initiated maturation is separable from the prevention of cell death. The inability of J 2E-NR cells to mature in response to erythropoietin was not due to a defect in the erythropoietin receptor sequence, although surface receptor numbers were reduced. Both the receptor and Janus kinase 2 were phosphorylated after erythropoietin stimulation of J 2E-NR cells. However, protein interactions with the erythropoietin receptor and Grb2 were restricted in the mutant cells. Subsequent investigation of several other signaling molecules exposed numerous alterations in J 2E-NR cells; phosphorylation changes to phosphatidylinositol 3-kinase, phospholipase Cγ, p120 GAP, and mitogen-activated protein kinases (p42 and p44) observed in erythropoietin-stimulated J 2E cells were not seen in the J 2E-NR line. These data indicate that some pathways activated during erythropoietin-induced differentiation may not be essential for the prevention of apoptosis.

Erythropoietin (Epo) is the principal regulator of red blood cell development, which acts primarily on immature erythroid precursors to maintain cell viability and promote proliferation and terminal differentiation (1–3). There are generally fewer than 1000 Epo receptors (Epo-R) on the surface of erythroid precursors (4) and the intracellular signaling events following the receptor/ligand interaction are rapidly being deciphered. The Epo-R and its associated protein Janus kinase 2 (JAK2) are phosphorylated (5–8), while the membrane protein, pp43 is dephosphorylated (9). Shc is also phosphorylated after Epo stimulation and binds to Grb2 (10). Activation of Ras, increased phosphorylation of GDP-ase-activating protein (GAP), and enhanced kinase activity of Raf-1 have also been documented (11, 12). In addition, mitogen-activated protein (MAP) kinases are stimulated in TF-1 and Ba/F3 cells, although their role in Epo-stimulated proliferation is unclear (13–15). Epo also initiates phosphorylation of p92-raf (16), and phosphatidylinositol (PI) 3-kinase associates with the phosphorylated Epo receptor via its Src homology 2 domains (17–19). Down-regulation of the receptor is achieved via association of the Src homology-PTP1 phosphatase and subsequent dephosphorylation (20).

The J 2E cell line was generated by transforming immature erythroid cells with the raf/myc-containing J 2 retrovirus (21). These cells proliferate and differentiate in response to Epo; hemoglobin is synthesized and the cells undergo morphological alterations, which culminate in a proportion of cells enucleating (21–31). However, a mutant subclone J 2E-NR, which arose spontaneously from a J 2E culture, fails to replicate faster, or mature, in response to Epo despite binding and internalizing the hormone (22). Unlike J 2E cells, GATA-1 or globin transcripts do not rise in J 2E-NR cells after Epo stimulation (27, 28); nevertheless, the hemoglobin-producing machinery is intact since exogenous hemin is able to initiate synthesis of a modest amount of the oxygen carrier. These observations indicated that a signaling defect may exist in the J 2E-NR cells which prevents Epo-induced terminal differentiation (22).

In this study we examined the capacity of Epo to (i) support the viability of J 2E and J 2E-NR cells and (ii) activate a number of signaling molecules in these cells. We report that Epo was able to enhance the survival of both J 2E and J 2E-NR cells in the absence of serum. Furthermore, following ligand binding, the Epo-R and JAK2 were phosphorylated in both lines, but significant differences were observed with several other signal transduction proteins. It was concluded that, in this system, distinct pathways may be involved in Epo-induced differentiation and cell survival.

MATERIALS AND METHODS

Cell Culture—J 2E (21) and J 2E-NR (22) cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.) at 37 °C in a humidified atmosphere of 5% CO2. Cells were stimulated with Epo (5 units/ml), sodium butyrate (500 μM), or serum (10%). Viability was determined by eosin

*To whom correspondence should be addressed: Dept. of Biochemistry, Royal Perth Hospital, G. P. O. Box X2213, Perth, Western Australia 6001, Australia. Tel.: 61-9-224-2426; Fax: 61-9-224-2491.
†Recipient of a Richard Walter Gibbon fellowship.
‡Recipient of a German Academic Exchange Service (DAAD) fellowship.
¶Recipient of a University of Western Australia postgraduate research award.

**This work was supported in part by National Health and Medical Research Council Grants 91-0171 and 93-0098 and by grants from the Raine Medical Research Foundation, the Cancer Foundation of Western Australia, and the Faculty of Medicine (University of Western Australia). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

††Recipient of a Richard Walter Gibbon fellowship.
‡‡Recipient of a German Academic Exchange Service (DAAD) fellowship.

1 The abbreviations used are: Epo, erythropoietin; PI 3-kinase, phosphatidylinositol 3-kinase; Epo-R, erythropoietin receptor; GAP, GTPase-activating protein, JAK2, Janus kinase 2; PLCγ, phospholipase Cγ; MAP, mitogen-activated protein.

2 S. J. Busfield and S. P. Klinken, unpublished observations.
RESULTS

Epo Protects J2E and J2E-NR Cells from Apoptosis—Epo stimulates erythroid precursor cells to proliferate and differentiate into erythrocytes (1–3). In addition, Epo has been shown to play an important role in maintaining the viability of immature red blood cells (36, 37). The J2E cell line undergoes enhanced replication and maturation in response to the hormone (21–31). To determine whether Epo could also protect J2E cells from programmed cell death, the hormone was added to serum-depleted cultures. Fig. 1 shows that the J2E cells died rapidly when serum was withdrawn from the culture medium. Cytochemical changes to serum-deprived J2E cells included nuclear condensation and fragmentation, membrane ruffling, and eventually cellular degeneration (Fig. 2). In addition, DNA degradation to oligonucleosomal repeat bands was observed with the removal of serum (Fig. 3). These morphological changes, together with DNA damage, are features characteristic of apoptotic cells (38, 39). Ultrastructural alterations have confirmed that these cells were undergoing apoptosis.3

Significantly, the data presented in Fig. 1 show that Epo was able to inhibit the death of J2E cells, although not as efficiently as serum. The protective effect of Epo was dose-dependent, with maximum protection from apoptosis occurring between 0.1 and 10 units/ml (data not shown). This is the same concentration range that maximally stimulates proliferation and differentiation (23). Cytocentrifuge preparations (Fig. 2) revealed that fewer apoptotic cells were present in the cultures and that the cells appeared much healthier; membrane ruffling was reduced, cells were rounded, and nuclei intact. Moreover, Epo was able to restrict the breakdown of DNA evident in serum-deprived cultures (Fig. 3). Others have also reported that Epo can reduce, but not completely prevent, DNA damage and programmed cell death of immature erythroid cells (36, 37). Thus, Epo not only induced the maturation of J2E cells in the presence of serum (21–31), but also restricted apoptosis when serum was withdrawn.

The capacity of Epo to act as a viability factor was also tested with J2E-NR cells. Despite the inability of the hormone to promote terminal differentiation (22), Epo was able to maintain the viability of the J2E-NR clone (Fig. 1). Cellular morphology was also protected (Fig. 2) and degradation of DNA suppressed (Fig. 3). These unexpected results demonstrated that Epo could still affect J2E-NR cells; although the hormone failed to elicit a proliferative/differentiative response, it was able to restrict cell death.

Epo-R and JAK2 Are Phosphorylated in J2E-NR Cells—To compare the signals delivered within Epo-stimulated J2E and J2E-NR cells, cell lysates were prepared 0–60 min after hormonal activation and tyrosine phosphorylation patterns determined. Fig. 4 shows that many more proteins were phosphorylated in J2E cells than J2E-NR cells, suggesting that some aspects of the signaling cascade were not being transmitted in the mutant clone. We speculated that the inability of the J2E-NR cells to transduce a differentiation signal lay with a
defect in the Epo-R. It had previously been shown that the number of surface receptors in J2E-NR cells was approximately half of J2E cells, although ligand affinity was unaltered (22). Immunoblotting confirmed that the J2E-NR cells synthesized much less Epo-R protein than J2E cells (Fig. 5A). Densitometric analyses from several experiments showed that there was on average 2.4 times more Epo-R protein in J2E cells than J2E-NR cells. Tyrosine phosphorylation of the receptor occurred within 5 min in the mutant clone (Fig. 5B). Significantly, maximum phosphorylation of the Epo-R in J2E cells was 2.5-fold greater than that of J2E-NR cells, indicating that the degree of receptor phosphorylation was the same in both cell types. However, the receptor was dephosphorylated more rapidly in the J2E-NR line (Figs. 4–6). JAK2 was also phosphorylated rapidly in both J2E and J2E-NR cells (Fig. 5C). Thus, the Epo-R and JAK2 were phosphorylated in J2E-NR cells upon hormonal stimulation, but the kinetics of receptor dephosphorylation differed slightly from the parental cells.

Epo-R Sequence Is Normal in J2E-NR Cells—One explanation of these data was that a mutation had occurred to the Epo-R in J2E-NR cells, which allowed phosphorylation events to occur, but prevented other interactions with signaling proteins. To ascertain whether a mutation had occurred to the Epo-R, transcripts from J2E and J2E-NR cells were converted to cDNA and sequenced. However no mutations in the J2E-NR Epo receptor were detected (data not shown). Both J2E and J2E-NR lines had sequences that were identical to that published by D’Andrea et al. (40), and nucleotide variations described by Kuramochi et al. (41) were not observed. These results indicated that the inability of J2E-NR cells to transmit signals for proliferation and differentiation was not due to a change in the sequence of the Epo-R.
Alterations to Proteins Associated with the Epo-R and Grb2 in J2E-NR Cells—To determine whether proteins associated with the Epo-R were different between J2E and J2E-NR cells, extracts were immunoprecipitated with anti-Epo-R antibodies and immunoblotted with anti-phosphotyrosine antibodies. Fig. 6 (A and B) shows the increased phosphorylation of the 72- and 75-kDa Epo-R in both lines. In addition, a 69-kDa phosphoprotein co-precipitated in both J2E and J2E-NR samples. However, two major phosphoproteins (53 and 56 kDa), associated with the receptor in J2E-NR cells, were absent from J2E cells. A 55-kDa molecule associated with the Epo-R was consistently phosphorylated 15-60 min after hormonal stimulation of J2E cells but was not seen in J2E-NR cells, even when increased amounts of protein were immunoprecipitated. In contrast, two proteins of approximately 32 and 34 kDa reproducibly appeared linked with the receptor in J2E-NR cells, but not in J2E cells.

Grb2 associates with the Epo-R (42); hence, proteins linked with this molecule were studied in J2E and J2E-NR cells after Epo stimulation. Fig. 7A shows that shortly after addition of Epo to J2E cells, phosphoproteins with approximate molecular masses of 53, 55, 56, 72, 75, 145, and 150 kDa were associated with Grb2. The 72- and 75-kDa proteins were most probably phosphorylated forms of the Epo-R, and Grb2 binding to a 145-kDa protein has been reported in Epo-stimulated DA-3 cells (42). Of all the protein interactions involving Grb2 identified in differentiating J2E cells (Fig. 7A), only the 55-kDa molecule and the Epo-R associated with Grb2 in J2E-NR cells (Fig. 7B). Increasing the amount of protein in the immunoprecipitation did not reveal any further molecules in J2E-NR extracts. It was concluded from these experiments that differences existed between J2E and J2E-NR lines with respect to the proteins associating with the Epo-R complex.

Signaling Differences between J2E and J2E-NR Cells—Since differences were observed in the proteins associating with Epo-R and Grb2 in J2E-NR, other signaling molecules were investigated. As part of the Epo-activated Ras signaling pathway, p120 GAP undergoes enhanced phosphorylation with hormonal stimulation (11). Fig. 8A shows that phosphorylation levels of p120 GAP were elevated within minutes of hormone addition to J2E cells, before declining. In contrast, no phosphorylation of p120 GAP could be detected in J2E-NR cells (Fig. 8A). Phosphorylation of PI 3-kinase was investigated next because this protein has also been associated with Epo signaling (17-19). The data presented in Fig. 8B demonstrate that PI 3-kinase also underwent increased phosphorylation in J2E cells after hormonal stimulation. Once again, however, no phosphorylation was evident in J2E-NR cells (Fig. 8B). Alterations to PLCγ were then compared in both cell lines. While Epo induced a steady increase in the phosphorylation of PLCγ in J2E cells (Fig. 8C), this molecule was unexpectedly phosphorylated in J2E-NR cells prior to exposure to the hormone (Fig. 8C). The apparent hyperphosphorylation compared with J2E cells was also associated with a 5-fold increase in PLCγ protein present in J2E-NR cells (Figs. 8C and 9C).

MAP Kinase Is Not Activated in J2E-NR Cells—MAP kinases are pivotal in the regulation of cell division and maturation (43). To determine the role of p42 and p44 MAP kinases in J2E cells, the protein content and levels of activation were examined after stimulation with either Epo or sodium butyrate, a chemical agent that also initiates differentiation (but not proliferation) of these cells (23). J2E cells expressed both p42 and p44 MAP kinases, but the protein content of p42 was approximately 5-fold greater than p44 (Fig. 10, A–C). High levels of intrinsic MAP kinase phosphorylation were seen in J2E cells for both p42 and p44 (Fig. 10A). This may be due in part to the v-raf oncogene present in the cells, as MAP kinases are activated in v-raf-transfected Swiss 3T3 cells (44). However, serum deprivation of the cells for 5 h eliminated the phosphorylated form without altering the overall protein content (Fig. 10A). This indicated that serum factors may also have contributed to MAP kinase phosphorylation, or that phosphatases were activated after serum withdrawal. Once the MAP kinase was dephosphorylated, both Epo and sodium butyrate were shown to induce rapid phosphorylation of the molecule; Epo acted within 10 min and sodium butyrate within 1 min (Fig. 10, B and C). The enhanced phosphorylation of the

Fig. 7. Proteins associated with Grb2 in J2E and J2E-NR cells. J2E cells (A) or J2E-NR cells (B) were serum-starved for 5 h (O) and then stimulated for 2-60 min with 5 units/ml Epo. Lysates (3 mg) were immunoprecipitated with anti-Grb2 antibodies and immunoblotted with anti-phosphotyrosine (PY) or anti-Grb2 (Grb2) antibodies. — indicates unique phosphoproteins, while — indicates phosphoproteins common to both cell lines. Molecular mass markers (46–200 kDa) are shown at left. Time (min) after Epo stimulation is shown above each lane.

Fig. 8. Epo induces tyrosine phosphorylation of GAP, PI 3-kinase and PLCγ in J2E cells. Cells were serum-starved for 5 h (O) and then stimulated for 2-60 min with 5 units/ml Epo. Lysates (3 mg) were immunoprecipitated with anti-phosphotyrosine (A and B) or anti-PLCγ (C) antibodies and immunoblotted with anti-GAP (A), PI 3-kinase (B), or phosphotyrosine (C) followed by PLCγ (C) antibodies. Time (min) after Epo stimulation is shown above each lane.
MAP kinases were also demonstrated by immunoprecipitation with anti-phosphotyrosine antibodies, followed by immunoblotting with antibodies recognizing the MAP kinases (Fig. 10D). To confirm that the rise in the phosphorylated MAP-kinase correlated with increased enzyme activity, in-gel kinase assays were performed. Fig. 10E shows that Epo-induced phosphorylation of p42 and p44 MAP kinases produced a concomitant increase in kinase activity.

To assess the importance of the MAP kinase pathway in maintaining cell viability, the phosphorylation status of p42 and p44 MAP kinases was determined in Epo-stimulated J2E-NR cells. Following dephosphorylation of MAP kinase with serum deprivation (Fig. 11A), the cells were exposed to Epo. Unlike J2E cells, no activation of the p42 and p44 MAP kinases was observed when Epo or sodium butyrate were added to J2E-NR cells (Fig. 11, B and C). Immunoprecipitation with anti-phosphotyrosine antibodies also failed to detect an increase in the phosphorylation status of the MAP kinases (Fig. 11D). Since Epo inhibits the death of J2E-NR cells and the MAP kinase pathway was not activated by the hormone, we suggest that this pathway is not involved in maintaining the viability of these cells.

DISCUSSION

The mutant J2E done J2E-NR binds and internalizes Epo, but does not proliferate or differentiate in response to the hormone (22). We show here that Epo still has an effect on the J2E-NR cells by supporting viability in the absence of serum. Therefore, some signals from the Epo-R must be transmitted within these cells to prevent apoptosis. As no faults were identified in the receptor and the Epo-R was phosphorylated with Epo stimulation, it appeared that some essential signaling protein was defective, thereby preventing the maturation message from being transduced.

J AK2 was considered as a candidate molecule that may be defective in the J2E-NR cells. We have shown elsewhere that this protein is important for transmitting both proliferation and differentiation signals.4 However, J AK2 was phosphorylated in J2E-NR cells and also appeared unlikely to be responsible for the defect. Interestingly, it has recently been shown that phosphorylation of J AK2 is not sufficient for Epo-induced mitogenesis as mutation of the "extended box 2" domain of the Epo-R can prevent a growth signal from being transmitted, despite J AK2 phosphorylation (45).

Significantly, differences were observed in the Epo-R complex of J2E and J2E-NR cells. Several proteins consistently

4 P. A. Tilbrook, B. Callus, T. Bittorf, S. J. Busfield, and S. P. Klinken, submitted for publication.
co-precipitated with the Epo-R in J2E cells, and these appeared similar to previous reports (7, 46, 47). However, distinct differences were reproducibly seen in the J2E-NR receptor complex. These changes to the Epo-R complex in J2E-NR cells appear to have initiated numerous downstream signaling alterations. Whereas J2E cells underwent the anticipated phosphorylation of GAP, PI 3-kinase, and MAP kinases with Epo (11, 13–15, 17–19), similar modifications did not occur in J2E-NR cells. An additional change was observed with PLCγ; although the phosphorylation status of this protein rose steadily in J2E cells with hormonal stimulation, it was hyperphosphorylated in J2E-NR cells prior to exposure to Epo. Thus, major changes in intracellular signaling were observed in J2E-NR cells. These alterations clearly affected the ability of the J2E-NR cells to proliferate and differentiate, but had no impact on the cells’ capacity to survive in the presence of Epo.

It has been suggested that cytokine-mediated protection from apoptosis is not necessarily linked to the ability to promote growth (48, 49). Moreover, it has been proposed that the full-length Epo-R, and not a truncated form of the molecule, can transmit a signal to prevent apoptosis that is distinct from a mitogenic signal (50, 51). These observations corroborate the suggestion of Koury and Bondurant (3) that “the survival effect of Epo during terminal differentiation appears to be separate from any mitotic signal.” Our results support, and extend, these hypotheses to show that it is possible for Epo to maintain the viability of erythroid cells, in the absence of proliferation and differentiation. Independent signaling pathways probably exist within cells for survival, mitosis, and maturation. It is noteworthy that Epo alone did not completely protect the cells from DNA damage and death, as reported previously (36, 37), and other factors are probably required to fully maintain viability.

The MAP kinase family of enzymes are obviously very important in cellular signaling. MAP kinase activation has been associated with enhanced mitogenic activity (43, 52), but some recent publications have indicated that these proteins are also involved in cellular differentiation (53, 54). Although Epo-induced activation of MAP kinases has been shown previously (13–15), the results shown here indicate that MAP kinase activation may be linked with differentiation, rather than proliferation. Significantly, the J2E-NR cells remained viable with Epo without MAP kinase activation, indicating that this pathway is not essential for maintaining cell survival. This result supports the observation that suppression of apoptosis by v-abl is not associated MAP kinase activation (55).

PI 3-kinase has been shown to be necessary for a signaling pathway that prevents cell death in neurons (56). The data presented in this report demonstrate that PI 3-kinase was not activated in J2E-NR cells, yet the cells remained viable. It appears, therefore, that phosphorylation of the p85 subunit of PI 3-kinase is not essential for Epo-induced protection from apoptosis. Several groups have shown an association between PI 3-kinase and the activated Epo-R (17–19), but it has been suggested that this association is not linked with proliferation (19). Thus, the precise role of PI 3-kinase in Epo signaling requires further elucidation.

Two other explanations were entertained for the inability of J2E-NR cells to proliferate and differentiate after Epo stimulation. One possibility was that J2E-NR cells have reduced Epo-R numbers and the levels are too low to transmit the maturation signal. This suggests that signal transduction may be a quantitative, and not necessarily a qualitative, phenomenon (57). However, based on the observation that phosphorylation of only 15% of Epo-R was sufficient to induce maximum differentiation of J2E cells, this proposition appears unlikely. Another possibility was that the J2E-NR cells may have reverted to a less mature form of erythroid precursor. J2E cells occasionally mutate and can display an altered phenotype (58). If so, the J2E-NR cells may only respond to Epo as a viability factor, and not as a maturation agent. In support of this notion, the loss of the erythroid-specific surface marker, Ter 119, by J2E-NR cells (57) indicates these cells may have acquired a more primitive phenotype (59). Nevertheless, the J2E-NR done together with the parental J2E cells provide us with an opportunity to dissect signaling pathways within erythroid cells leading to viability, mitosis and differentiation.

Acknowledgments—Recombinant human Epo (Eprex) was a generous gift from Dr. J. eff Adams (Janssen-Cilag), and anti-Epo-R antibodies were kindly donated by Dr. D. Hilt (Walter and Eliza Hall Institute, Melbourne, Australia). We thank L. Conte for preparing this manuscript.
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J. Biol. Chem. 1996, 271:3453-3459.
doi: 10.1074/jbc.271.7.3453

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