Myeloid Leukemia Cell Growth and Differentiation Are Independent of Mitogen-activated Protein Kinase ERK1/2 Activation*

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The mitogen-activated protein kinase ERK1/2 pathway is essential in the control of cell proliferation and differentiation in most cellular systems. As such, it has been considered a potential target for antineoplastic therapy. For this purpose, we have examined the role of ERK activation in myeloid leukemia cell growth and differentiation. Using a representative set of myeloid leukemia cell lines, we show that cell proliferation was not accompanied by increases on ERK1/2 activation, and mitogenic stimulation did not enhance ERK activity. Moreover, abolition of ERK function by the inhibitor PD98059 or by a dominant inhibitory mutant ERK2 had no significant effects on proliferation. With the aid of various differentiation inducers, we found that within the same cell line, differentiation to a given lineage could occur with and without ERK1/2 activation, depending on the stimulus. Also, a differentiator could have the same effect in the presence or absence of ERK stimulation, depending on the cell line. ERK inhibition did not affect the differentiation elicited by stimuli whose effects were accompanied by ERK activation. Finally, constitutive ERK activity was also ineffective on proliferation and differentiation. Thus, our results indicate that ERK1/2 activation is not an essential requirement for leukemic cell growth and differentiation.

Our current understanding of the processes governing myeloid differentiation and leukemogenesis are mainly based upon studies utilizing pluripotent cell lines that can be induced to terminally commit to the different myeloid lineages by the addition of a wide array of differentiating agents (1). As such, cell lines derived from patients with chronic myelogenous leukemia (CML), like K562 (2), KU812 (3), and KBM5 (4), and from acute promyelocytic leukemias, such as HL-60 (5) and U937 (6), are blocked at different stages of their respective differentiation pathways. These lines can be growth-arrested and terminally differentiated by culturing them in the presence of a broad spectrum of differentiation inducers including tumor promoters, antibiotics, and DNA synthesis inhibitors among others (7–9), thereby providing an invaluable tool for the study of the molecular mechanisms underlying in proliferation, differentiation, and oncogenic transformation.

Mitogen-activated protein kinases (MAPKs)/extracellular signal-regulated kinases (ERKs) have been shown to be pivotal elements in the processes that govern cell destiny upon the reception of an external stimuli, either promoting proliferation, switching on the genetic guidelines that will lead to terminal differentiation, or directing cells into programmed cell death (10). Activation of p44 ERK1 and p42 ERK2 is a key step in the conveyance of signals from surface receptors to the nucleus through a route that includes the small GTPase Ras, Raf-1 serine/threonine kinase, and MEK dual specificity kinase as upstream components (11), and it has been proposed that the duration and amplitude of the ERK signal is determinant in the decision between proliferation and differentiation (12–14).

It is now widely documented that ERKs play an essential role in fibroblasts proliferation and neoplastic transformation (14–17). In this context, ERKs are also found to be constitutively activated in some human neoplasias (18–20). On the other hand, ERKs also function actively in differentiation processes during development, like photoreceptor cell patterning in Drosophila melanogaster (21), vulvar development in Caenorhabditis elegans (22), and mesoderm induction in Xenopus laevis (23). Likewise, activation of the ERK pathway has also been shown to be critical in the control of cellular differentiation in myocytes (24), melanocytes (25), and epithelial cells (26) and to induce neuronal differentiation in PC12 rat pheochromocytoma cells (14, 27). With regard to the hemopoietic system, it is known that ERK1/2 are activated by cytokines like thrombopoietin (28). It has also been established that ERK activation controls T-cell selection (29) and that sustained stimulation of the ERK pathway is required for megakaryocytic differentiation in cell lines like K562 and CMK (30–32). However, an overall picture of the involvement of ERKs in hemopoiesis and leukemogenesis is still lacking.

During these past years, and due to its unquestionable importance in the upbringing of oncogenic transformation and its deep implication in the events that will ultimately decide cell fate, much attention has been focused on the ERK pathway as a possible target for newly designed antineoplastic drugs (33). Therefore, it is essential to establish the role that the activation of the ERK pathway plays in the processes that lead to malignant transformation within a specific tissue or cell type. For this purpose, we have investigated the role of ERK1/2 in several myeloid leukemia cell lines when proliferating and subjected to the effects of a wide array of well known antileukemic

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‡ The abbreviations used are: CML, chronic myelogenous leukemia(s); MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; TPA, 12-O-tetradecanoylphorbol-13-acetate; MBP, myelin basic protein; HA, hemagglutinin; PDGF, platelet-derived growth factor; LPA, lysophosphatidic acid; ara-C, 1-β-D-arabinofuranosylcytosine; WB, Western blot; ERK, extracellular signal-regulated kinase.
and differentiating agents. Our results indicate that myeloid leukemia cells responded to mitogenic stimuli and grew without pronounced variations on ERK1/2 activation and that blockade of ERK1/2 activity, both pharmacologically and genetically, did not affect the cell proliferation rate. Likewise, differentiation of leukemic cells, regardless of the lineage they were committed to, could take place with and without ERK stimulation. Moreover, a differentiating stimulus like the phorbol ester 12-O-tetradecanoylphorbo-13-acetate (TPA), which invariably induces ERK1/2 activation, may or may not induce differentiation, depending on the cell line. Also, leukemic cell lines expressing an active mutant of MEK (34) in which ERKs are constitutively activated do not exhibit alterations in their proliferation and differentiation behaviors. Overall, our results indicate that ERK activation is not an essential process in leukemic cell proliferation and differentiation, and therefore the ERK pathway would not be a suitable target for antileukemic therapy.

MATERIALS AND METHODS

Cell Culture—Cells were regularly grown in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 8% fetal calf serum. Cells were supplemented with 10% interleukin-3-rich WEHI-conditioned medium. Cell densities were kept below 10^6 cells/ml. When indicated, exponentially growing cells at a concentration of 2.5 × 10^5 cells/ml were treated for 3 days with the different differentiating agents: TPA (Sigma), 1-ß-arabinofuranosylcytosine (ara-C) (Upjohn), hydroxyurea (Roche Molecular Biochemicals), busulfan (Sigma), staurosporine (Roche Molecular Biochemicals), MeSO (Sigma), and daunomycin (Sigma). The MEK inhibitor PD98059 was from New England Biolabs. When indicated, cells were growth-arrested by serum starvation for 48 h in most cases or 48 h in the case of 32D. Growth arrest and routine cell growth and viability were assayed by hemocytometer and trypan blue exclusion test counts.

MAPK Assays—Endogenous ERK1/2 activity was determined as described previously (36), using a rabbit polyclonal anti-ERK1/2 antibody (C-14) (Santa Cruz Biotechnology) as the precipitating antibody and myelin basic protein (MBP) as a substrate. pCDNAHA-ERK2 and -ERK1 were transfected by electroporation, after which cells were incubated for 48 h and serum-starved, and kinase assays were performed as described above using anti-HA antibody for immunoprecipitation.

RESULTS

ERKs Are Not Activated by Growth or Mitogenic Stimuli in Myeloid Leukemia Cells—In this study, we have chosen a series of cell lines representative of the two main types of myeloid leukemias, including K562, KBM5, and KU812, typical Philadelphia-positive, chronic myelogenous leukemia lines, and HL60 and U937 archetypes of acute myeloid leukemias (9). We also included murine 32D cells that are diploid, nontumorigenic, interleukin-3-dependent myeloid progenitor cells (37, 38).

As a first approach to study the role of ERKs in myeloid leukemia cell proliferation, we investigated the level of activation of ERK1/2 in actively growing cells. To do so, cultures of the different cell lines were serum-starved for different periods, ranging from 4 h in 32D cells to 48 h in the case of K562 and KU812, until a complete growth arrest took place, and ERK activation was compared with that found in exponentially growing cultures by means of an immunocomplex assay using MBP as a substrate, as described under “Materials and Methods.” Because ERK activities in leukemic cells are extremely low, films had to be overexposed to detect the minimal signal. However, under this prolonged exposure, the signal emitted by the control 293T epithelial cells would be saturating. To avoid this effect, kinase assays on 293T cells were performed with one-third of the cellular lysate. As shown in Fig. 1, top panel, basal ERK1/2 activities under growth arrest varied extensively among the cell lines tested, from almost undetectable levels in 32D and U937 cells to higher levels, such as those present in the CML lines KBM5 and KU812. However, no significant differences in ERK activity were found between proliferating and growth-arrested cells in most cases. Slight elevations were detected in growing U937 cells, although small when compared
with the increase on ERK1/2 stimulation found in growing 293T cells, known to require ERK activation for proliferation (39). This result was further substantiated utilizing defined mi-

eral cells, only the U937 leukemic cell line responded with a

activity. For this purpose, cells were growth-arrested by serum

stimulation with an elevation on ERK1/2 activation, as described previously (30–32),

and data not shown). While under the same experimental set-

from (Fig. 5) and the basal ERK activity levels were diminished (Fig. 3, inset, and data not shown), did not significantly affect the growth kinetics of most cell lines tested (Fig. 3), with the exception of HL60 cells, in which a 60% reduction in cell number was observed after 4 days of treatment. Increasing PD98059 concentration to 50 μM, however, completely blocked proliferation in the case of KRB5 or HL60 cell lines or reduced it by 40–50% in the rest of the cell lines tested in the absence of any detectable cell toxicity (data not shown).

As a parallel attempt to study the effects of ERK inactivation on leukemic cell growth, we followed a genetic approach by the use of a catalytically inactive ERK2 mutant K52R, suggested to act as a dominant inhibitory molecule on the endogenous ERK1 and ERK2, most probably by competitively inhibiting its interaction with upstream activators or by interfering with its nuclear translocation (15). To study the effects of the ERK2 mutant (hereafter referred to as ERK2 DK) in leukemic cell proliferation, we first stably transfected K562 and KU812 cells with an expression vector carrying an HA-tagged ERK2 DK to facilitate its detection, and individual colonies selected on the basis of their resistance to Geneticin were isolated. To check if ERK2 DK had growth-inhibitory properties, we compared the number of viable colonies arising after Geneticin selection of ERK2 DK-transfected cells with those yielded by the transfection of an empty vector and found no significant differences (data not shown). Ten clones were selected for each cell line, based on the stable expression of HA-ERK2 DK during extended culture conditions. In order to avoid misinterpretations due to clonal variations, these clones were pooled, resulting in mass cultures, named K562DK and KU812DK, in which HA-ERK2 DK was stably expressed as shown by anti-HA immunoprecipitation and anti-ERK immunoblotting (Fig. 4A and data not shown).

To confirm the functionality of the transfected ERK2 DK, we assessed its ability to interfere with the endogenous ERK activity. As shown in Fig. 4B, top panel, TPA-induced ERK activation was markedly diminished in KU812DK cells in comparison with the response detected in TPA-treated KU812 parental cells. Moreover, upon prolonged exposure, a reduction of the basal ERK activity could be detected in the ERK2 DK transfectants in comparison with the parental cells (Fig. 4B, lower panel), thus verifying its inhibitory effects. Expression of the dominant inhibitory ERK2 mutant, however, did not have a negative effect on the proliferation kinetics of both cell lines tested. As seen in Fig. 4C, both K562 DK and KU812 DK cell
lines grew at the same rate and reached similar cellular densities after 4 days in culture as their respective parental cell lines, suggesting that active ERKs are not required for leukemic proliferation.

Inhibition of ERK Activity Does Not Affect TPA-induced Differentiation of KU812 Cells—It has been shown that activation of ERK2 is a requisite for TPA-induced differentiation of the CML cell line K562 (30, 31, 43). To determine if this is a common feature to all of the cell lines of this type of leukemia, we investigated the effect of the blockade of ERK activation on the differentiation of KU812 cells. In a similar fashion to what has previously been shown in K562 cells (30), stimulation of KU812 cells with TPA induces a fast, potent, and sustained activation of ERKs, remaining at peak levels for more than 3 h after stimulation (Fig. 5A). On the other hand, a 30-min pretreatment with PD98059 at concentrations as low as 10 μM completely abolishes TPA-induced ERK activation (Fig. 5B). Likewise, 10 μM PD98059 inhibited to the same extent the specific ERK1 and ERK2 activities, determined in cells transfected with HA-ERK1 and HA-ERK2 (data not shown).

Like K562, the KU812 cell line was characterized as a blast crisis CML, in which basophilic precursors are predominant (3) and closely resembles K562 in exhibiting the potential to differentiate into mature hematopoietic lineages such as basophils, monocytes, erythrocytes, and megakaryocytes in response to various agents (44). Untreated cells showed a homogeneous population of typical blastic, lobulated cells with rounded nuclei and a diameter of 8–10 μm (Fig. 6A). After 3 days of TPA treatment, the cells had a more heterogeneous nature, 36% of them showed a monocytic-macrophagic morphology with a high cytoplasm/nucleus ratio and eccentrically located kidney-shaped nuclei (Fig. 6B), scored positive in nitro blue tetrazolium and β-naphthyl acetate esterase tests, and presented CD14 and CD64 surface markers (data not shown). Cells exhibiting a megakaryocytic morphology were also abundant (21%), characterized by their large, 30–40-μm size and multiple nuclei (Fig. 6B). Culture in the presence of PD98059 at a concentration of 10 μM, at which ERK activation is entirely blocked, had little effect in the morphology or the proportions of the different lineages present in the TPA-differentiated population (Fig. 6C and Table I), suggesting that ERK activation is not necessary for TPA-induced differentiation of KU812 cells. Surprisingly, raising PD98059 concentration to 50 μM increased the proportion of cells differentiated to the megakaryocytic lineage (Fig. 6D and Table I), as ascertained by β-naphthyl acetate esterase test negativity and an increase of the CD61
inhibitory mutants, KU812DK and K562DK. Cells were seeded at 10^5
K562 and KU812 cells and cultures expressing the ERK2 dominant
prolonged exposition of the kinase assay. C

anti-ERK immunoblotting as described under “Materials and Methods.”

ERK activities in parental (P)
were determined in total lysates (TL) and immunoprecipitates (IP)
were immunoprecipitated using an anti-HA antibody, and ERK levels
HA epitope-tagged ERK2 dominant inhibitory mutant (KU812DK)
treated (T)
were immunoprecipitated using an anti-HA antibody, and ERK levels
expression vector encod-
were expressed in KU812 and KU812DK cells left untreated (−) or stimulated with 10 nM TPA for 5 min. Lower panel, basal ERK activities in parental (P) and KU812DK cells (DK) revealed after prolonged exposition of the kinase assay. C, growth kinetics of parental K562 and KU812 cells and cultures expressing the ERK2 dominant inhibitory mutants, KU812DK and K562DK. Cells were seeded at 10^5
cells/ml, and their proliferation rate was followed for the next 4 days. Data show the average ± S.E. of five independent experiments.

surface marker (data not shown), while the number of cells showing a monocytic morphology dropped (Fig. 6D), verified by a decrease on CD14 and CD64 markers.

Induction of ERK Activation in Leukemic Cell Lines by Differentiating Agents—To further test the necessity for ERK activation during the differentiation process of leukemic cells and not to restrict our study to a one-line/one-stimulus situation that could lead us to a partial interpretation, solely based on a specific situation, we investigated the response of a representative set of myeloid leukemic cell line to the exposer to a broad spectrum of well known in vitro differentiating agents and widely used antileukemic drugs. Most agents failed to induce any ERK activation in any of the cell lines regardless of the duration of the stimulation, as ascertained by ERK activation time courses (data not shown). It was found that only TPA induced ERK activation in all of the cell lines tested, with the exception of 32D (Fig. 7). However, stimulation of ERKs could not be correlated with the differentiation to a specific lineage, since TPA induced mainly monocytic differentiation in K562, KU812, HL60, and U937 cells but did not cause any differentiation in KBM5 cells, despite potently inducing ERK activation (Fig. 7). Moreover, monocytic differentiation was also brought about in the absence of any detectable ERK activation by other compounds, such as staurosporine in KBM5, HL60, and 32D cells and Me3SO in U937, 32D, and also HL60 cells, which proceed to granulocytes if culture is sustained. Megakaryocytic differentiation could also take place accompanied by ERK activation, such as that induced by TPA in K562 and staurosporine in KU812 cells, or without ERK activation such as in the case of K562 differentiated by busulfan, daunomycin, or staurosporine and in U937 by ara-C and daunomycin. Likewise, erythroid differentiation induced by busulfan or daunomycin in KU812 cells occurred with a slight ERK activation or completely without ERK stimulation such as that induced by hydroxyurea, as was the case in other cell lines as K562 differentiated by ara-C, hydroxyurea, or daunomycin. Thus, indicating that ERK activation is not strictly associated with the commitment to a specific lineage in leukemic cells.

Next, we tested whether the abolition of ERK stimulation would alter the pattern of differentiation induced by compounds whose effects were accompanied by an increase on ERK activity. For this purpose, we selected KU812 cells, in which monocytic differentiation brought about by TPA, daunomycin-induced erythroid differentiation, and staurosporine-induced megakaryocytic differentiation all took place in the presence of ERK activation. In addition, we also included K562 cells subjected to the same treatments. Like before, blockade of ERK activation by the addition of 10 μM PD98059 had no significant effect on the differentiation of KU812 or K562 cells irrespective of the stimulus (Table I). Interestingly, the proportions of the resulting lineages were very similar to those found when KU812DK and K562DK were treated with TPA and stauros-
porin, verifying that the genetic blockade of ERK activation closely mimics ERK pharmacological suppression. On the other hand, increasing the concentration of PD98059 to 50 μM caused a shift to the megakaryocytic lineage accompanied by a 35–45% drop in the number of monocytes in those cells treated with TPA. The effects of high concentrations of PD98059 were more heterogeneous in staurosporine-treated cells, causing a 40% drop on the total number of differentiated cells in KU812 and a 50% shift from monocytic to erythroid differentiation in K562 cells.

It is conceivable that the abrogation of ERK activity could result in a different outcome, depending on the stage of differentiation the cells are in. To test if this was the case, K562 and KU812 differentiation was stimulated by the addition of TPA and staurosporine, and 10 μM PD98059 was added at different intervals during differentiation. As shown in Table II, the addition of PD98059 1, 24, or 48 h after stimulation did not alter significantly the differentiation pattern when compared with that exerted by TPA and staurosporine alone, or by the addition of PD98059 1 h prior to stimulation, with the exception of a slight increase on monocytic morphology on TPA-differentiated cells treated with PD98059 after 24 and 48 h of stimulation. Thus, suggesting that ERK activation is dispensable throughout the differentiation process.

Effects of ERK Constitutive Activation on Leukemic Growth and Differentiation—It has been recently shown that sustained stimulation of the ERK pathway elicits megakaryocytic differentiation of K562 cells (30, 31). To ascertain if this is a generalized feature in leukemic cell biology, we explored the effects of ERK constitutive activation in other CML lines similar to K562. For this purpose, and following the procedures used to generate ERK2 K562 stable cell lines outlined before, we transfected K562 and KU812 cells, with an expression vector encoding a constitutively active mutant of MEK (MEK E), in which the regulatory serine residues Ser218 and Ser222 had been sub-
stituted for glutamic residues (30, 34). During the G418 selection process, K562 cells transfected with MEK E grew with difficulty (data not shown). However, after prolonged culture, we were able to obtain a pool of several clones in which MEK E was stably expressed, resulting in an evident constitutive ERK activation, as verified by phospho-ERK levels (Fig. 8A). On the other hand, G418-resistant KU812 cells transfected with MEK E grew readily, and pools of clones were obtained in which constitutive ERK activation was present (Fig. 8A).

The morphological appearance of the MEK E transfectants was indistinguishable from the original lineages (data not shown), and no major differences were detected in the growth kinetics of parental and MEK E transfectants in either cell line (Fig. 8A), suggesting that constitutive ERK activation does not alter growth or differentiation per se. Likewise, when subjected to differentiation by the addition of TPA or staurosporine, MEK E transfectants behaved almost identically to the untransfected parental cell lines, giving differentiation patterns very similar to those shown in Table II for TPA and staurosporine-treated K562 and KU812 cells (data not shown), further demonstrating that constitutive ERK activation does not affect leukemic cell differentiation.

DISCUSSION

In the past few years, great efforts have been dedicated to the identification of cellular components susceptible of being utilized as molecular targets for antineoplastic therapy. Due to its paramount role in the control of cell growth and differentiation, the ERK pathway has attracted much attention. However, because of its multiple and sometimes antagonistic effects, depending on the cellular background we are referring to (10), a concise study of its functions in a given cell, tissue, or type of tumor should precede and validate any initiative with therapeutic aims. For this purpose, we have investigated the role of ERKs in myeloid leukemia cell growth and differentiation, using a set of cell lines representative of the two main types of myeloid leukemias: chronic myelogenous leukemia and acute myeloid leukemia.

Our data indicate that, unlike most other cell types in which proliferating cells have much higher levels of ERK activity than growth-arrested cells (13–16, 39), no significant differences are found in the majority of leukemic cell lines. Moreover, with the exception of U937, none of the cell lines tested responded with an increased activation of ERKs to stimulation by serum or mitogens. In the case of CML cell lines, this could be attributed to the constitutive Bcr-Abl activity saturating the pathway that leads to ERK1/2 activation, since basal phosphotyrosine levels are high and do not change in response to mitogens. However, the fact that TPA potently activates ERKs and induces the appearance of new tyrosine-phosphorylated proteins argues against it. In this respect, it has been shown that Bcr-Abl does not activate ERKs (45). At present, the factors that render ERKs unresponsive to serum and mitogen stimulation in leukemic cells remain unknown, but the fact that leukemic cells can grow without any detectable ERK activity clearly indicates that ERK function can be dispensable for leukemic cell proliferation. In agreement with our ex vivo results, a recent clinical study indicates that only 40% of the samples of primary cells extracted from acute myeloid leukemia patients presented activation of ERKs (46).

We have also made use of the inhibitor PD98059, known to
TABLE I
Effects of inhibition of ERK2 on the differentiation induced by ERK2-activating agents

K562 and KU812 cells at a concentration of 2.5 × 10^5 cells/ml were treated with 10 nM TPA, 20 nM daunomycin, and 100 nM staurosporine in the presence of low and high concentrations of PD98059 (PD). Cells expressing the ERK2 dominant inhibitory mutant K562DK and KU812DK were also treated with the same differentiating agents. Differentiation was evaluated after 3 days in culture as described before. Data show the average of five independent experiments.

| Cells/treatment | PD 10 | PD 50 | ERK2 DK |
|-----------------|------|------|--------|
|                 | %    | %    | %      |
| K562 TPA        |      |      |        |
| Blasts          | 40   | 20   | 10     |
| Monocytes       | 26   | 16   | 5      |
| Megakaryocytes  | 34   | 34   | 34     |
| Daunomycin      |      |      |        |
| Blasts          | 23   | 23   | 23     |
| Monocytes       | 7    | 7    | 7      |
| Megakaryocytes  | 4    | 4    | 9      |
| Erthrocytes     | 29   | 29   | 55     |
| Staurosporine   |      |      |        |
| Blasts          | 7    | 5    | 3      |
| Megakaryocytes  | 93   | 95   | 99     |
| KU812 TPA       |      |      |        |
| Blasts          | 43   | 48   | 45     |
| Monocytes       | 36   | 34   | 36     |
| Megakaryocytes  | 21   | 34   | 38     |
| Daunomycin      |      |      |        |
| Blasts          | 19   | 49   | 40     |
| Monocytes       | 4    | 4    | 2      |
| Megakaryocytes  | 17   | 6    | 13     |
| Erthrocytes     | 60   | 58   | 44     |
| Staurosporine   |      |      |        |
| Blasts          | 4    | 1    | 6      |
| Megakaryocytes  | 96   | 99   | 99     |

Inhibit ERK activation (42). PD98059 has been shown to block the proliferation of different cancer cell lines (47–49) and can also prevent growth of the acute promyelocytic leukemia cell line KG1 (50). However, we found that PD98059 had no significant effects on the proliferation of most of the leukemic cell lines used in this study. This lack of response could be reproduced by the expression of a dominant inhibitory mutant ERK2. It is noteworthy that the cell line most affected by PD98059 is HL60, whose dependence on ERK activity has been previously suggested (51). On the other hand, increasing PD98059 concentration to 50 μM elicited a 50% reduction on the growth rate of the leukemic cells, although the possibility cannot be discarded that this could be the consequence of an unspecific interaction with another unidentified kinase. The fact that ERK activation is completely abrogated at low concentrations of PD98059, at which it has no effects on proliferation, strongly suggests likewise. Therefore, our data showing that mitogenic stimulation is not accompanied by ERK activation and that abolition of ERK activity does not affect proliferation represent evidence that ERKs are not necessary for leukemic cell growth.

Recent reports indicate that constitutive activation of ERKs is necessary and sufficient to induce megakaryocytic differentiation of K562 (30). In light of our results, this could be attributed to the individualized response of K562 cells to extremely high levels of ERK stimulation. In this context, while our data clearly show that K562 cells can sustain constitutive ERK activity without affecting its growth and differentiation, transfection of MEK mutants that yield ERK activations 270–2100-fold over basal levels induce megakaryocytic differentiation (30). Although the physiological relevance of such high levels of activation is debatable, this may imply that K562 cells would have a threshold of maintained ERK activation over which differentiation would be triggered. This, however, would be a particular characteristic of K562 cells rather than being a general rule for all leukemia cells, because KU812 cells are unaffected by high levels of constitutive ERK activation, and TPA treatment of KBM5 cells, despite potently stimulating ERK1/2, does not induce any differentiation. This clearly indicates that ERK activation is not sufficient to cause megakaryocytic differentiation in all leukemic cells. Moreover, busulfan, daunomycin, and staurosporine induce a megakaryocytic phenotype on K562 cells in the absence of any detectable ERK activation, thus proving that ERK stimulation is not an essential requirement for megakaryocytic differentiation.

In the same line, our results indicate that there is no connection between ERK1/2 activation and the commitment to a given myeloid lineage, based on the following: 1) Myeloid-monocytic, erythroid, and megakaryocytic differentiation can occur in the presence or absence of ERK activation, depending on the cell line and stimulus utilized. 2) Even in the same cellular context, differentiation to a specific lineage can occur with and without ERK activation, depending on the stimulus, as is the case in KU812 cells, in which erythroid differentiation can be brought about by daunomycin or busulfan accompanied by...
**TABLE II**

Effects of the addition of PD98059 at different intervals on the differentiation of K562 and KU812 cells

| Cells/treatment | No PD | PD 1 h before | PD 1 h after | PD 24 h after | PD 48 h after |
|-----------------|-------|---------------|--------------|---------------|---------------|
| K562 TPA        |       |               |              |               |               |
| Blasts          | 53    | 58            | 44           | 41            | 51            |
| Monocytes       | 13    | 13            | 18           | 23            | 23            |
| Megakaryocytes  | 34    | 29            | 38           | 36            | 26            |
| Staurosporine   |       |               |              |               |               |
| Blasts          | 7     | 5             | 2            | 4             | 5             |
| Megakaryocytes  | 93    | 95            | 98           | 96            | 95            |
| KU812 TPA       |       |               |              |               |               |
| Blasts          | 58    | 48            | 53           | 50            | 50            |
| Monocytes       | 16    | 14            | 16           | 23            | 23            |
| Megakaryocytes  | 26    | 38            | 31           | 27            | 27            |
| Staurosporine   |       |               |              |               |               |
| Blasts          | 4     | 5             | 6            | 5             | 6             |
| Megakaryocytes  | 96    | 95            | 94           | 95            | 94            |

**FIG. 8.** Effects of constitutive ERK activation on leukemic cell growth and differentiation. A, expression of ERK1/2 and phospho-ERK levels in lysates from parental and MEK E-expressing KU812 and K562 cells. B, growth kinetics of parental K562 and KU812 cells and cultures expressing the MEK E-activated mutant. Cells were seeded at 1.5 x 10^5 cells/ml and their proliferation rate was followed for the next 4 days. Data show the average ± S.E. of three independent experiments.

ERK activation or without detectable ERK activity such as that induced by ara-C and hydroxyurea, or in HL60 cells, in which TPA-induced monocytic differentiation occurs with an increase on ERK activity, as described previously (51), while staurosporine or Me2SO-induced monocytic commitment takes place without any stimulation of ERKs. 3) A given stimulus can have the same differentiating effect with and without ERK activation, depending on the cell line, as evidenced by the effects of staurosporine, which induces megakaryocytic differentiation accompanied by ERK activation in KU812 and without any ERK stimulation in K562 cells.

Moreover, the abolition of ERK activity, both genetically and pharmacologically, did not affect the outcome of those stimuli whose differentiating effects were accompanied by ERK activation, which suggests that the process is independent of ERK function, and only a high concentration of PD98059 could alter the results, a response that could, again, be attributed to some unspecific effect of the inhibitor. In this respect, the data showing that high concentrations of PD98059 produce changes in the ERK-independent differentiation induced by daunomycin in K562 cells point to the pleiotropic nature of the inhibitor at high doses. Our data indicating that in K562 TPA-induced differentiation is unaffected by the inhibitor PD98059 may seem to be in opposition to previous reports (30, 31, 43). However, it is noteworthy that the inhibitor concentrations utilized in these studies are in the range at which we have observed the aforementioned pleiotropic effects.

Although our data indicate that ERK activation is not essential for myeloid leukemia differentiation and regardless of the fact that other cell types, like adipocytes, can also differentiate by a process that does not require ERK (52, 53), our results do not rule out the possibility that ERKs could play some important role in normal hematopoiesis and myeloid differentiation. As such, many hematopoietic cytokines are known to be strong activators of ERKs (54). In this line it could be argued that, like most highly transformed cells in which genetic instability is a hallmark, leukemia cells have sustained a continuous accumulation of genetic errors, making their proliferation/differentiation independent of cytokines and enabling them to circumvent the necessity for ERKs.

Overall, the fact that neither activation nor inhibition of ERKs may necessarily lead to growth arrest or terminal differentiation of myeloid leukemic cells argues against the ERK pathway as an efficient molecular target in antileukemic therapy. Nevertheless, this and other studies (32, 46, 50) show that variable degrees of ERK activity can be present in leukemias. Whether ERK activation could determine the susceptibility to different therapies or serve as a prognosis marker in myeloid leukemia merits some attention.

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