Flow Cytometric Detection of Drugs Altering the DNA Methylation Pattern

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ABSTRACT

We have developed a model system for assessing the demethylating potential of external agents. Disruption in the DNA methylation pattern was evaluated at the translational level of the Echerichia coli β-galactosidase coding gene (lacZ). We have constructed a clonal cell line (A4/4 cells) derived from the adenovirus-transformed human embryonic kidney 293 strain. The A4/4 cells contain the E. coli lacZ gene under the control of the mouse metallothionein I promoter which is down-regulated by a natural DNA methylation pattern. Furthermore, the lacZ transcription is also regulated by the E. coli lac operator/repressor system and by mouse metallothionein I metal responsiveness offering a wide range in lacZ expression. In this system, the β-galactosidase activity was only recovered in the presence of a demethylating agent such as S-azacytidine. The demethylating potential of S-azacytidine, 5-aza 2'-deoxycytidine and sodium butyrate was rapidly assessed by a flow cytometric method using fluorescein di-ß-o-galactopyranoside as a fluorescent probe. A tremendous induction of lacZ expression was triggered by these drugs. Analysis of cell cycles showed little disruptions with 5-azacytidine and sodium butyrate, but an important blockage in the S-phase following 5-aza 2'-deoxycytidine treatment was observed. This approach allows a rapid identification and study of environmental demethylating agents.

INTRODUCTION

Several lines of evidence indicated that 5-methylcytosine is implicated in the fine tuning of gene expression (1, 2). Methylation of 5′CpG3′ sequences of many vertebrate genes may be part of a general gene regulation involved in tissue and developmental stage-specific expression (3, 4), cellular differentiation (5), X chromosome inactivation (6), genomic imprinting (2), or endogenous retrovirus induction (7). This transcriptional control is somatically inherited through DNA synthesis followed by specific DNA methylases (8). An inverse correlation between extent of DNA methylation and level of gene expression has been demonstrated for many but not all viral or cellular genes (9–12).

In recent years, alterations in methylation patterns at specific promoter sites of various genes have received a great deal of attention in the study of oncogenesis (for review see Ref. 13). Lowered DNA methylation levels could be involved in the manifold activation of oncogene expression (14, 15) and could increase the rate of tumor progression (16). External agents interfering with the DNA methylation pattern might give rise to perturbed programs of gene expression impairing normal cell proliferation (17, 18). The demethylating potential of various carcinogens has been assessed with in vitro systems (19–21; for review see Ref. 22). It turned out that some of these agents could lead to perturbations in the “maintenance” DNA methylation activities and reduce the DNA methylation level. It should also be noted that DNA hypomethylation induced by a demethylating agent, such as 5AzCd3, during repair synthesis of damaged DNA might be a key event for initiation of carcinogenesis (23–25). For these reasons, we have focused our attention on the rapid detection of demethylating agents in using a relevant cell system.

As part of a larger study to determine the demethylating potential of xenobiotics, we have developed an experimental tool based on a clonal cell line (A4/4 cells). In this system, the E. coli β-galactosidase coding gene, lacZ, served as a reporter gene because its expression could be rapidly analyzed in a single cell by flow cytometry (26, 27). In the A4/4 cells, the specific lacZ gene reactivation induced by demethylating agents, coupled with the very low spontaneous βgal activity of these cells, should allow us to detect small variations occurring at the transcriptional level. We report the use of flow cytometry for the detection of demethylating agents such as 5AzCd3, 5AzCd and Btr.

MATERIALS AND METHODS

Cell Lines. A4/4 cells were derived from the 293 human cells. They contained two Epstein-Barr virus based plasmids integrated into the genomic DNA (28). The pEBVAdyadCMVlacI (p189) plasmid carries the lacZ gene downstream of the human CMV (29) and the pEBVMTlacOClacZ (p145) vector bears the mouse metallothionein-I promoter (mMT-I) upstream of the lacZ gene (30). A symmetrical lac operator 5′ATTGTGAGCGCTCACAAT3′ lies between the mMT-I promoter and the lacZ gene (31). The state of the two lac building blocks inside the A4/4 cells has been previously analyzed (28) and it can be summarized as indicated in Fig. 1. One copy per cell of the p189 plasmid is integrated within its EBNA-1 sequence, while one copy/cell of the p145 plasmid is integrated between the 3′ end of the Dyd sequence and 5′ part of the mMT-I promoter. The control cell line is the 293/145 line which carries the pEBVMTlacOClacZ (p145) vector and expresses the lacZ gene in the absence of demethylating drugs.

A4/4 and 293/145 cells were maintained in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal calf serum, 100 units/ml of penicillin and 100 μg/ml of streptomycin (minimum essential fetal calf serum medium), under 5% CO2. Culture medium was either supplemented with hygromycin B (125 μg/ml; Sigma) and Geneticin (250 μg/ml; G418; Sigma) for the A4/4 cells or with hygromycin alone for the 293/145 cell population. The A4/4 cells and 293/145 control cell lines were seeded at 2.10⁶ cells/dish and 5AzCd (Sigma), 5AzCd (Sigma), Btr (Sigma), heavy metal cocktail (ZnCl2 and CdCl2), or IPTG (10 μg; Gibco-BRL) was eventually added after a 3-day culture period. The lacZ gene expression was analyzed 2 or 4 days after treatment.

Enzymatic Assay for β-Galactosidase Activity. lacZ gene expression was quantified as described by Nielsen et al. (32) using ONPG (Sigma) as substrate. βgal activities were expressed as units (nmol of βgal activity/minute) per mg of protein.

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The abbreviations used are: 5AzCd, 5-aza 2′-deoxycytidine; Btr, sodium butyrate; CMV, cytomegalovirus immediate-early promoter; mMT-I, mouse metallothionein-I; ONPG, o-nitrophenyl β-D-galactopyranoside; HBSS, Hanks’ balanced salt solution without magnesium and calcium; FACS, fluorescence-activated cell sorter; FDG, fluorescein β-D-galactopyranoside; IPTG, isopropylthiogalactoside; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride.
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**RESULTS**

Main Feature of the A4/4 Cells. In A4/4 cells, the lacZ gene was inserted downstream of the metal-inducible mMT-I promoter. Its transcription was repressed by the lacI protein. The lacZ gene expression was also hindered by a specific 5'CpG3' sites DNA methylation process in its 5' part (28). In the absence of demethylating drugs, the lac-specific inducer (IPTG) and heavy metal ions could not alleviated this transcriptional hindrance. Conversely, in the presence of demethylating agents, of IPTG and of heavy metal, the methylation-imposed inhibition of the mMT-I promoter could be efficiently relieved triggering a tremendous increase of lacZ gene expression. In 293/145 control cell line, heavy metal ions remained the most efficient inducers to trigger the mMT-I regulated lacZ expression. Fig. 2 shows a scheme of the A4/4 experimental model outlining different induction pathways including the quantification methods used to evaluate βgal activities.

**Effect of Demethylating Agents on βgal Activities using Conventional Quantification.** In this approach, βgal activities were measured in total cell lysates by using the chromogenic substrate ONPG. In order to determine the demethylating potential of 5AzCyd, A4/4 cells were treated for 2 days with 5AzCyd concentrations ranging from 0 to 10 μM together with IPTG and heavy metal ions. A lacZ gene expression enhancement was observed with 5AzCyd doses as low as 0.5 μM (Table 1, Numbers 1-4). We observed a synergistic response with all inducers (IPTG, ZnCd and 5AzCyd) leading to a large increase of lacZ expression. This induction was 5AzCyd dose-dependent and could reach a 146-fold increase over the basal level. In the
Table 1  

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<tr>
<td>1 + IPTG</td>
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<tr>
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<td>2.4</td>
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<tr>
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<td>8 + IPTG</td>
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Table 2  

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<tr>
<td>2 + IPTG</td>
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<tr>
<td>3 + IPTG</td>
<td>2.4</td>
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<tr>
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<td>5 AzCyd (μM)</td>
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<td>8 AzCyd (μM)</td>
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Effect of Demethylating Agents on βgal Activities Using Fluorometric Quantification. βgal activity was scored in single cells using the FACS/FDG protocol of Nolan et al. (26). A4/4 cells were maintained for 4 days in presence of 5AzCyd, 5AzCdCyd, or Brt. Heavy metals were introduced into the culture medium in order to trigger an increased lacZ expression. Fig. 3 shows FACS profiles obtained 4 days after the treatment of A4/4 cells with 5AzCyd, 5AzCdCyd, and Brt. The abscissas correspond to an arbitrary scale which denote the logarithm of the fluorescence, and the ordinates represent the relative cell number. We obtained a tremendous shift in the fluorescence profile towards.

Fig. 3. Quantification of cells expressing β-galactosidase activity after 5-azacytidine, 5-aza 2'-deoxycytidine, and sodium butyrate treatments. After treatment with 5AzCyd, 5AzCdCyd, or Brt at various concentrations with or without ZnCd (50 μM ZnCl2 and 0.5 μM CdCl2) cells were loaded with FDG substrate and analyzed by flow cytometry as described in "Materials and Methods". Ordinates, relative fluorescence units in a logarithmic scale. To quantitate lacZ+ cells, gates (vertical arrows) were set so that 99 to 100% of mock-treated cells were considered lacZ-.
higher intensities with increased concentrations of drugs, which indicated a high β-gal induction after demethylating treatments. It is noteworthy to mention a bimodal lacZ gene expression in the A4/4 cells after demethylating treatments coupled with heavy metal induction (Fig. 3). Bimodal lacZ gene expressions were also obtained with several control cell lines overexpressing the lacZ gene with either the mMT-I or CMV promoter driving transcription (data not shown).

When the gates determining the number of lacZ+ cells were set in histograms to include all cells expressing the lacZ gene (between 26 and 255 fluorescence units) we obtained 4 days after a 5AzCyd (10 μM)/ZnCd cotreatment 70% of lacZ+ cells depending on the Btr concentration. The induction rate increased up to 132 and 255-fold with SAzCyd and SAzdCyd concentrations ranging from 0 to 10 μM (Table 3, Numbers 1–4). With the higher SAzdCyd concentrations the fraction of lacZ+ cells reached a plateau while the fraction of cells expressing the lacZ gene with either the mMT-I or CMV promoter driving transcription (data not shown).

We observed no variation in the Msp I digestion patterns between mock-treated cells and cells treated with demethylating agents because the enzyme is insensitive to the methylation state of 5′ CpG3′ sites (Fig. 4). Conversely, we clearly observed the appearance of a roughly 700-base pair fragment in DNA from A4/4 cells treated with 5AzCyd, 5AzCd, or Btr and cut with the methylation sensitive HpaII enzyme. This fragment corresponded to the 764 base pair one spanning the coding region of the lacZ gene (data not shown). Previous results obtained with A4/4 cells showed that the mMT-I promoter was methylated at HpaII and HhaI sites (28). An interesting site was particularly investigated; it spanned inward one of the mMT-I metal responsive elements. This site was methylated as evidenced by its HpaII digestion insensitivity, and the presence of 5AzCyd entailed its hydrolysis and the resumption of a 200 base pair fragment. From these data it appeared that the mMT-I promoter and the 5′ part of the lacZ gene were methylated and 5AzCyd, 5AzCd, or Btr treatment restored the HpaII sensitivity. Hence, these drugs could efficiently demethylate the lacZ transcriptional cassette in the A4/4 cells, and the lacZ gene reactivation was rapidly analyzed by dosing β-gal activities.

Effects of Demethylating Agents on Cell Cycle. To analyze effects of drugs on A4/4 cell cycle we have investigated the cell cycles with DAPI as DNA fluorochrome (Table 4). 5AzCyd slightly disturbed the cell cycle whereas 5AzCyd entailed an extensive perturbation. The 5AzCyd led to a lowered number of cells in the S-phase (21% for control to 13% with 10 μM of 5AzCyd) and to an increased number in the G2-M phase (15% for control to 22% with 10 μM of 5AzCyd). 5AzCd treatment caused an important blockage of the cells in S-phase (20% for control to 40% with 10 μM of 5AzCd) coupled with a decreased G0-G1 phase (65% for control to 42% with 10 μM of 5AzCd). These results were difficult to compute because of
the extensive perturbation of the cell cycle. This could be related to the high toxicity of the 5AzCdCyd encountered in different assays when we have tried to maintain the A4/4 cells in presence of this drug for several weeks. Before FACS analysis, the surviving cell fractions were analyzed as percentage of living cells in culture 4 days after 5AzCdCyd or 5AzCdCyd treatment. We obtained values greater than 90% as determined after propidium iodide and fluorescein diacetyl staining followed by fluorescent microscopy quantification. Analysis of cell cycle disruption following Btr treatment led us to the conclusion that Btr entailed a blockage of the cells in the G1-phase (66% for control to 74% at 3 mM Btr) and a subsequent reduction of the S-phase (20% for control to 11% with 3 mM Btr).

DISCUSSION

We have developed a promising approach to rapidly detect xenobiotic agents interfering with DNA methylation pattern using the up-to-date FACS/FGD technique for a rapid and easily quantified assay. We have used a clonal cell line (A4/4 cells) in which lacZ gene expression was recovered only in the presence of demethylating agents (28). Because lacZ gene transcription was repressed by the lacI protein and was driven by the inducible mMT-I promoter, a synergistic response could be reached in presence of IPTG and heavy metal ions (Table 1). In A4/4 cells the lacZ gene expression was not significantly recovered after IPTG and heavy metal treatment alone. The demethylation-imposed inhibition was only relieved in the presence of a demethylating drug. The FACS/FGD method was applied as a mean of studying the lacZ gene reactivation after a demethylating treatment on a cell-by-cell basis and in order to relate βgal activities to other cellular parameters, such as distribution of cells within cell cycle phases.

The great magnitude of induction of the lacZ gene expression allows us to easily detect drugs hindering the DNA methylation process (e.g., 5AzCd and 5AzCdCyd). In particular, the very low level of uninduced lacZ gene expression improved the detection of drug entailing small variations in the βgal activity (e.g., Btr; Table 3). With the cell-by-cell quantification devoted to cytometry analysis we observed a dramatic increase in the number of lacZ+ cells triggered by 5AzCd and 5AzCdCyd treatments in presence or absence of heavy metal ions (Fig. 3; Table 3). LacZ gene reactivation was correlated with DNA demethylation at the 5′CpG3′ sites in the 5′ region of the lacZ gene transcriptional unit (Fig. 4). 5AzCdCyd and 5AzCdCyd are known to be potent inhibitors of DNA methylation (5) by impeding the transmethylation process and inhibiting the processive DNA methylase enzyme (35). 5AzCd is known to be a strong inducer for numerous “silenced” genes (8) and it could, for example, induce the mouse metallothionein I gene in the cadmium-sensitive W7 mouse thymoma cell line (10). In the FACS/FGD technology, the ability to detect positive lacZ expression in single viable cells without previous selection considerably simplifies the βgal quantification and the lacZ+ cell isolation. Our approach could bring new insights in the action of demethylating drug because the lacZ gene expression could be related to other cell characteristics such as cell cycle or sites of DNA demethylation (Table 4).

The results obtained with the Btr had to be considered carefully because this fatty acid is known to generate pleiotropic effects on cultured mammalian cells including inhibition of histone deacetylation and DNA replication, disruption of cell morphology, and alterations in the level of certain gene products (for review see Ref. 36, 37). Btr treatment causes hypermethylation of DNA cytosines in W1–38 human embryonic lung fibroblasts (38) and HeLa cells (36), but it also entails DNA hypomethylation in CBT human cells (36). In our system, 4 days after the Btr treatment with doses which did not modify the cell morphology, we observed a significant increase of the number of lacZ+ cells (Fig. 3; Table 3, Numbers 5–6) related to the DNA demethylation of the 5′ part of the lacZ gene (Fig. 4). It was noteworthy to observe that Btr could induce the demethylation of the DNA in human 293 cells at similar extent as 5AzCdCyd did. As a subsequent result, extent of DNA demethylation was not directly correlated with the magnitude of the lacZ gene reactivation for different drugs. Furthermore, Btr treatment slightly reduced cell growth by arresting a small fraction of cells in the G1 phase of the cell cycle (Table 4). A similar observation was mentioned elsewhere (for review see Ref. 38).

In the aforementioned FACS/FGD results, we stimulated the lacZ gene expression through the heavy metal responsiveness of the mMT-I promoter (Fig. 3; Table 3) because IPTG was not suitable to relieve the lacI repression and to enhance the assessment of demethylating drugs. Indeed, IPTG entailed lower fluorescence intensities when cells were treated with IPTG and ZnCd than with ZnCd alone (data not shown). It seemed likely that IPTG was a competitor of the FDG substrate for the βgal enzyme.

Histograms shown on Fig. 3 revealed a bimodal pattern of lacZ expression. It seemed likely that lacZ gene expression is slightly dependent upon the cell cycle phase. We have found that lacZ expression was slightly greater when the cells were positioned in the G2–M phase than in other phases (data not shown). Furthermore, in a control clonal cell line where lacZ gene transcription was driven by the strong CMV promoter we never obtained 100% of lacZ+ cells as evidenced by Xgal staining or flow cytometric analysis (data not shown). Another explanation might emerge from a recent study of Fiering et al. (27). Using the FACS to quantitate βgal activity in stimulated T-lymphocytes, these authors observed a bimodal lacZ expression. The authors ruled out the possibility of a cell cycle dependence but envisaged that the T-cells remained inactivated until
induced transcription factors reached threshold concentrations to control inducible genes. This bimodal expression occurred with multiple copies of NF-AT or NFкB responsive elements to monitor the lacZ gene transcription. Similar results were obtained by Karttunen and Shastri (39). We could envisage the existence of a threshold in transcription factor concentrations to trigger the entry of A4/4 cells in an overactivated state.

In conclusion, molecular genetics allows us to develop relevant cellular tools, such as A4/4 cells, for detecting demethylating agents or other genetic disruptions. Our future aim is to focus our attention to the methionine-methylation metabolism. Indeed, treatments entailing a starvation for methyl groups (e.g., L-ethionine, cordycepin, or 2-deoxycadenosine) could induce reexpression of certain embryonic genes and cellular differentiation subsequently to hypomethylation of the DNA (for review see Ref. 17). These treatments could contribute to the evolution towards a fully developed neoplastic phenotype. The demethylating properties of these antimetabolites and of environmental agents susceptible to be in contact with humans can be detected with our cell system.

REFERENCES

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