Activation of Erythroid-Specific Promoters During Anthracycline-Induced Differentiation of K562 Cells

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Anthracycline antitumor drugs such as aclacinomycin (ACM) and doxorubicin (DOX) used in subtoxic concentrations induce erythroid differentiation of the erythroleukemic cell line K562. To elucidate the possible role of erythroid genes of the erythropoietin receptor (EpoR) and the transcription factor GATA-1 in this effect, the regulatory regions of the above genes and human $\gamma$- and $\epsilon$-globin and porphobilogen deaminase (PBGD) genes were fused to the firefly luciferase gene. The resulting reporter constructs were tested in a transfection assay of the erythroleukemic cell line K562 stimulated to differentiate by treatment with the anthracycline drugs ACM and DOX or hemin (HEM). The results showed activation of the tested promoters after cell treatment with ACM, but not with DOX or HEM. In contrast to the mouse EpoR gene promoter, the activity of the human EpoR gene promoter (–659/–60) in the reporter construct was not modified by addition of the first intron sequence. In ACM-treated K562 cells, EpoR gene promoter activity completely correlated with EpoR and GATA-1 mRNA levels and the degree of erythroid maturation. In addition, ACM strongly activated the erythroid gene promoters that contain GATA binding sites. Nevertheless, less activation was also observed for the GATA-1 gene promoter (–312/–31) lacking any known GATA binding sites. Insertion of the GATA-1 gene enhancer with two canonic GATA binding sites, stimulated the ACM activation effect for EpoR and GATA-1 promoter–containing constructs. Mutation of the enhancer GATA binding sites abolished this effect. All the regulatory regions tested (except $\gamma$-globin promoter) were completely inactive in nonerythroid COS7 cells. These data indicate that (1) two structurally different anthracycline analogues, DOX and ACM, differ in their differentiation mechanisms, and (2) ACM switches on the erythroid program of K562 cells, at least in part because of interaction with a factor(s) that recognizes the GATA binding sites in the promoter region of erythroid genes leading to their activation.

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W E HAVE PREVIOUSLY shown that treatment of the human erythroleukemic cell line, K562, with the anthracycline drugs aclacinomycin (ACM) and doxorubicin (DOX) in subtoxic concentrations stimulated a differentiation-like process resulting in depletion of cell proliferation and accumulation of hemoglobinized cells.1, 2 This was accompanied by an increase of late erythroid gene mRNAs such as $\gamma$-globin and porphobilogen deaminase (PBGD). However, accumulation of mRNAs of the abundant erythroid transcription factors, GATA-1 and NF-E2, was observed during ACM but not during DOX treatment.3, 4 Likewise, erythropoietin receptor (EpoR) mRNA was also shown to increase only in ACM-differentiated K562 cells, in accordance with the previously observed accumulation of EpoR on the cell surface.5

To determine whether accumulation of erythroid gene mRNA may involve specific activation of gene regulatory regions and to understand better the difference in the mechanism of action of ACM and DOX, we used transfection assays to analyze the expression of GATA-1, EpoR, PBGD, and $\gamma$- and $\epsilon$-globin regulatory regions. The results obtained clearly indicate that ACM but not DOX activated the erythroid regulatory regions. For ACM, such activation appears to occur via GATA-1–dependent and –independent mechanisms.

MATERIALS AND METHODS

Cell lines. The human erythroleukemic cell line, K562, was obtained from the American Type Culture Collection (Rockville, MD) and grown in RPMI 1640 medium with 10% heat-inactivated fetal calf serum (Institut Jacques Boy, Reims, France). The fibroblast-like cell line, COS7, was kindly provided by Dr. P. Mayeu (Hôpital Cochin, Paris, France) and grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 2 mmol/L L-glutamine. Both lines were cultivated at 37°C in a 5% CO2 humidified atmosphere.

Induced differentiation of erythroleukemic cells. K562 cells at an exponential growth phase were induced to differentiate by addition of 10 to 40 nmol/L ACM, 50 nmol/L DOX, or 30 µmol/L hemin (HEM) in culture medium. Inducers were added after completing DNA capture and washing steps in transfection experiments with K562 cells (discussed later). Erythroid differentiation was scored with the benzidine test on day 3. Inhibition of growth and viability of cells were calculated as previously described.6

Construction of plasmids. Plasmids pGL2-Basic and pCAT-Basic (Promega, Charbonnieres, France) containing luciferase (LUC) and chloramphenicol acetyltransferase (CAT) were used for preparation of reporter constructs. All promoter regions were introduced in the appropriate site of the polylinker sequence located upstream of the reporter genes LUC or CAT. Enhancer regions were placed in BamHI or SalI sites located downstream of the polyadenylation site of the reporter gene. The mouse GATA-1 gene promoter spanning nucleotides –312 to –31 relative to the translation start site (+1),

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the enhancer (−856/−655) and the mutated version of the enhancer (with destroyed GATA binding sites) with HindIII or BamHI linkers, respectively,7 were subcloned in corresponding sites of pGL2-Basic. The human EpoR gene promoter region (−659/−59) was amplified by polymerase chain reaction (PCR) with primers (sense, −659/−639; antisense, −59/−79) containing HindIII linkers, and was subsequently cloned in pGL2-Basic. The identity of the amplified fragment to the published sequence8 was confirmed by sequencing. To test the potential enhancer activity of the first intron of the EpoR gene,9 the 0.4-kb BamHI-XhoI or 1.5-kb BamHI fragments were introduced into BamHISalGI sites of pGL2-Basic that contained the EpoR promoter. Promoter regions of late erythroid genes were introduced into the reporter construct as 0.24-kb (−221/+18) and 0.33-kb (−299/+55) HindIII fragments of c- and γ-globin genes, respectively,10,11 or as a 0.8-kb BamHI fragment of the PBGD gene (−714/+76 relative to transcription start site).12 A control construct containing the LUC gene linked to the EpoR promoter and GATA-1 gene enhancer was also created. Two reporter constructs containing the ubiquitous β-actin promoter, differing only in the reporter gene, were prepared. The BgIII-HindIII fragment (−340/+35) of the β-actin gene promoter13 was first subcloned in pGL2-Basic. Secondly, the LUC reporter gene (HindIII-BamHI fragment) of the resulting constructs was substituted for the CAT reporter gene of pCAT-Basic.

RNA analysis. Isolation of RNA was performed as previously described.14 RNA electrophoresis and Northern blot analysis were performed according to the method of Maniatis et al.15 Conditions for reverse transcriptase (RT)-PCR analysis and the control reactions and the primers for amplification of GATA-1 mRNA and the S14 ribosomal protein gene mRNA (standard) were the same as described previously.16 Primers for EpoR mRNA evaluation were selected using the program PCRBASE17 and the following published EpoR cDNA sequence: 5'-AGCCTGTGTCGGTGCTGAGC-3' (sense, 818-838) and 5'-GGTTCCGTGAAGGGGGTGC-3' (antisense, 1064-1084). Aliquots of PCR product that exhibited similar intensities of the amplified control bands of S14 ribosomal gene were loaded on native 4% polyacrylamide gel and analyzed for EpoR and GATA-1 after electrophoresis by scanning on a GS-363 Molecular Imager (Bio-Rad, Ivry sur Seine, France).  

Transient transfection of eukaryotic lines and analysis of reporter gene signal. DNAs of recombinant plasmid were prepared by column chromatography (Bio 101, Montigny le Bretonneux, France) and additionally purified by CsCl gradient ultracentrifugation. Two independent DNA preparations of the same construct were used for transfections. DNA was introduced into the COS7 line by the DEAE-dextran method, as described previously.18 K562 and nonerythroid COS7 cells were transfected by the transfemnfection technique (Transferinfection kit, Serva/Bio Whittaker, Fontenay sous Bois, France). Briefly, 6 μg plasmid DNA was mixed with 10 μg Fe-loaded transferrin-polylysine complex in 0.5 mL 200-mmol/L HEPES buffer (pH 7.2) and incubated for 30 minutes at room temperature. This mix was added to 6 × 10⁵ pretreated (24 hours’ incubation in RPMI medium containing 50 μmol/L desferoxamine) K562 cells in 2 mL RPMI with 50 μmol/L desferoxamine and 100 μmol/L chloroquine. Cells were placed in a CO₂ incubator at 37°C for 6 hours for DNA capture. Cells were washed once with RPMI, divided into equal parts, and then cultivated in the same medium with or without inducers for 6 to 72 hours before analysis of the reporter gene signal. The pcAT β-actin (1 to 2 μg) plasmid was cotransfected with tested plasmids. Cell extracts were prepared in three cycles of freezing-thawing of the cell suspension in 0.25 mol/L Tris hydrochloride (pH 7.5). The amount of protein in the extracts was determined by the Bradford method.19 LUC activity in the extracts was measured with the Luciferase Assay Kit (Promega) in accordance with the manufacturer’s instructions, using the Lumac-3M luminometer (Bertold, Wildbad, Germany). An absolute signal was determined as the maximal rate of the sample luminescence during the first 2 minutes of

Fig 1. Structures of pGL2-based reporter constructs containing regulatory regions of tested genes and their relative LUC activities in transfection assays of untreated K562 and COS7 cell lines. P, E, and I represent promoter, enhancer, and intron sequences of tested genes, respectively. Activity of the construct containing the promoter of the ubiquitously expressed β-actin gene was arbitrarily set at 100%. ND, not determined. Arrows indicate the presence of BamHI sites.

RESULTS

Specific expression of the different erythroid reporter constructs tested in K562 cells. Constructs containing an extremely sensitive firefly LUC reporter gene fused to the regulatory regions (promoters and enhancers) of key erythroid genes were prepared. LUC activity of the resulting constructs was measured in transient transfection assays in erythroid K562 and nonerythroid COS7 cells (Fig 1). For GATA-1 LUC fusion constructs, we selected two regulatory regions (−312/−312 and −655/−856) that are localized upstream of the GATA-1 gene start codon. It was previously shown that
these regions are strictly conserved among mouse and human GATA-1 genes,21 functioning like a promoter and a GATA-dependent enhancer, respectively.7 For EpoR LUC fusion constructs, we used a PCR-amplified 0.6-kb fragment of the human EpoR promoter region (−59/−659) containing a unique canonic GATA-1 binding site60 and other binding sites for ubiquitous transcription factors, all of which are important for tissue-specific expression of the EpoR gene.22,23 Reporter plasmids containing erythroid-specific parts of the promoter regions of late erythroid genes (γ- and ε-globin and PBGD genes) were also tested.

Our preliminary experiments showed a low efficiency and reproducibility of the electroporation transfection procedure for the analysis of reporter construct signals, particularly in anthracycline-treated K562 cells. Indeed, electroporation followed by drug treatment of the erythroleukemic cells increased mortality to an unacceptable level. We chose an alternative gentle transfection procedure via the transferrin receptor–mediated capture of DNA-transferrin-polylysine complexes by K562 cells,18 and modified it slightly for our conditions (as detailed earlier).

In untreated K562 cells, all the tested erythroid constructs produced strong LUC signals higher than the background signal of pGL2-Basic. In contrast, such constructs did not show any significant LUC activity in nonerythroid COS7 cells, except for the γ-globin promoter, the signal of which was 20-fold less than that observed with the SV40 regulatory regions. In K562 cells, the signal was minimal for the construct containing the PBGD gene promoter and maximal for the constructs containing the GATA-I or γ-globin gene promoter, in agreement with the previously found abundance of corresponding mRNAs.4,5 Addition of the GATA-I enhancer increased the signal of the reporter construct containing the GATA-I promoter by 5.7-fold in untreated K562 cells.

To examine the possible enhancer activity of the human EpoR gene first intron, as previously reported in murine cells,24 we used two reporter constructs containing any of two fragments covering the first EpoR intron (+303/+699; +303/+1896). The results show that such constructs did not increase the reporter construct signal (Fig 1). Since the human EpoR intron did not seem to present any enhancer activity (although it possesses noncanonic GATA binding sites), we then created a construct containing both the EpoR gene promoter and the GATA-I gene enhancer. This led to a significant stimulation of the EpoR promoter (4.6-fold) in K562 cells.

In conclusion, the above data indicate that (1) the regulatory regions tested displayed erythroid specificity, and (2) in contrast to the murine system, the human EpoR first intron did not present any enhancer activity in our cell line. They also indicate that K562 cells can be used as a convenient in vitro model for studying the involvement of these regulatory regions during chemical stimulation of erythroid differentiation.

**Specific activation of erythroid promoters in ACM-differentiated K562 cells.** To select the optimal time for analysis of LUC activity during the differentiation process, we measured the signals of the GATA-1 and EpoR promoter constructs in K562 cells, induced or not, to differentiate by ACM (Fig 2). In undifferentiated cells, transient LUC activity may be detected from 24 until at least 72 hours after transfection, and a stable high-level expression of constructs can be observed between 24 and 48 hours. ACM-treated cells showed an increase of LUC activity for the GATA-1 construct, with a maximum difference at 48 hours in comparison to untreated cells (Fig 2A). In contrast, maximal activation of the EpoR construct by this drug was achieved within 24 hours of treatment and maintained for at least 48 hours (Fig 2B). Consequently, we decided to perform further analysis of construct activity on day 2 of incubation with or without the presence of inducers.

The observed activation of erythroid promoters during ACM treatment was confirmed and extended in subsequent experiments with additional constructs. This was compared with the effect of another differentiating anthracycline, DOX, as well as HEM, which is commonly used as a classic erythroid inducer of K562 cells. ACM stimulated LUC activity of all erythroid constructs tested (Table 1). In contrast, DOX and HEM did not modify the activity, suggesting that mechanisms of DOX- and HEM-induced differentiation in K562 cells differ from those of ACM. Indeed, with ACM, we obtained a significant increase of EpoR, PBGD-, and γ and ε-globin promoter activity (~threefold to fourfold).
GATA-1 gene promoter exhibited less activation (~two-fold), but it was higher than that of the nonerythroid β-actin gene promoter (1.4-fold).

To determine a possible influence of the vector sequences on the observed effect, we performed the above experiments with a BamHI fragment of reporter plasmid containing only the GATA-1 promoter and the reporter gene with the polyadenylation signal (Fig 1). For such a cassette, we obtained the same results as for the whole reporter construct (data not shown). It is also necessary to note that the signal of the original promoterless pGL2-Basic plasmid never exceeded 0.1% of the signals obtained for recombinant constructs in all of the tested conditions.

Contribution of the GATA-1 enhancer element to GATA-1 and EpoR promoter activity in ACM-differentiated K562 cells. As mentioned earlier, GATA-1 and β-actin promoters exhibited the lowest degree of ACM-mediated activation. In contrast to other tested erythroid promoters, they do not contain any known GATA binding sites. Therefore, it was reasonable to suggest that the presence of these sites was involved in ACM-mediated promoter activation. To test this, we used the GATA-1 gene enhancer, which contains two inverted canonic GATA binding sites. Insertion of this GATA-1 enhancer in the constructs resulted in an additional multiplication (~two-fold) of ACM-mediated stimulation of GATA-1 and EpoR promoter activity (Table 1). As with promoters alone, DOX and HEM did not modify the response of constructs containing the GATA-1 enhancer. In addition, we checked the activity of the construct containing the enhancer that was mutated at the GATA-1 binding sites (TTCTAAGACTTACAT). With this construct, the degree of activation by ACM was decreased to the low level found with the construct containing only the GATA-1 promoter, indicating that GATA-1 sites in the enhancer are responsible for much of the observed ACM effect.

Differential activity of EpoR and GATA-1 promoters in ACM-differentiated K562 cells. To determine whether the expression of EpoR and GATA-1 promoters was dependent on the appearance of hemoglobinized cells, we compared LUC activity of the corresponding reporter constructs as a function of increasing ACM concentrations (20 to 35 nmol/L) on day 2 of culture (Fig 3). We found a clear difference in the behavior of the tested promoters. The activity of the EpoR promoter directly correlated with the differentiation (percent of benzidine-positive cells), whereas the GATA-1 promoter linked to the enhancer continued to increase at drug concentrations (>20 nmol/L) that inhibited differentiation and EpoR promoter activation. Moreover, the decrease in GATA-1 promoter activity observed with ACM at 35 nmol/L could be explained by the cytotoxic effect appearing at this concentration (~10% of dead cells). To test whether the activities of the transfected promoters correlate with those of the endogenous genes, GATA-1 and EpoR transcripts were measured by RT-PCR. Figure 4 shows that the lowest ACM concentration tested strongly increases the EpoR mRNA level relative to untreated cells, whereas higher concentrations tend to reduce the effect. Thus, ACM has an effect on the transfected EpoR construct similar to that on the endogenous gene. In contrast, GATA-1 mRNA levels are only moderately increased at ACM concentrations that greatly stimulate the GATA-1 reporter construct. The observed discrepancy may indicate the existence of additional regulatory mechanisms that prevent excess accumulation of GATA-1 mRNA in ACM-treated K562 cells.

### DISCUSSION

Anthracylcine antitumor drugs have been shown to be potent erythroid differentiation inducers of human leukemic K562 cells. Mechanisms by which such drugs activate specific expression of erythroid genes are poorly understood. In
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In this study, we therefore investigated the effects of ACM and DOX on the activity of regulatory regions of erythroid genes in K562 cells. Reporter constructs containing the extremely sensitive LUC reporter gene were transiently transfected by means of the reporter-mediated transfection technique. As reported previously, this novel procedure is more efficient and reproducible and significantly less cytotoxic than electroporation, which is of great advantage when cells must be treated with antitumor drugs.

Erythroid constructs were transcriptionally active in erythroid K562 cells and not in nonerythroid COS7 cells (Fig 1), except for the γ-globin promoter, which was highly trans-activated in COS7. However, the activity of this promoter in K562 cells was close to that of the other erythroid genes, and the γ-globin gene was silent in COS7 cells (data not shown). This suggests that unknown positive transcriptional factors present in SV40-transformed COS7 cells may nonspecifically activate this γ-globin reporter construct, or that another part of the γ-globin promoter, which blocked gene expression in COS7 cells, is absent in our construct. ACM, at its optimal differentiating effect, induced promoter activation (~threefold to fourfold) of the late erythroid genes, γ- and ε-globin, and PBGD, a key enzyme of the heme pathway. This was associated with activation of the GATA-I gene, which occurred via both regulatory elements: the promoter and the upstream GATA-dependent enhancer. The scale of the GATA-I promoter/enhancer construct activation (~fourfold) was in accordance with the previously described GATA-I mRNA accumulation after ACM-mediated differentiation, and was confirmed by RT-PCR. It is likely that the ACM-mediated GATA-I increase was able to transactivate late erythroid genes, as previously shown in Epo-treated J2E cells transfected with the βEpo globin promoter. It is also consistent with previous results that showed an elevated threshold level of GATA-I can determine the commitment of transformed hematopoietic cells toward the erythroid lineage.

ACM-induced activation of the GATA-I gene occurred mainly but not exclusively via a GATA-I–dependent mechanism. The promoter region of the GATA-I gene (~312/−31), which neither contains canonic GATA-I binding sites nor binds the GATA-I protein in gel-shift or DNase protection assay, was induced by twofold, i.e., only slightly more than the ubiquitous actin promoter (1.4-fold). In this case, the presence of binding sites for the erythroid Krüppel-like factor and ubiquitous Ets or Sp1 transcription factors in the GATA-I promoter region and a modification of their activity by ACM might be reasons for the observed effect. Addition of the GATA-I enhancer (~856/−655) amplifies both the absolute LUC signal of the resulting construct in untreated cells and the promoter activation effect in ACM-treated cells. Both these features were completely dependent on the intact structure of the GATA binding sites in the enhancer (Table 1), proving the participation of these sites in the ACM-mediated activation effect. This conclusion is also supported by the observation that the erythroid promoter of the GATA-I gene lacking GATA-I binding sites is activated less by ACM than erythroid promoters with such sites (γ- and ε-globin or EpoR; Table 1). In addition, among enhancer-containing reporter constructs, the maximal response to ACM was found for the EpoR promoter construct bearing the maximal number of GATA binding sites (Table 1). Since ACM has been shown to enhance the EpoR number and since the appearance of membrane EpoR is an essential event during erythroid differentiation, we analyzed the activity of EpoR promoter (~659/−59) in differentiated cells. Data obtained here show that the EpoR gene promoter was more responsive than the GATA-I gene promoter, since the saturation level of EpoR promoter activation was achieved more rapidly (Fig 2) and at a lower drug concentration (Fig 3). This suggests that activation of the EpoR gene may be the first target in ACM-treated cells.

Recently, it has been shown that the murine EpoR first intron (ISV1) plays the role of an enhancer. In human K562 cells, two fragments (+303/+699; +303/+1896) of the human ISV1 that we tested did not exhibit any enhancer activity or any effect on ACM-mediated promoter activation. This could be explained by the absence of canonic GATA-I binding sites in such regions. Nevertheless, addition of the GATA-I enhancer (~856/−655) containing two GATA-I binding sites to the human EpoR led to activation of the resulting construct in K562 cells (~twofold v EpoR promoter alone). For the ACM concentration corresponding to an optimal induction, we observed a maximal accumulation of EpoR mRNAs and activation of the EpoR promoter. In contrast, GATA-I mRNA reached its maximum, whereas promoter activation required a higher concentration close to 100% of growth inhibition. At this concentration, ACM may act via additional targets that could affect the mRNA level.

In contrast to ACM, DOX, a structurally different anthracycline analog, does not activate the tested erythroid promoters. This finding confirms the existence of a DOX-specific differ-
entiating mechanism, which takes place without transactivation of the GATA-1 or the EpoR gene. In DOX-treated cells, accumulation of mRNA of the late erythroid genes was observed without corresponding promoter activation (Table 1), so the DOX-mediated stabilization of these mRNAs may be suggested, and preliminary experiments now in progress strongly support this idea. We found that another known inducer of erythroid differentiation, HEM, used here as a reference inducer, as well as DOX, does not stimulate the tested promoters. In contrast to the anthracyclines, no growth inhibition (until 72 hours) of HEM-treated cells was observed. Therefore, we suggest that HEM follows a differentiation mechanism different from that of ACM or DOX.

In conclusion, our data show that ACM stimulates erythroid differentiation of K562 cells via activation of erythroid gene promoters. This activation mainly occurs because of the presence of GATA binding sites in these promoters. This is the first indication that an antitumor drug can positively stimulate the regulatory regions of genes involved in the differentiation program of a tumor cell.

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Activation of erythroid-specific promoters during anthracycline-induced differentiation of K562 cells

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