Differential Expression of ras Protooncogenes during in Vitro Differentiation of Human Erythroleukemia Cells

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ABSTRACT

We have compared the expression of the ras protooncogene family (H-, K-, and N-ras) in leukemia cell differentiation utilizing as a model K562 and HEL erythroleukemia cells treated either with 1-β-arabinofuransylcytosine or 12-O-tetradecanoylphorbol-13-acetate (TPA). 1-β-Arabinofuransylcytosine induced terminal erythroid differentiation of K562 cells, while TPA induced myeloid differentiation of K562 and HEL cells, resulting in myelomonocytic-like cells expressing macrophagic and megakaryocytic markers. H-ras mRNA levels showed a dramatic decrease in K562 cells subjected to erythroid and myelomonocytic differentiation. The same result was found at the protein level for p21

ABSTRACT

INTRODUCTION

The three members of the mammalian ras gene family, H-ras, K-ras, and N-ras, are highly homologous and encode similar Mr, 21,000 proteins (p21). These proteins belong to the small GT-Pases superfamily and are suspected to act as signal transducers. Mutated ras genes carrying point mutations in codons 12, 13, or 61 are the most prevalent oncogenes found in human cancer (1, 2).

The function of mammalian ras genes was originally related to the control of cell proliferation. However, a large body of evidence gathered over recent years indicates that these and other protooncogenes are also involved in cell differentiation (reviewed in Ref. 3). Thus, activated H-ras induces differentiation of embryonal carcinoma cells (4) and has been suggested to play a role in the differentiation of epithelial cells in vivo (5).

Mutated versions of ras genes can induce neuronal differentiation (6–8) or adipocyte differentiation (9) in vitro.

The involvement of ras function in myeloid differentiation is suggested by the high frequency of ras activation in acute myeloid leukemia. N-ras is the ras oncogene most frequently activated in human acute myeloid leukemia, with frequencies ranging from 14% to 27% (10–15) as well as in myelodisplastic syndrome (20% to 45%) (16–19). K-ras activation in myeloid leukemias is more infrequent, while H-ras activation is very rare. Despite these data, little research has been carried out to unveil the role that the different ras genes may play in myeloid differentiation. As a first approach, we have studied the expression pattern of the three genes in a myeloid differentiation model system.

A widely used model is the chemically induced differentiation of several myeloid leukemia cell lines (20). The K562 and HEL cell lines were originally isolated from patients with chronic myeloid leukemia (21) and erythroleukemia, respectively (22). K562 and HEL cells are considered multipotent hematopoietic cells, since erythroid, megakaryocytic, and macrophagic differentiation can be induced by several compounds. Agents inducing erythroid differentiation include hemin and ara-C (23–25). The phorbol ester TPA has been reported to induce both megakaryocytic and macrophagic differentiation of K562 and HEL cell lines (26–30). As an approach to studying a possible differential involvement of the ras genes in myeloid differentiation, we have compared the mRNA levels of the three members of the ras gene family during in vitro differentiation of K562 and HEL cell lines. A decrease in K-ras expression in tiazofurin-treated K562 cells has been reported (31). However, a systematic study on the expression of all three ras genes in myeloid differentiation has not been carried out. Our results indicate that there are significant differences in the expression of the three ras genes in response to myeloid cell differentiation, the decrease in H-ras expression being much larger than for K-ras and N-ras.

MATERIALS AND METHODS

Cell Culture and Differentiation Protocols. K562 and HEL cell lines were obtained from the American Type Culture Collection. Cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum and gentamycin (80 μg/ml) at cell densities below 10^4/ml. Cell growth and viability were assayed by the trypan blue dye exclusion test. To induce differentiation, exponentially growing cells at a concentration of 3 × 10^5/ml were exposed to 1 μM ara-C or 10 nM TPA.

Assessment of Differentiation. Morphological differentiation was monitored by examining fixed slide preparations stained with May-Grünwald Giemsas and assessed using established cytological criteria. The fraction of hemoglobin-producing cells was scored by benzidine staining as previously described (25). Nitroblue tetrazolium dye reduction was used to monitor monocyte/granulocytic differentiation (32). Latex phagocytosis and adherence to plastic plates were assayed as described (33). The expression of cell surface markers was measured by quantitative fluorescence analysis using an Epics Profile II flow cytometer (Coulter Electronics). The cells were incubated for 45 min at 4°C with the appropriate monoclonal antibodies. About 10,000 cells were analyzed for each antibody for light scattering and fluorescence intensity. The antibodies used were: anti-glycoprotein Ila (CD61; Dako; fluorescein isothiocyanate-conjugated) and anti-glycoprotein A (Immunotech; indirect immunofluorescence). Nonspecific fluorescence was determined by using isotypic negative control antibodies.

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The abbreviations used are: ara-C, 1-β-arabinofuransylcytosine; TPA, 12-O-tetradecanoylphorbol-13-acetate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SDS, sodium dodecyl sulfate; cDNA, complementary DNA.
RNA Analysis by Northern Blot Hybridizations. Total RNA was isolated from cells by the acid guanidine thiocyanate method (34). Northern blots were prepared essentially as described (35). Total RNA (20 μg/lane) was electrophoresed through a 1% agarose-formaldehyde gel and transferred to nitrocellulose membranes (Millipore), and the blots were hybridized at 42°C in the presence of 40% formamide. The probes were labeled with [α-32P]dCTP (Amersham Multiprime labeling kit). Filters were washed to a final stringency of 0.5x standard saline citrate and 0.1% sodium dodecyl sulfate at 65°C and exposed to Amersham MP film at —70°C with intensifying screens. The densitometric analysis was carried out with a Hoeffer GS300 densitometer.

The probes used were purified restriction fragments isolated from the following plasmid clones: for histone H4, a 0.4-kilobase EcoRI fragment from plasmid PSPH41NV (mouse gene) (36); for GAPDH, a 1.4-kilobase PstI fragment from rat cDNA (37); for ε-globin, a 1.4-kilobase EcoRI-PstI fragment from plasmid pSPI6-5epsi (human gene); for γ-globin, a 0.56-kilobase EcoRI-HindIII fragment from plasmid cHuViml (American Type Culture Collection); for H-ras, a 0.7-kilobase BamHI-SalI fragment from plasmid pSP64-γ (human gene) (T. Enver and P. Constantoulakis, University of Washington, Seattle); for vimentin, a 1.1-kilobase EcoRI-EcoRI fragment from plasmid cHuViml (American Type Culture Collection); for GAPDH, a 1.4-kilobase PstI fragment from human cDNA (38).

Analysis of p21 Proteins. Cells (10⁶) from control cultures or treated for 3 days with ara-C or TPA as above were harvested, washed with phosphate-buffered saline, and lysed with 1 ml of a solution containing 1% Nonidet NP-40, 0.5% deoxycholic acid, 10 μg/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride, 150 mM NaCl, and 50 mM Tris (pH 7.5). Protein content of lysates was determined by the Bradford method, and 300 μg of protein were immunoprecipitated with monoclonal antibody Y13-259 (from the American Type Culture Collection) essentially as described (39) using protein A-agarose as solid support. Immunoprecipitated proteins were electrophoresed on 12% SDS-polyacrylamide gels (35) and transferred to Immobilon membranes (Millipore). Membranes were incubated one of two mouse monoclonal antibodies, anti-p21 ras or anti-c-H-ras (Oncogene Science) were used as controls. The anti-c-H-ras antibody used showed some reactivity against c-K-ras protein (not shown).

RESULTS

ras Gene Family Expression in Erythroid Differentiation of K562 Cells. Erythroid differentiation of K562 cells was induced by treatment with 1 μM ara-C. As previously reported (24, 40), ara-C treatment resulted in the induction of glycoporphin A expression and an increase in the percentage of benzidine-positive cells from about 5% to 60% (Table 1), and the treated cells resembled basophilic erythroblasts (not shown). The erythroid differentiation of K562 was also demonstrated by the increase in ε-globin mRNA levels, as shown by Northern blot hybridization. Uninduced K562 cells expressed ε-globin, but the mRNA level of ε-globin increased dramatically after 72 h (Fig. 1A). The addition of ara-C also resulted in a rapid arrest of cell proliferation, as shown by cell counting and the decrease of histone H4 mRNA levels (Fig. 1A). The viability of

![Figure 1](https://cancerres.aacrjournals.org)
To show whether H-ras down-regulation was a direct effect of the agent used rather than an effect related to the state of differentiation, cells were washed to remove ara-C from the medium after 24 h of treatment. The cells maintained the differentiated phenotype (high percentage of benzidine-positive cells and erythroid morphology) and did not regrow for at least 6 days after ara-C removal. As shown in Fig. 2, levels of H-ras mRNA remained low in these cells in the absence of ara-C.

**ras** Gene Expression in Myelomonocytic Differentiation of K562 and HEL Cells. Treatment of K562 cells with 10 nM TPA resulted in myeloid differentiation with expression of both macrophagic and megakaryocytic markers. The morphology of the treated cells resembled that of myelomonocytes (not shown). DNA synthesis and histone expression (Fig. 3A) were rapidly inhibited by TPA, and cell growth became irreversibly arrested. The cells formed clusters, attached to the plate surface, showed reduced nitroblue tetrazolium (Table 1), and expressed the intermediate filament vimentin (Fig. 3A) as described (41). These phenotypic characteristics are typical for the monocyte/macrophage lineage. The expression of glycoprotein IlA (a megakaryocytic marker) was also induced by TPA (Table 1). Interestingly, the expression of erythroid markers present in uninduced K562 cells such as \( \epsilon \)-globin (Fig. 3A) and glycoporphin A (Table 1) was extinguished when the cells were differentiated with TPA.

As shown in Fig. 3B, the steady-state mRNA levels of ras genes decreased in K562 cells with TPA-induced differentiation, although H-ras and K-ras were down-regulated to a much larger extent than N-ras. After 3 days of treatment the cells showed a fully differentiated phenotype, and their proliferation was irreversibly arrested. At this time N-ras transcripts were abundant (a signal intensity of 60% with respect to controls), while K- and N-ras maintained mRNA levels of about 60% with respect to uninduced cells in terminally differentiated cells. Ethidium bromide staining and hybridization to a GAPDH probe demonstrated that the above-mentioned differences were not due to unequal loading and transfer of RNAs (Fig. 1B). Consistent with the result observed at the mRNA level, ara-C treatment resulted in a drastic reduction in the content of p21H-ras (Fig. 1C). Densitometric analysis of Western blots from three separate experiments showed that the signal from cells treated for 3 days with ara-C was about 18% with respect to controls.

As shown in Fig. 3B, the steady-state mRNA levels of ras genes decreased in K562 cells with TPA-induced differentiation, although H-ras and K-ras were down-regulated to a much larger extent than N-ras. After 3 days of treatment the cells showed a fully differentiated phenotype, and their proliferation was irreversibly arrested. At this time N-ras transcripts were abundant (a signal intensity of 60% with respect to controls), while K-ras transcripts were still detectable. By contrast, the expression of H-ras became almost undetectable after 48 h of treatment. In agreement with this result, H-ras protein levels were very low after 3 days of treatment with TPA (Fig. 1C). The p21H-ras signal intensity in the immunoblots was about 10% with respect to controls. As occurred with ara-C treatment, H-ras mRNA levels remained very low once TPA was removed.

**Fig. 2.** Continued down-regulation of H-ras mRNA after transient exposure to ara-C or TPA. RNA samples correspond to cells treated for 48 h with 1 mM ara-C (Lane 1) or 10 nM TPA (Lane 3) and cells treated for 48 h with the indicated inducer, washed, and further incubated for 24 (Lanes 2 and 6), 48 (Lanes 3 and 7), and 72 h (Lanes 4 and 8). Control (Lane C) is RNA from uninduced K562 cultures. Ethidium bromide staining of the RNA on the filter was performed to assess RNA integrity and loading. The position of the 18S rRNA is indicated.

**Fig. 3.** Expression of ras genes in K562 cells in response to TPA-induced differentiation. A. Northern analysis of GAPDH, histone H4, \( \epsilon \)-globin, and vimentin genes. Ethidium bromide staining of the gel (top) and hybridization to GAPDH probe were performed to assess RNA integrity and loading. B. Northern analysis of H-, K-, and N-ras genes. The same filter was consecutively hybridized to the different probes as indicated in "Materials and Methods." The positions of the 28S and 18S rRNAs are indicated.
from the medium after 24 h (Fig. 2). Washed cells did not regrow and maintained the differentiated phenotype for at least 4 days after TPA removal.

HEL cells differentiated with TPA displayed phenotypic traits of megakaryocytes and macrophages (27, 28). Overall, TPA induced similar phenotypes in K562 and HEL cells (growth inhibition, myelomonocytic morphology, cell aggregation, adherence to plastic and phagocytosis) (Table 1), although TPA did not induce vimentin expression in HEL cells (not shown). As in K562 cells, TPA treatment of HEL cells decreased the basal expression of globin as well as of histone H4 gene, indicating the arrest in DNA synthesis (Fig. 4A).

The pattern of ras gene family expression in HEL cells differentiated with TPA paralleled that of K562. As demonstrated in the Northern blot hybridizations of Fig. 4B, H-ras undergoes a dramatic down-regulation, while N-ras is the most prevalent ras gene in HEL cells differentiated with TPA.

**Comparison of H-ras, K-ras, and N-ras Expression in ara-C versus TPA-induced Differentiation.** A summary of the densitometric analysis of Northern blot hybridizations of three different sets of experiments is shown in Fig. 5. The signals corresponding to ras mRNAs are normalized with respect to the GAPDH signals. Data are arranged for a better comparison of the expression of the response of ras genes to TPA and ara-C in the cell lines used. There was a significant difference between the expression pattern of ras genes when K562 cells were induced to differentiate with either ara-C or TPA. Differentiated cells resulting from either treatment maintained relatively high levels of K- and N-ras mRNA. In contrast, H-ras mRNA levels are very low or undetectable in differentiated K562 and HEL cells (less than 20% with respect to controls by densitometric analysis). Differential amplification of any of the ras genes in K562 and HEL cells was ruled out by Southern blot hybridizations (not shown).

**DISCUSSION**

As an approach to detecting possible functional differences among ras genes on myeloid differentiation, we carried out a systematic study of ras mRNA levels in differentiating erythroleukemia cells. We chose these cells for our studies because they can be differentiated *in vitro* into two clearly different hematopoietic cell types. This is in contrast to other myeloid cell lines broadly used in differentiation studies that can only be differentiated into monocytes (U937, THP-1, KG1) or to related cell types such as granulocytes and monocytes-macrophages (HL-60) (20, 41). We used the bipotent K562 cells as a model system of differentiation along two different pathways by using ara-C

![Fig. 4. Expression of ras genes in HEL cells in response to TPA-induced differentiation. A, Northern blot hybridization analysis of GAPDH, histone, and γ-globin genes. Ethidium bromide staining of the gel (top) and hybridization to GAPDH probe were performed to assess RNA integrity and loading. B, Northern blot hybridization analysis of H-, K-, and N-ras genes, as indicated on the left. The same filter was consecutively hybridized as indicated in "Materials and Methods." The positions of the 28S and 18S rRNAs are indicated.](image1)

![Fig. 5. Relative mRNA levels of RAS gene family in differentiated K562 and HEL cells. Columns, mean values of densitometric measurements of autoradiographic signals from three separate experiments. Data correspond to control (C), TPA-treated cells for 72 h (T), and ara-C-treated cells for 72 h (A).](image2)
and TPA. ara-C irreversibly differentiates K562 cells into erythroid lineage (24, 25). This effect is cell line-dependent, since the same compound induces monocytic differentiation in the pro-myelocytic cell line HL-60 (42). It is noteworthy that ara-C is a widely used drug for myeloid leukemia treatment and therefore an effect on leukemia cell differentiation cannot be ruled out as a part of its therapeutic action. Further work should be aimed at demonstrating this effect in vivo. While ara-C differentiates the cells into erythroid lineage, TPA does so into the megakaryocytic/macrophagic lineage (26, 29, 30, 43). Moreover, TPA treatment decreased the basal expression of globin and glycophorin in uninduced K562 cells. The TPA-treated cells also expressed megakaryocytic markers, but the cell morphology, positive nitroblue tetrazolium reaction, adherence to plastic, and phagocytic activity indicated that the myelomonocytic differentiation was more prominent in the K562 subline used in this work. Uninduced K562, HEL, and other erythroleukemia cell lines coexpress erythroid, myeloid, and megakaryocytic markers, as shown previously (44—47). However, the K562 cells provide a system of dual differentiation induced by either ara-C or TPA. On the other hand, K562 and HEL cells do not contain mutated (50, 51) or amplified ras genes that could make it difficult to interpret differences in gene expression. Overall, these features make these cell lines suitable models for studying the involvement of ras genes in myeloid differentiation.

Our results indicate in the first place that there is no correlation between the expression of K-ras and N-ras genes and cell proliferation in this model. Shortly after the addition of ara-C or TPA the proliferation of K562 and HEL cells stops, as deduced from the cell counts and the mRNA levels of histone H4, which expression is closely linked to DNA synthesis (52). Despite that, there is a significant K-ras and N-ras expression in cells arrested by both treatments. Although some experimental models have linked high ras expression to active tissue proliferation (53, 54), other reports demonstrate high ras expression in nonproliferating and differentiated tissues, such as brain (55—58).

We have also shown a clear differential expression of the three ras genes depending on the differentiating pathway. H-ras undergoes a dramatic and rapid down-regulation during differentiation in both K562 and HEL cells. This result is in contrast with the reported increased expression of H-ras during granulocytic differentiation of HL60 cells (59). In K562 the levels of H-ras mRNA remain low when the inducers are removed from the media. This is consistent with the differentiated phenotype persisting in the washed cells. Conversely to H-ras, significant levels of K-ras and N-ras transcripts are detected in terminally differentiated cells, N-ras being the most prevalently expressed gene. Recently the induction of N-ras and K-ras during the TPA-induced differentiation of a megakaryoblastic cell line has been reported, but H-ras was not detected in this system (60). It is unclear why H-ras mRNA levels decrease so much in differentiated K562 and HEL cells with respect to N-ras and K-ras. It is noteworthy that mutated H-ras is rarely detected in human and experimental leukemia (10—14, 61). However, infection of mouse hematopoietic cells by Harvey and Kirsten sarcoma viruses in vitro caused erythroid differentiation in addition to increased cell proliferation (62, 63), and the same viruses induce erythroleukemia in mice (64, 65). N-ras activation is a frequent finding in myelodysplastic syndrome (up to 45% of cases), a disorder of myeloid cell differentiation (16—19). Our findings, showing a significant level of steady-state N-ras mRNA in differentiated K562 and HEL cells, are consistent with a major role for the N-ras gene in signal transduction in myeloid lineage.

Despite previous studies showing ras gene involvement in experimentally induced differentiation (4—8), to our knowledge this is the first report showing a specific modulation of each of the ras genes in a differentiation model system. So far, no differences have been detected in the biochemical activities of ras genes. The total amount of p21 varies among the different tissues (55, 56). Furthermore, it has been shown that each of the ras genes is differentially expressed at the mRNA level depending on the tissue, albeit all three ras genes show concurrent expression in all mouse and human tissues tested (57, 58). Differential expression of the ras gene family was also found during mouse development (58). This diversified pattern of expression may reflect differences in the functions of the three ras products. Our results agree with those data, since they demonstrate the differential expression of ras genes when the same cells are induced to differentiate along two separate pathways (myelomonocytic and erythroidic). This would not necessarily imply that changes in ras expression trigger differentiation. However, differences in the ratio of the three ras gene products, depending on cell phenotype, may reflect certain specializations in their signal transduction functions.

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