Absence of Aminonucleoside-sensitive Steps in the Cell Cycle of SV40-transformed Human Fibroblasts

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SUMMARY

Puromycin aminonucleoside arrests cultured normal human lung fibroblasts in the G1 and G2 phases of the cell cycle but permits fibroblasts transformed by the oncogenic virus SV40 to continue their cell-cycle traverse. Aminonucleoside has no detectable effect on the entry of the transformed cells into mitosis but it does accelerate the rate of detachment of these cells from the growth surface. These differences were not due to an altered rate of penetration of the membrane of the transformed cells by aminonucleoside.

INTRODUCTION

Aminonucleoside of puromycin, a semisynthetic antibiotic, arrests the proliferation of diploid cell cultures from normal mammalian tissues at concentrations which permit continued growth of heteroploid cells derived from neoplastic tissues (11). Recently, we showed that the sensitivity of normal lung fibroblasts to this inhibitor is due to the presence of aminonucleoside-sensitive steps in the G1 and G2 phases of their generation cycle (12). HeLa cells, of neoplastic origin, do not appear to have any cell cycle points with special sensitivity to aminonucleoside (12). Since RNA synthesis, and in particular rRNA synthesis, is inhibited by aminonucleoside in both normal and neoplastic cells (2, 10), these findings suggest that replication of neoplastic cells is less dependent on RNA synthesis than is that of normal cells. However, the attempt to contrast HeLa cells and lung fibroblasts as examples of normal versus neoplastic cells suffers from the fact that these cells are of different histological types and differ in tissue of origin. The hypothesis that neoplastic conversion causes emancipation from a dependence of replication on unpaired RNA synthesis can be tested directly by comparison of pairs of cell lines with common origin. We therefore compared the effects of aminonucleoside on the proliferation of WI38 and WI26 cells derived from human embryonic lung (4, 6) with that of their SV40 transformants, WI38-VA13 and WI26-VA4 (3, 7).

MATERIALS AND METHODS

Tissue Culture. WI38, WI38-VA13, WI26, and WI26-VA4 cells were grown as monolayers in plastic bottles in antibiotic-free Eagle's basal medium supplemented to 10% with fetal calf serum (heated for 30 min at 56°C) and to 1% with glutamine. For measurements of the rates of cell proliferation and for autoradiographic experiments, the cells were seeded into replicate “ring” cultures (14), WI38 cells between Passages 17 and 25, WI26 between Passages 28 and 35, WI38-VA13 between Passages 196 and 205, and WI26-VA4 between Passages 177 and 189. Mycoplasma contamination was excluded by weekly monitoring of the cultures by colony formation on Mycoplasma agar (5) and by a more convenient autoradiographic method (13). All tissue culture materials were obtained from Flow Laboratories, Rockville, Md.

Cell Number and Volume Determinations. The procedures for cell counting and cell volume determinations have been described in detail previously (12). Completeness of cell detachment from the monolayer and the absence of cell clumps were verified by phase microscopy prior to enumeration of the cell number by an electronic particle counter.

Correction of Growth Curves for Cell Loss from Monolayers. A number of replicate “ring” cultures were exposed to nutrient medium containing 0.1 μCi of thymidine-3H (specific activity, 6.7 Ci/mmole) for 48 hr immediately preceding the start of the experiment. The medium was then removed, and a cell count and measurement of radioactivity by scintillation counting were performed to obtain the baseline for further studies. Different concentrations of aminonucleoside were then applied to groups of ring cultures, and measurements of the cell population number and total remaining 3H radioactivity were performed daily for 3 days.

Cytochemistry. Glass coverslips containing the cell monolayers were rinsed in 0.14 M saline, fixed in Carnoy’s fluid, stained with 0.5% aqueous toluidine blue, dehydrated in tertiary butanol, and mounted in Permount. The proportion of mitotic figures was then determined under oil immersion. In each coverslip culture, 1000 cells were examined for evidence of mitosis.

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Other Procedures. Methods for radioactive labeling and liquid scintillation counting have been detailed in a recent publication (12).

RESULTS

The untreated WI38 and WI38-VA13 cells had similar culture doubling times (Chart 1). When high concentrations of aminonucleoside were present (30 μg/ml or greater), cultures of normal fibroblasts became stationary after 24 hr of treatment, but transformed cells continued to increase in number throughout this experiment, although at a reduced rate. When treated with 100 μg of aminonucleoside per ml, cells in WI38-VA13 cultures increased at approximately 40% of the rate of those in untreated cultures for at least 72 hr. Similar results were obtained for WI26 and WI26-VA4 cells (not shown). WI26 cells at the passage levels available grew at lower rates than WI38 cells but responded in a similar manner to aminonucleoside. Their transformed counterparts, WI26-VA4 cells, were not fully inhibited by aminonucleoside at concentrations of 30 and 100 μg/ml. This showed that virally transformed fibroblasts resemble other neoplastic cell lines in their low sensitivity to the replication-inhibitory action of this compound.

The lowered sensitivity of transformed cells to aminonucleoside was not due to the inability of the inhibitor to penetrate the cells (Table 1). The data in Table 1 show that the amounts of aminonucleoside associated with cells after exposure to isotopically tagged aminonucleoside were proportional to cell volumes. Similarly treated cultures subjected to autoradiography showed no concentration of aminonucleoside at cell periphery, indicating that we were not dealing with surface binding of the inhibitor. Therefore, as indicated in Table 1, the intracellular concentration of isotopically tagged aminonucleoside appears to be the same in normal and transformed fibroblasts.

The relative insensitivity of WI38-VA13 cells to aminonucleoside was also shown by the effect of this inhibitor on the frequency of mitotic figures in the cultures (Chart 2). In normal fibroblasts treated with the inhibitor, there was a transient rise in mitotic index that could have been due to a prolongation of mitosis by the inhibitor but, in 6 hr, mitotic figures in aminonucleoside-treated WI38 cultures became more infrequent than in control cultures, due to a previously demonstrated G2 block in normal fibroblasts (12) and, in 24 hr, mitotic figures were almost absent. In cultures of transformed fibroblasts, on the other hand, mitotic index showed little change after treatment with the inhibitor, indicating that either the duration of mitosis became prolonged in proportion to the elongation of the entire cell cycle or the decrease in the rate of accumulation of cells in treated cultures was due to an increased loss of cells from the monolayer induced by the inhibitor, rather than the result of inhibition of the cell cycle. Data shown in Table 2 indicate that a significant loss of cells does take place from the monolayers of transformed fibroblasts and that this loss is accelerated by aminonucleoside. Thus cell density alone does not give a true indication of the growth-inhibitor effectiveness of aminonucleoside, and it is apparent that WI38-VA13 cells can continue to multiply for several cell generations, at only slightly reduced rates, in the presence of aminonucleoside.

DISCUSSION

It appears from experiments presented here that treatment with aminonucleoside produces the same differential effect on normal and transformed fibroblasts as does cell crowding in the so-called contact inhibition. In both crowded or aminonucleoside-treated cultures, the normal cells cease dividing and become arrested at specific points of the cell cycle, mainly G1 phase, while SV40 transformants continue the cell cycle traverse, but cell detachment and loss from the monolayer limits growth of the cultures. Thus a defined chemical substance seems to produce the same effects as the poorly understood consequences of “topoinhibition.” It remains to be discovered whether aminonucleoside acts in this situation by virtue of its known inhibitory activity on rRNA synthesis.
Entry of tritiated AMS* into the cells

Exponentially growing monolayer cultures of human lung fibroblasts and their SV40-transformed counterparts were exposed to 1 μCi puromycin AMS•H (The Radiochemical Centre, Amersham, England; 4 Ci/m mole) per 1 ml of Eagle’s basal medium for 1 hr at 37° in a CO2 incubator. Five μg of unlabeled AMS per ml were also present, so that the total amount of AMS in the system was approximately 6 μg/ml. The medium containing AMS was withdrawn and the monolayers were washed 3 times with 1-ml aliquots of 0.2 N TCA at 0°. The TCA washes were pooled and referred to as the acid-soluble fraction. The ring culture was then dismantled and the coverslip with acid-insoluble material was dried at room temperature, broken in half, and placed in a vial for counting of radioactivity. Both acid-soluble (0.2-ml aliquots) and acid-insoluble fractions were mixed with 1.0 ml of NCS tissue solubilizer (Amersham/Searle Corp., Arlington Heights, Ill.) for 1 hr at 37°, followed by the addition of 10 ml of scintillation fluid (Spectrafluor; Amersham/Searle). The samples were cooled in the dark, and the radioactivity was measured in a Nuclear-Chicago Mark 1 liquid scintillation counter, calibrated for approximately 20% efficiency of tritium counting. The values given in the table are totals of acid-soluble and -insoluble counts of radioactivity. Cells from monolayer cultures grown in parallel but not exposed to tritiated AMS were harvested by trypsinization, 3 aliquots were taken for cell counting, and the remaining cells were placed in 1-ml Winthrobe hematocrit tubes and centrifuged at 3000 x g for 10 min. The volume of 10⁶ cells was read off the calibration of the tube.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>dpm/10⁶ cells</th>
<th>Volume of 10⁶ cells in ml</th>
<th>dpm/ml of cell volume x 10⁻⁵</th>
</tr>
</thead>
<tbody>
<tr>
<td>WI38</td>
<td>48.260 ± 5.185</td>
<td>0.26 ± 0.03</td>
<td>18.6</td>
</tr>
<tr>
<td>WI38-VA13</td>
<td>71.000 ± 4.890</td>
<td>0.41 ± 0.03</td>
<td>17.3</td>
</tr>
<tr>
<td>WI26</td>
<td>35.391 ± 2.714</td>
<td>0.23 ± 0.02</td>
<td>15.4</td>
</tr>
<tr>
<td>WI26-VA4</td>
<td>56.490 ± 4.151</td>
<td>0.35 ± 0.05</td>
<td>16.1</td>
</tr>
</tbody>
</table>

* The abbreviations are: AMS, aminonucleoside of puromycin, TCA, trichloroacetic acid.
* Mean ± S.E.

to aminonucleoside is similar to the response of HeLa and other neoplastic cells to this drug.

The finding that SV40-transformed human fibroblasts show marked cell loss from monolayer cultures is similar to the observation of Macieira-Coelho (8), who found significant numbers of detached cells in supernatant fluids of cultures of SV40-transformed fibroblasts derived from adult human skin but not in supernatant fluids from cultures of normal fibroblasts. What is more important, we found that exposure to the inhibitor leads to increasingly greater cell loss from the treated cultures, thus showing that cell counts alone may not be sufficient for assessment of the inhibitory effects of chemicals on SV40-transformed human fibroblasts, and perhaps on other transformed cells.

The published data indicate that the resistance of SV40-transformed human fibroblasts to aminonucleoside is not a manifestation of a general property of lessened sensitivity to antimetabolites. Wright and Hayflick (15) reported that WI38 and WI38-VA13 cells are equally sensitive to inhibition of cell division by cytochalasin B, and Miedema et al. (9) found that these cells were equally resistant to methandrostenolone, although cells derived from malignant tissues were markedly inhibited by this steroid. Little more is known about comparative biochemistry of these cells, except that alkaline phosphatase content but not acid phosphatase or lactic dehydrogenase content is reduced in the transformed fibroblasts (1).

The experiments described here were performed on cells many generations after their transformation with the oncogenic virus, so an objection could be raised that the differences in their response to aminonucleoside need not...
Table 2
Assessment of cell loss by measurement of retention of radioactive DNA

Cultures were exposed to 0.1 µCi tritiated thymidine per ml (6.7 Ci/mmole) for the 48 hr preceding the treatment with AMS, and cell and radioactivity counts were made daily of cells remaining on the monolayer. The values are arithmetical means of 3 determinations. The spread of individual values did not differ by more than ±8% from the mean.

<table>
<thead>
<tr>
<th>Culture</th>
<th>W138 cells</th>
<th>W138-VA13 cells</th>
<th>Corrected cell count (x 10^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cell no./culture (x 10^4)</td>
<td>H remaining (%)</td>
<td>Cell no./culture (x 10^4)</td>
</tr>
<tr>
<td>0 hr</td>
<td>30</td>
<td>100</td>
<td>43</td>
</tr>
<tr>
<td>24 hr</td>
<td>Control</td>
<td>57</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>30 µg AMS</td>
<td>45</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>100 µg AMS</td>
<td>36</td>
<td>100</td>
</tr>
<tr>
<td>48 hr</td>
<td>Control</td>
<td>110</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>30 µg AMS</td>
<td>48</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>100 µg AMS</td>
<td>34</td>
<td>105</td>
</tr>
<tr>
<td>72 hr</td>
<td>Control</td>
<td>205</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>30 µg AMS</td>
<td>46</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>100 µg AMS</td>
<td>35</td>
<td>95</td>
</tr>
</tbody>
</table>

* The abbreviation used is: AMS, aminonucleoside.

necessarily be related to the neoplastic transformation, but perhaps to some characteristic acquired during subsequent passages. This is made unlikely by the finding that fibroblasts transformed and propagated independently, W138-VA13 and W126-VA4, differ from the parent cell strains in exactly the same way as regards their response to aminonucleoside and by the similarity of the effects of aminonucleoside on both these cell lines and on all other neoplastic lines tested (11).

We conclude that neoplastic transformation liberates normal fibroblasts from aminonucleoside-sensitive steps in the G1 and G2 phases of their life cycle. The mechanism of aminonucleoside action and the relevance of these findings to the acquisition of neoplastic properties by the transformed cells are the subject of a continuing investigation.

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REFERENCES

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