Inhibition of Dimethyl Sulfoxide Induced Erythropoietic Differentiation of Murine Erythroleukemia Cells in Culture

Magda Foresti, Luciano Gaudio, Giuseppe Geraci, and Paola Manduca

ABSTRACT

The dimethyl sulfoxide induced erythropoietic differentiation of murine erythroleukemia cells, as determined by scoring benzidine positive cells, is inhibited by mitomycin C at concentrations that have no effect on cell proliferation. The inhibition occurs only when cells are treated with mitomycin C during induction and has a limit value of about 50%, independent of mitomycin C concentration. This limit value does not depend on cell heterogeneity since genetically homogeneous subclones, derived from DS19 clone, show levels of mitomycin C inhibition between 16 and 50%. Treatment with mitomycin C at different times after dimethyl sulfoxide addition shows that cell sensitivity to inhibition is not homogeneous during the induction period; it is maximal between 18 and 24 h from the start of induction and is observed with a concentration of mitomycin C as low as 25 μM. The inhibition of the benzidine positive phenotype expression appears irreversible since this effect is observed on cells even several generations after those which were actually treated.

INTRODUCTION

Murine erythroleukemia cells are virus transformed erythroid cells blocked in a relatively early stage in the pathway of differentiation (1). Erythroid differentiation can be induced in them in vitro by culturing in the presence of one of the several known inducers such as dimethyl sulfoxide (2), hemin (3), hemagglutinin (4), or butyric acid (5). Some inhibitors of DNA and RNA synthesis synergistically stimulate erythroid differentiation (6). The induction causes chains of events that eventually lead to synthesis and accumulation of hemoglobin. Increase of globin mRNAs synthesis, production of enzymes of heme biosynthesis, and erythrocyte specific membrane alterations are common effects of the induction, although different transitory metabolic changes might occur depending on the inducer used (7). The use of DMSO (8) as an inducer causes a transient arrest of the cells in G<sub>1</sub> before they become definitively committed (8) and it affects the chromatin structure (8, 9). It has already been reported that the intercalating dye propidium iodide differentially binds to the chromatin of MEL cells cultivated with and without DMSO (8). MEL cells induced to differentiate with DMSO are a convenient model system to investigate the step(s) involved in alterations of the chromatin states during the differentiation process. MitC has been chosen in this study because it interacts with DNA, as a single or double strand reagent (10), with preferential target sequences, leading to alterations in the DNA structure (11). MitC causes accumulation of hemoglobin (6) in the T3-C12 clone of murine leukemia cells at concentrations much higher than those compatible with survival of the MEL cell clone we use in this study. We show here that MitC inhibits induction of the benzidine positive phenotype of MEL cells at concentrations that do not affect cell viability and proliferation. The inhibition value is about 30–50% in the original DS19 clone and ranges between 16 and 50% in all its subclones tested. This effect appears to be irreversible. A well defined time period during the induction process has been identified in which the cells show maximal sensitivity to MitC. The high efficiency of the inhibition and the noncorrelation with the concentration or with the duration of cell exposure to the drug are discussed as evidence that MitC, under the conditions used, interferes with MEL cell commitment.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. Murine erythroleukemia cells were originated in Dr. Friend’s laboratory (1). Clone DS19 was grown in modified Dulbecco’s minimal essential medium (Gibco Laboratories, Paisley, Scotland) supplemented with 7.5% fetal bovine serum (Flow Laboratories, Irvine, Scotland), penicillin (550 units/ml) and streptomycin (75 units/ml) at 37°C in 10% CO<sub>2</sub> humidified atmosphere. Cell growth was monitored daily by counting cells in a Burker chamber. Cell viability was measured as the ability to exclude trypan blue (Burr, BDH Chemicals, Poole, England). Cloning of DS19 was performed in 0.4% agar (Difco Bacto-agar). Single colonies, picked up with Pasteur pipets, were enlarged over a period of 30 days and then tested for the expression of the benzidine positive phenotype in the presence or absence of MitC. Experiments were performed also on subclones of individual clones. Inductions were performed by subculturing 5 x 10<sup>3</sup>–1 x 10<sup>5</sup> cells/ml in fresh medium containing either 280 μM DMSO (Riedel, Hannover, Germany) or 0.1 mM hemin (Sigma Chemical Co., St. Louis, MO). Solutions of MitC (Sigma) in water were filter sterilized and stored at −20°C. Cultures containing MitC were manipulated in dim light to avoid the drug decay. At the end of MitC treatment, cells were washed once by centrifugation and then were resuspended in prewarmed fresh culture medium.

Determination of Benzidine Positive Cells. Percentage values of benzidine positive cells were determined with the following procedure. An aliquot of the cell suspension in the culture medium was treated with an equal volume of 0.2% benzidine HCl (Sigma) in 3% acetic acid; then 10% (v/v) of 40 volumes H<sub>2</sub>O<sub>2</sub> was added. When hemin was the inducer, cells were washed twice in 0.9% NaCl solution before adding the benzidine stain. The percentage of benzidine positive cells was determined from the actual number of cells scored which exceeded 200/ determination. Each value is the average of at least duplicate determinations on duplicate samples. Spontaneous benzidine positive cell induction, in the presence or absence of MitC, was lower than 2%.

Statistical Test. Student’s t distribution for paired values has been applied for the statistical analysis of the data.

RESULTS

MitC interferes with the induction of the benzidine positive phenotype of MEL cells only if present during DMSO treatment. There are no statistically significant differences in the percentage of induced benzidine positive phenotype between control and cells treated for 24 h with 25 μM MitC before DMSO induction was initiated (Table 1A). Increase of MitC concentration decreases cell viability and proliferation and eventually causes cell death but has no effect on benzidine positive phenotype expression of the surviving cells (data not shown). Concentrations of MitC, which have no effect if added...
Table 1  Effect of MitC on the expression of the DMSO induced benzidine positive phenotype in MEL cells

A. Cells were cultivated in the presence of 25 pm MitC for 24 h and then resuspended in fresh medium without MitC and induced with DMSO.
B. Cells were treated with 25 pm MitC during the initial 24 h from the start of induction with DMSO and then were resuspended in fresh medium containing only DMSO. Values are percentage of cells expressing the benzidine positive phenotype between days 4 and 8 following DMSO induction and are the average of at least 4 independent determinations. In A, the differences between control and MitC treated cells are not statistically significant: (P > 0.01); in B, the differences are statistically significant: (P < 0.01).

<table>
<thead>
<tr>
<th>% of benzidine positive cells</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 4</td>
<td>34.5</td>
<td>33.0</td>
</tr>
<tr>
<td>5</td>
<td>55.3</td>
<td>51.5</td>
</tr>
<tr>
<td>6</td>
<td>67.7</td>
<td>57.5</td>
</tr>
<tr>
<td>7</td>
<td>82.1</td>
<td>69.0</td>
</tr>
<tr>
<td>8</td>
<td>73.0</td>
<td>72.0</td>
</tr>
</tbody>
</table>

Fig. 1. Effect of MitC on percentage of MEL cell benzidine positive (B+) phenotype expression induced by hemin. Control: cells grown in the presence of 100 μM hemin as inducer (●); test: cells grown as in control plus 25 pm MitC (▲). Percentage of benzidine positive cells is determined from days 3 to 6 of treatment. Differences in percentage of benzidine positive cells between control and test are not statistically significant (P > 0.05). Top, growth curves. to the culture before induction with DMSO, quite effectively inhibit the expression of the benzidine positive phenotype when present for the same duration in the cell culture for the initial 24 h of induction with 2% DMSO (Table 1B). Replicas of the experiments give consistent results, showing statistically significant differences between control and treated groups, with confidence limits better than 1%. Similar experiments, performed with hemin as inducer, show no appreciable difference in the percentage value of induced benzidine positive phenotype between control and MitC treated cells, even when the drug is continuously present in the culture medium during the induction period (Fig. 1).

Fig. 2 shows the data when MitC is present during the initial 4 days of DMSO induction. In this case the inhibition of the benzidine positive phenotype is evident but the level of this inhibition is practically identical to that observed when MitC is present in the culture medium for only the initial 24 h (see Table 1). Hence, there is no additional effect on the inhibition of the benzidine positive phenotype expression by increasing 4-fold the duration of exposure of cells to MitC. MEL cells exposed to MitC for different sequential 24-h time periods during the initial 4 days of DMSO induction show differential inhibition of the expression of the benzidine positive phenotype. Treatments during 0–24- and 24–48-h periods cause maximal and almost identical inhibitions while 48–72- and 72–96-h periods are much less sensitive to inhibition of the benzidine positive phenotype induction (Fig. 3). Differential sensitivity of MEL cells to MitC inhibition is present also during the initial 48 h. This is shown by experiments in which MEL cells are exposed to MitC for only 6-h periods during the initial 30 h of DMSO induction. The results of these experiments are reported
MitC INTERFERENCES WITH MEL CELL DIFFERENTIATION

in Fig. 4. It is evident that MEL cells are not equally responsive to MitC in the initial 48-h period of DMSO induction since there are clear differences in the inhibition during the different 6-h periods. Statistical analysis of the data, using matched pairs Student’s t distribution, shows that the benzidine positive phenotype is inhibited by MitC during all 6-h periods tested and the period between 18 and 24 h is by far the most effective.

MitC, at the concentrations used in the above experiments (2500–25 pm), influenced neither the cell viability, as indicated by trypan blue exclusion test, nor the cell growth rate as evidenced by the growth curves shown in the figure insets. No significant difference is observed in growth rate between control cells and those treated with MitC for 6-h periods, as determined every 24 h since drug treatment (Fig. 4). Experiments were performed with decreasing MitC concentrations in order to study the correlations between the drug dose and its effect (Fig. 5). The MitC effect is the same between 250 pm and 25 fm. At the lowest concentration value causing inhibition, it is in a ratio of a few hundred molecules per MEL cell. At 0.25 fm, the lowest concentration value tested, MitC did not affect viability, growth rate, and percentage of benzidine positive cells in DMSO induced cells. The effect of MitC was tested also on individual subclones derived from one of the clones of DS19 in our stock, by exposing the cells to 25 pm MitC for 6 h, between 18 and 24 h from the beginning of induction. We found that all clones are inhibited by MitC although to different extents (Table 2). The average inhibition value is about 30% and in all clones this value never reaches 100%.

DISCUSSION

The experiments on MEL cells reported here show that very low concentrations of MitC, between pm and fm, having no influence on cell proliferation and viability, cause a decrease in the induction of erythroid differentiation by DMSO. The decrease in the percentage of induction is observed in cells which are several generations later than that actually treated with MitC (see Fig. 3). This demonstrates that the MitC effect is permanent and not due to a lag in the expression of the benzidine positive phenotype. Other substances were reported to inhibit DMSO induced MEL cell differentiation but, in all those cases, the effects were reversible upon removal of the substance (12, 13). The irreversibility of MitC effect shown here may be due to modifications of DNA. In fact, MitC is known to interact in vitro with both single and double stranded DNA, showing preferential binding to specific short sequences (11). It causes in vivo the formation of single strand exchanges in chromatin, when used at m–mm concentrations (14). If the inhibitory effect is indeed due to DNA modifications, then the finding that low doses (hundreds of molecules per cell) of MitC are effective in inhibiting benzidine positive phenotype expression suggests that the targets for MitC are not randomly distributed on DNA (Fig. 5). Consequently, the MitC specificity is dependent on the chromatin structure rather than on DNA sequences (11, 15). The existence of preferential binding regions on DNA of active chromatin has been observed in in vitro studies of human lung cells treated with a nonspecific reagent such as benzopyrene (16). In our experiments, preferential interactions with chromatin altered in conformations are suggested by the fact that the benzidine positive phenotype expression is inhibited only in cells treated with MitC during DMSO induction. In fact no inhibition is seen when the cells are exposed to MitC for 24-h period prior to DMSO (Table 1). In line with this hypothesis, no effect on benzidine positive phe-

Table 2 MitC inhibition of the expression of the benzidine positive phenotype in individual subclones derived from a clone of DS19

<table>
<thead>
<tr>
<th>Clone</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>B</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>DS22-1</td>
<td>37</td>
<td>25</td>
<td>69</td>
</tr>
<tr>
<td>DS22-2</td>
<td>22</td>
<td>16</td>
<td>42</td>
</tr>
<tr>
<td>DS22-3</td>
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<td>52</td>
<td>91</td>
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<td>DS22-4</td>
<td>58</td>
<td>31</td>
<td>69</td>
</tr>
<tr>
<td>DS22-5</td>
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<td>41</td>
</tr>
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<td>49</td>
<td>35</td>
<td>52</td>
</tr>
<tr>
<td>DS22-7</td>
<td>26</td>
<td>23</td>
<td>76</td>
</tr>
<tr>
<td>DS22-8</td>
<td>26</td>
<td>11</td>
<td>55</td>
</tr>
</tbody>
</table>

AIC% indicates average percentage of benzidine positive cell inhibition for a specific clone in the whole period. AID%, average percentage of benzidine positive cell inhibition for all tested clones in each day. Differences between controls and MitC treated cells are statistically significant (P < 0.001).
mitC interferences with MEL cell differentiation

Phenotype expression is observed when hemin is used as an inducer (see Fig. 1). These data suggest the possibility that limited numbers of targets are formed in the chromatin of each cell, at specific times, as a consequence of exposure to DMSO. Studies on DNA binding with intercalating molecules (8) provide evidence that treatment of MEL cells with DMSO alters the chromatin organization. Evidence of chromatin alteration has also been obtained from studies of β-globin chromatin DNA sensitivity to micrococcal nuclease in differentiating MEL cells treated with DMSO. DNA coding for β-globins is specifically hydrolyzed by DNase I at hypersensitive sites between 24 and 36 h following DMSO treatment (17). Friend cells, induced with hexamethylenebisacetamide or DMSO, show between 6 and 48 h following induction, a Ca²⁺-Mg²⁺ dependent endonuclease activity that causes single strand DNA breaks in high molecular weight chromosomal DNA (18). Both nuclease sensitivity and endonuclease activation are not observed when hemin is used as the inducer.

The finding of the existence of a defined time window between 18 and 24 h following DMSO treatment (see Fig. 4), in which the cells are particularly sensitive to MitC, supports the prevalence of a mechanism by which DMSO treatment would alter the chromatin structure and the availability of specific DNA sites at specific times during the induction. The inhibition of benzidine positive phenotype expression caused by MitC is limited to about 50% and this maximum value of inhibition does not increase by increasing MitC concentration or by prolonging the treatment time. This type of response, blurring results of analyses attempting to demonstrate temporal order of biochemical events (19), has been found when analyzing commitment of Friend erythroleukemia cells to erythroid differentiation (19, 20). This has led to the proposal of stochastic mechanisms of cell commitment. Indeed, intrinsic within a stochastic commitment program is an inevitable asynchrony in the expression of functions. The limit value of the benzidine positive phenotype inhibition of MitC treated cells, observed in the experiments presented here, cannot be ascribed to a heterogeneity in the cell population of the DS19 clone as clearly demonstrated by our data (Table 2). All the individual subclones of DS19 clones were sensitive to MitC inhibition with an average limit value of about 30%. The number of subclones tested is small, but the development of genetic heterogeneity in all of them during the short time of clone expansion seems impossible. The consequence is that the limit in MitC inhibition is indeed intrinsic within the cell. This result is in accordance with that expected in the case of MitC interaction with chromatin at specific sites exposed stochastically on the chromosomes during the process of DMSO induced commitment. In summary all the results strongly suggest that the effect of MitC is due to interference with cell commitment.

Acknowledgments

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References

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