Drug-induced revertants of adenovirus-transformed cells: retransformation by 5-azacytidine without reactivation of E1a

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We have isolated drug-resistant variants from adenovirus-transformed rat cells that had concomitantly lost their transformed phenotype. Our aim was to determine the reason for reversion, to attempt retransformation with 5-azacytidine (5-AzaC) and to study the mechanism of retransformation. Of the three cell lines studied, one (G4F) had lost the integrated Ela genes, whereas the other two (G2a and G5) failed to synthesize Ela RNA or proteins. Incubation of these cell lines with 3 μM-5-AzaC for 2 days, followed by passaging in the absence of drug, gave rise to transformed foci in all of the cell lines. The efficiency of transformation was typical of each cell line. Surprisingly, retransformation was not accompanied by the reappearance of detectable levels of Ela gene activity in the G2aAza and G5Aza cell lines. In search of a mechanistic explanation for the loss of gene activity in the revertants and its reappearance in the retransformants, we examined the state of methylation of the Ela gene region in these cells. Neither the Ela promoter nor its upstream region was methylated in the revertants or the 5-AzaC retransformants. These results suggest that Ela transcription was suppressed by mechanisms other than DNA methylation and that 5-AzaC could retransform these cells without lifting the Ela-suppressed state.

Introduction

There is considerable evidence that DNA methylation represents an epigenetic mechanism for controlling gene expression. Transcriptionally active genes or their flanking sequences frequently show evidence of hypomethylation when compared to their inactive form. Furthermore, alterations in the profile of DNA methylation are often heritable in somatic cells, thus providing a mechanism for epigenetic alterations in gene expression (Bird, 1986; Wigler et al., 1981). There is evidence to suggest that abnormalities in DNA methylation may be specifically related to carcinogenesis (Jones, 1985; Riggs & Jones, 1983). A decreased level of DNA methylation has been found in a number of tumours and tumour cell lines (Gama-Sosa et al., 1983; Kuhlmann & Doerfler, 1983; Lapeyre et al., 1981; Nyce et al., 1983). The ras and myc oncogenes were found to be hypomethylated in tumours compared to the corresponding normal tissues (Cheah et al., 1984; Feinberg & Vogelstein, 1983).

A well known inhibitor of DNA methylation, 5-azacytidine (5-AzaC), is a useful tool for assessing the role of this type of epigenetic phenomenon in cancer, because several studies have shown that the agent is not demonstrably mutagenic in eukaryotic cells (Delers et al., 1984; Kerbel et al., 1984; Landolph & Jones, 1982). The drug is capable of direct transformation of cultured cells (Benedict et al., 1977; Harrison et al., 1983; Hsiao et al., 1985), but the mechanism of action is not clearly defined. Samid et al. (1987) have successfully retransformed revertants by treatment with 5-AzaC. We have isolated drug-resistant variants from adenovirus-transformed rat cells that had concomitantly lost the transformed phenotype (Sircar et al., 1987; Sircar & Weber, 1988). Our aim was to determine the reason for reversion and to establish whether these cells could be retransformed with 5-AzaC. It was hoped that a study of these mechanisms might shed light on the molecular basis for reversion and retransformation of mammalian cells.

Methods

Cells. F4 rat cells were obtained from Bruce Stillman (Cold Spring Harbor Laboratory). They were derived from embryonic rat brain cells transformed by adenovirus 2 (Gallimore et al., 1977). F4 cells were recloned three times in succession and variants resistant to methylglyoxal bis(guanylhydrazone) (MGBG) were isolated from these subclones (Sircar et al., 1987). These isolates were named G2, G4 and G5 and have been described before (Sircar et al., 1987; Sircar & Weber, 1988). Variant subclones derived from G2 and G4 were named G2a and G4F, respectively, and both of these cell lines maintained MGBG resistance and a detransformed phenotype. G2a and G5 contained integrated E1 genes indistinguishable from those of the parental F4 cells. However, the G4F line had undergone a major deletion event, which removed any detectable traces of Ela genes, leaving about one
copy of the late region intact. G4F could therefore serve as an excellent transformation-negative control cell line. The transformation-positive control cell lines were the parental F4 cells and G4NF, a transformed derivative of G4. The FR3T3 cell is a normal rat fibroblast cell line (Seif & Cuzin, 1977). All cell culture was in Petri dishes in Dulbecco’s minimal essential medium and 5% foetal calf serum in air-CO2 (Seif & Cuzin, 1977). All cell culture was in Petri dishes in Dulbecco’s

Azacytidine treatment. The cells were treated with freshly prepared 3 μM-5-AzaC (Sigma) or 3.0 μM-6-azacytidine (6-AzaC; ICN Biochemicals) at 48 h after plating. Cells were subcultured thereafter in the absence of the nucleosides and observed for phenotypic alterations. Oncogenic transformation was determined by focus formation, growth in soft agar or tumorigenicity in nude mice.

For in vivo treatment 6- to 8-week-old nude mice were inoculated subcutaneously with 5 x 10⁶ cells/site. Twenty-four h later 400 μg of freshly prepared 5-AzaC in 200 μl of phosphate-buffered saline was administered intraperitoneally to each treated animal (20 mg/kg).

Analysis of DNA, RNA and protein. Total intracellular DNA was extracted from NP40-isolated nuclei by the SDS-protease K-phenol method and Southern blot analysis, performed according to standard procedures (Maniatis et al., 1982). Cytoplasmic RNA was purified according to Maniatis et al. (1982) and separated on a 1% agarose gel containing 0.66 M-formaldehyde and blotted onto Hybond nylon membranes. Western blots of total cell lysates were from SDS-mercaptoethanol denaturing gels, as described before (Sircar & Weber, 1988).

Results

Reversion due to loss of Ela gene expression

The G2a, G4F and G5 cell lines had been in culture continuously for over 6 months prior to treatment with 5-AzaC and had remained stable, flat revertants during this time. Southern blot analysis showed no changes in integrated adenovirus sequences, except in the case of G4F, which had lost all traces of Ela genes at the time of isolation (unpublished results). To determine whether loss of Ela gene expression caused the detransformation we verified the synthesis of Ela proteins and RNA. Western blotting showed that none of these three cell lines contained detectable levels of Ela proteins (Fig. 1, lanes 4, 6 and 8). Northern blotting of cytoplasmic RNA confirmed this absence of Ela gene expression (compare Fig. 2 lanes 1 and 2 with lanes 3, 4 and 6). It is therefore presumed that the loss of Ela gene expression was responsible for the revertant phenotype in the case of the G2a and G5 cells. This transcriptional silencing had occurred at the time of the initial isolation of the G5 cells subsequent to mutagenesis (Rodrigues et al., 1987; Sircar & Weber, 1988). In the case of the G2a cell line this had happened more recently and occurred spontaneously during subcloning of the Ela-positive detransformed cell line. The expression of Elb genes was also verified by Western blotting. Elb transcription is absolutely dependent on the presence of Ela proteins (Flint, 1981), so it was not surprising that no Elb proteins were detected in cells other than F4 (results not shown).

Retransformation of revertants with 5-AzaC

The simplest explanation for the loss of Ela gene expression in the transformation revertants G2a and G5 cells is transcriptional silencing by methylation. To test this hypothesis monolayers of cells were exposed to 3 μM-5-AzaC for 48 h and then serially passaged in its absence. This treatment did not cause observable cytopathic
Table 1. Effect of 5-AzaC on the morphology and cloning efficiency of revertant cells in soft agar*

<table>
<thead>
<tr>
<th>Cell</th>
<th>Morphology†</th>
<th>Cloning efficiency (%) in soft agar with colony diameters‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Morphology</td>
<td>Small</td>
</tr>
<tr>
<td>F4</td>
<td>T</td>
<td>6</td>
</tr>
<tr>
<td>G2a</td>
<td>F</td>
<td>0-08</td>
</tr>
<tr>
<td>G2a/5-AzaC</td>
<td>T</td>
<td>1-4</td>
</tr>
<tr>
<td>G5</td>
<td>F</td>
<td>0</td>
</tr>
<tr>
<td>G5/5-AzaC</td>
<td>T</td>
<td>1-3</td>
</tr>
<tr>
<td>G4F</td>
<td>F</td>
<td>0</td>
</tr>
<tr>
<td>G4F/5-AzaC</td>
<td>F/T</td>
<td>0-2</td>
</tr>
<tr>
<td>FR3T3</td>
<td>F</td>
<td>0</td>
</tr>
<tr>
<td>FR3T3/5-AzaC</td>
<td>T</td>
<td>1-2</td>
</tr>
<tr>
<td>G2a/6-AzaC</td>
<td>F</td>
<td>0</td>
</tr>
</tbody>
</table>

* The cells were tested 16 passages after treatment with 5-AzaC.
† T, Transformed; F, flat; T/F, mixed morphology.
‡ Colony size was small (0-03 to 0-09 mm), medium (0-15 to 0-3 mm), large (0-45 to 0-9 mm) or giant (1-5 mm or greater).

effects. After the fifth passage transformed foci composed of strikingly rounded cells began to be observable in the G2a monolayer of cells (Fig. 3b). The number of foci progressively increased in the course of subsequent passages, such that by about 30 passages the cell population appeared to be composed entirely of transformed cells (Fig. 3c). Morphological transformation was slower in the other cell lines tested; G5 and FR3T3 developed foci after 16 passages and G4F only after 27 passages. However, these cells did not develop the rounded morphology seen in G2a cells. That morphological transformation was induced by 5-AzaC and not merely by serial passaging was shown by the fact that these cells have been passaged continuously for 1 (G4F, G2a) to 3 years (G5, FR3T3) without such changes. Furthermore, a similar experiment using 6-AzaC treatment of G2a cells did not result in transformation, even after 16 passages (Table 1).

Sixteen passages after treatment with 5-AzaC all the cell lines were tested for growth in soft agar and for tumour formation in nude mice. The efficiency of colony formation and the morphology of the colonies in agar was quite different for the different cell lines tested. A representative sample of colonies is illustrated in Fig. 4.

Fig. 3. Morphological transformation of G2a with 5-AzaC. Monolayers of G2a cells (a) were exposed to 3 μM-5-AzaC for 48 h, then washed and the cells were cultured in the absence of drug. Transformed foci were photographed after eight passages (b) and 30 passages (c).
difference in this experiment we determined the relative proportion of small, medium, large and giant colonies in agarose for each cell line (Table 1). These data show that the efficiency of 5-AzaC retransformation is variable and highly dependent on the nature of the starting cell line. This conclusion was corroborated by the differential tumorigenicity of the cell lines in nude mice (Table 2). Tumours developed with all cell lines tested, including the E1a gene-negative G4F line and the normal rat fibroblast line FR3T3. The analogue 6-AzaC, which does not inhibit methylation (Jones, 1986), did not induce tumours with G2a cells, the cell line most responsive to 5-AzaC.

To determine whether 5-AzaC would also retransform G2a cells in vitro, nude mice were inoculated subcutaneously with the MGBG-resistant G2a cells, and intraperitoneally the next day with 5-AzaC. Tumours developed at the sites of cell inoculation after a somewhat longer interval than in the case of in vitro treated cells (Table 2). Cells explanted from a tumour were resistant to MGBG, confirming that the tumours originated from G2a cells.

Table 2. Effect of 5-AzaC on the tumorigenicity of revertant cells*

<table>
<thead>
<tr>
<th>Cells</th>
<th>Tumour-free (days)</th>
<th>Tumours/site</th>
<th>Diameter (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F4</td>
<td>21/24</td>
<td>1.5-2</td>
<td></td>
</tr>
<tr>
<td>G2a</td>
<td>60/33</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>G4F</td>
<td>0/0</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>G5</td>
<td>0/0</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>FR3T3</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>5-AzaC treatment in vitro</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G2a/5-AzaC</td>
<td>14-28</td>
<td>14/18</td>
<td>1.5-2.5</td>
</tr>
<tr>
<td>G4F/5-AzaC</td>
<td>24-30</td>
<td>2/9</td>
<td>0.2-0.5</td>
</tr>
<tr>
<td>G5F/5-AzaC</td>
<td>30-50</td>
<td>4/9</td>
<td>1.0-1.5</td>
</tr>
<tr>
<td>FR3T3/5-AzaC</td>
<td>40</td>
<td>7/9</td>
<td>0.7-1.0</td>
</tr>
<tr>
<td>G2a/6-AzaC</td>
<td>43</td>
<td>0/9</td>
<td>-</td>
</tr>
<tr>
<td>5-AzaC treatment in vitro†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G2a</td>
<td>43</td>
<td>6/9</td>
<td>1.5-2.0</td>
</tr>
</tbody>
</table>

* Five × 10⁵ cells/site were injected subcutaneously (at three sites) in nude mice 16 passages after treatment with 5-AzaC. Tumour size was recorded at day 60. Cells were considered non-tumorigenic when no evidence of tumour formation was seen 2 months after injection.

† Twenty-four h after inoculation of nude mice with untreated cells the animals were injected with 5-AzaC (20 mg/kg) intraperitoneally.
Mechanism of retransformation

Retransformation of the revertants after 5-AzaC treatment could have occurred via several possible mechanisms. One such mechanism in the case of G2a and G5 cells could have been the reactivation of E1a expression, which was tested by checking for the synthesis of E1a proteins and mRNA at approximately the same passage number as in the transformation tests. As shown by the Western blot (Fig. 1) and the Northern blot (Fig. 2) there appeared to be no significant expression of the E1a genes before or after 5-AzaC treatment. The slight RNA signal observed in G5/5-AzaC (Fig. 2, lane 5) measured 6% of that expressed by the transformed F4 cells and was not sufficient to induce a detectable level of E1a proteins (Fig. 1, lane 7). It was conceivable that the apparent lack of E1a expression was merely a reflection of the heterogeneous nature of the mixed cell populations. This possibility was unlikely because the G2at/5-AzaC cells, which were derived from a nude mouse tumour induced by G2a cells, did not express detectable levels of E1a proteins.

The reason for the lack of reactivation of E1a gene expression was also investigated by determining the state of methylation of these genes before and after 5-AzaC treatment.

Cellular DNA was digested with XbaI followed by HpaII, and Southern blots were probed with the BglII E fragment of adenovirus-2, representing the E1 region (Fig. 5). This procedure would be expected to reveal the state of numerous CG sequences flanking and within the E1a region. Surprisingly, there was no evidence of methylation in this region in any of the cells tested (Fig. 5b). In a second experiment the analysis was restricted to a smaller region of E1a encompassing the promoter and part of the E1a coding region (see Fig. 5 map for details). Again no difference was found between the transformed F4 cells and the G2a or G2a/5-AzaC cells (Fig. 5c). The same conclusion was drawn from a similar experiment using an AvaI–AccI (nucleotides 192 to 757) fragment, which encompassed both E1a enhancers and also included G5 and G5/5-AzaC cells (data not shown).

These results indicated that reversion and 5-AzaC-mediated retransformation were due to changes in genes other than E1a. This conclusion is consistent with the observed 5-AzaC-mediated transformation of the G4F and FR3T3 cells, which do not possess E1a genes.

Discussion

One of the objectives of cancer research is to identify means of arresting or reversing the transformed phenotype. It is therefore interesting that stable reversion can be obtained by selection for resistance to a cancer chemotherapeutic agent such as MGBG (Sircar et al., 1987, 1988). In this report we have shown that these revertants, which no longer express the oncogene E1a, were retransformable by 5-AzaC without reactivation of E1a. In fact two cell lines which do not carry E1a genes were also transformed by 5-AzaC.
In two of the revertants, G2a and G5, reversion to the non-transformed phenotype occurred as a result of transcriptional arrest of the Ela genes. It should be recalled that these cells contain 16 tandemly integrated copies of the left 62% joined to the right 4% of the adenovirus chromosome (Flint, 1981; Fig. 5). Surprisingly this loss of Ela expression of most or all 16 copies was not accompanied by any detectable increase in methylation of this region.

The treatment of these revertants with 5-AzaC could not, and in fact did not result in increased cleavage with HpaII. What might then have been the cause of retransformation? The broad hypomethylating effects of 5-AzaC could have activated cellular oncogenes or cellular effector genes. Although 5-AzaC is not demonstrably mutagenic in mammalian cells, the drug has been shown to have variable effects on tumour progression (Frost et al., 1984; Jones, 1986; Kerbel et al., 1984; Olsson et al., 1985; Olsson & Forchhammer, 1984). It may activate genes necessary for progression in some cases, or serve to activate genes that repress progression in others (Babiss et al., 1985). Our results support previous reports that 5-AzaC is capable of the direct transformation of cultured cells (Benedict et al., 1977; Harrison et al., 1983; Hsiao et al., 1985), as well as being tumorigenic in animals (Carr et al., 1984; Samid et al., 1987). These studies not only cast a grave doubt on the use of demethylating drugs in cancer therapy, but also point to the need for more investigation of cotransforming factors or transformation effector genes, which would also be operative in tumour progression.

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References


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