Multiple Mechanisms of N-(Phosphonomoacetyl)-L-aspartate Drug Resistance in SV40-infected Precrisis Human Fibroblasts

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ABSTRACT

Normal and SV40-infected human fibroblasts were grown in the presence of the drug N-(phosphonomoacetyl)-L-aspartate (PALA) and examined for evidence of genetic instability. Both cell populations were precrisis and showed a normal, diploid karyotype at early passage. In contrast to the normal IMR-90 cells, which showed growth arrest and did not form colonies in PALA, the SV40-infected IMR-90 cells formed colonies at a very high frequency and continued to cycle in the drug. The drug-resistant colonies senesced after continued growth in culture, indicating that this change in ability to amplify preceded immortalization. This is the first observation of mortal human cells overcoming the drug-induced growth arrest. Although all previously isolated PALA-resistant colonies demonstrated CAD gene amplification as the mechanism of the drug-resistant phenotype, these SV40-infected human cells also showed alternative mechanisms, including increases in gene copy number by aneuploidy and formation of an isochromosome 2p.

INTRODUCTION

The infection of normal cells with SV40 has long been known to destabilize the host genome, causing numerous chromosomal aberrations (1, 2), including tetraploidy and chromosomal rearrangements. These dramatic effects of viral infection are due to a specific viral gene, the large tumor antigen, or T-antigen (3). T-antigen expression is also responsible for the increased lifespan observed after viral infection and for the immortalization of a rare fraction of infected cells (4, 5). Among its activities, this protein binds and inactivates wild-type p53 (6) and binds the retinoblastoma gene product (7) as well as a recently identified Mr 300,000 cellular protein and others (8). It is likely that some of the genetic instability caused by addition of either the whole SV40 virus or T-antigen alone is due to interaction with these and possibly other cellular components.

Previously, a clonogenic assay demonstrated a high gene amplification potential in several immortal human cell lines, both tumorigenic and nontumorigenic, (9–11) but an undetectable frequency of amplification in normal (mortal) human fibroblasts (9, 12). When normal diploid human fibroblasts are placed in drug, the cells arrest in G1 and G2 of the cell cycle. In contrast, cells with a demonstrable amplification potential do not arrest and continue cycling in the presence of drug. Hybrids between normal diploid human cells and tumor cells that amplify at a high frequency were suppressed for the ability to amplify and arrested in the drug (10), suggesting that normal cells have a gene or genes that suppress the ability to amplify. The role of p53 in this process was examined in both human and mouse cells. We found that in the presence of either one copy or two copies of wild-type p53, we could not detect amplification in the malignant cells. In contrast, cells that had lost both copies of p53 readily generated drug-resistant colonies (11). This suggests that p53 is one component of the pathway regulating gene amplification.

In the present study, we continued our examination of the regulation of amplification in normal human fibroblasts. We report that the expression of SV40 large T-antigen is sufficient to allow normal cells to generate PALA3-resistant colonies. Previous to this work, all human cells which demonstrated a measurable frequency of gene amplification, as detected in the clonogenic assay, were immortal. Furthermore, all PALA-resistant colonies examined so far have shown amplification of the endogenous CAD gene. This work presents the first example of a human cell strain able to generate drug-resistant colonies at an extremely high frequency prior to immortalization. Furthermore, for the first time, resistance to the drug PALA was found in the absence of CAD gene amplification. Multiple types of genetic instability such as aneuploidy, chromosomal rearrangements including isochromosome 2p formation, and telomeric association are seen in these PALA-resistant SV40-infected human cells.

MATERIALS AND METHODS

Cell Lines. Cells were grown in a-minimal essential medium minus nucleosides and deoxynucleosides plus glutamine (Gibco) and 10% diazoyed fetal bovine serum at 5% CO2. IMR-90 normal human fetal lung fibroblasts were obtained from the National Institute on Aging Cell Repository and have a normal diploid karyotype. IMR-90 cells at PD30 were infected with SV40 to generate the diploid AGO3204 culture which we obtained from the National Institute on Aging Cell Repository at PD38. These precrisis AGO3204 cells had an extended lifespan and grew to approximately PD97. This number includes the generations prior to introduction to SV40. AGO3204C, a postcrisis cell line derived from AGO3204, was also obtained from the National Institute on Aging Cell Repository. Population doublings were determined by plating a known cell number and counting the cells when passaged (e.g., 2 x 106 cells plated, grown to 1.6 x 106 cells, added 3 PDs to the cumulative PD number).

The cell lines were tested for Mycoplasma and found to be free of contamination.

Karyotypic Analysis. Cell cultures at 50–80% confluence were incubated in medium containing Colcemid (0.25 µg/ml) for 4 hr. Mitotic cells were removed from plates, swollen in 0.075 M KG hypotonic solution, and fixed onto slides. Chromosomes were G-banded with 0.05% trypsin-0.02% EDTA (Boehringer Mannheim) and Giemsa stain (Fisher Scientific).

Clonogenic Assay. Cells were incubated in the presence of PALA, a drug which specifically inhibits the aspartate transcarbamylase activity of the multifunctional CAD enzyme (9). PALA was obtained from the Drug Evaluation Branch of the National Cancer Institute. Cells were plated at either 300 or 3000 cells/10-cm dish in increasing concentrations of the drug, and the plates were fixed and stained when colonies comprised at least 50 cells. The frequency of amplification is calculated as the number of PALA-resistant colonies occurring at 9 x LD50 of drug and is expressed relative to the number of colonies formed in medium without drug.

Cell Cycle Analysis. Cells were plated in regular growth medium or medium containing PALA at 9 x LD50 for the respective cell strain. On the fourth day, BrdUrd was added to a concentration of 10 µM, and after 4 to 5 hr the cells were trypsinized, counted, and fixed using 1.5 ml cold phosphate-buffered saline and 3 ml cold 95% ethyl alcohol per 106 cells. The fixed cells were processed as follows for propidium iodide staining. Two million fixed cells were centrifuged for 5 min at 1200 rpm using a Sorvall H1000B rotor at 4°C, and...
and the pellet was resuspended with vortexing in 3 ml 0.04% pepsin. After a 20-min incubation at 37°C, the nuclei were centrifuged as above and resuspended in 1.5 ml 2 n HCl while vortexing. After a 20-min incubation at 37°C, 3 ml 0.1 M sodium borate was added to neutralize the nuclei, and they were centrifuged for 5 min. The pellet was resuspended in 2 ml IFA with 0.5% Tween-20 and centrifuged as above. The nuclear pellet was resuspended in 100 μl 1:5 dilution of anti-BrdUrd fluorescein isothiocyanate in IFA (Becton Dickinson). After a 30-min incubation on ice in the dark, 2 ml IFA with 0.5% Tween-20 was added, and the tubes were centrifuged for 5 min. The nuclei were then suspended in 500 μl IFA, RNase A (Sigma) was added to 5 μg/ml, propidium iodide (Aldrich Chemical Co.) was added to 50 μg/ml, and suspension was incubated at 37°C for 15 min. The nuclei were then kept in the dark on ice for 15 min. Flow cytometry was then performed to distinguish cells in the G1, S, or G2 phase of the cell cycle using a Becton Dickinson FACScan instrument.

**Immunofluorescent Staining.** Cells were tested for the presence of T-antigen using the immunofluorescent staining procedure provided by Oncogene Science. The primary antibody, SV40 T-antigen (Ab-2; Oncogene Science), was used at 15 μg/ml. The secondary antibody was biotinylated goat anti-mouse IgG (10 μg/ml; Oncogene Science), and the fluorescein avidin was purchased from Vector Laboratories and was used at 2.5 μg/ml.

**RESULTS**

**Cell Characterization.** The growth potential, chromosome complement, and sensitivity to the drug PALA were determined for the normal human IMR-90 cells as well as for the SV40-infected derivative AGO3204. IMR-90 cells senesced after 63 PDs, while AGO3204 cells did not enter crisis until PD97 (Fig. 1). Both IMR-90 cells and early passage AGO3204 cells (PD46) displayed a normal, diploid karyotype (data not shown). As the SV40-infected cells increased in passage number, they became tetraploid with 67% of the metaphases having a tetraploid chromosome number at PD68 (Table 1). At PD82, the doubling time of these cells increased from 31-45 h, by PD93 the cells had slowed significantly in their growth, and by PD97 they were no longer dividing (Fig. 1). Morphologically, these late passage cells were very large with ruffled edges compared to the smaller fibroblast-like early passage cells. Immunofluorescent staining was done to confirm the presence of T-antigen in the SV40-infected cells. Strong nuclear staining was seen in AGO3204 but not IMR-90 cells (data not shown).

**PALA Resistance in Normal and SV40-infected Human Cells.** The ability to generate PALA-resistant clones was assayed in normal and SV40-infected IMR-90 cells at early and late passage levels using the previously described clonogenic assay. Both cell populations exhibited a similar sensitivity to the drug PALA with an LD50 of 1.5 μM for IMR-90 and 3 μM for AGO3204 cells. The IMR-90 cell strain, like other normal human fibroblasts examined, lacked a detectable frequency of amplification (i.e., no colony growth at 9 X LD50 of PALA, Fig. 2). The shape of the curve shown for IMR-90 is typical of that seen with nine other normal human fibroblasts (e.g., WI-38, NHI, GM2291; 9, 10) in that colonies are only detected at low concentrations of drug. In contrast, the SV40-infected IMR-90 cells were tested at PD46 and PD72, and at both early and late passages they showed an identical response, an extremely high frequency of colony growth in PALA (6.5 X 10^-2 at 9 X LD50). This frequency is 10-fold higher than any previously studied highly tumorigenic cell line. In addition to the high frequency, we also noticed an unusual dose-response curve. Past reports had documented reduced colony formation as the concentration of PALA (or methotrexate) increased. Formerly, in many tumorigenic cell lines that amplify at high frequencies (up to 10^-3), incubation in stringencies of drug that exceed 10- to 15-fold the LD50 yields no discernible colonies. In contrast, the frequency of PALA-resistant colonies in the SV40-infected cells plateaued at drug concentrations of 9 X LD50, and similar frequencies were detected at PALA concentrations as high as 100 X LD50. The postcrisis cell line AGO2804C also formed colonies at the same high frequency at 9 X LD50 as well as at higher concentrations of PALA (Fig. 2). Thus, immortalization of the AGO3204 cells does not alter the frequency of formation of drug-resistant colonies.

To determine whether SV40 T-antigen alone could also generate PALA resistance in NHI cells, the pSV3neo plasmid containing this gene along with a selectable neomycin resistance gene was transfected into NHI cells using Lipofectin reagent (BRL). These precrisis cells also formed colonies in the presence of drug at a high frequency (Fig. 2), indicating that expression of T-antigen alone in normal human fibroblasts is sufficient to allow these cells to become drug resistant. The y2xmet128 mutant of T-antigen, missing the first 127 amino acids, was also introduced into NHI cells. This mutant retained the p53-binding region but lacked Rb binding (13). Cells containing this plasmid generated drug-resistant colonies at a high frequency identical with that seen with the whole virus (data not shown).

**Characterization of Resistance Mechanisms.** To examine the mechanism of PALA resistance in the AGO3204 cells, 10 colonies selected in 27 μM PALA (9 X LD50) were isolated and expanded. Six additional subclones selected at higher concentrations of PALA (up to 130 X LD50) were also analyzed. Table 1 shows the PD level, chromosome count, and representative abnormalities seen in each clone. Particular attention was given to chromosome 2, since the CAD gene is located at 2p21. The two most striking differences between the normal IMR-90 cells and their SV40-infected counterparts were the chromosomal rearrangements and telomeric association observed in every SV40-infected clone examined. The high frequency of telomeric association was seen in late passage AGO3204 cell populations and subclones independently of exposure to drug.

To characterize the types of genomic instability seen with drug resistance, the subclones were categorized by gross examination of the chromosomes. As expected, induction of tetraploidy and aneuploidy by SV40 was observed (14). Of the 15 colonies examined, three of the clones (AG 27-10, 75-3, and 75-4) had a near-tetraploid chromosome number, two clones (AG 27-2 and 400-1) had an aneuploid number of chromosomes, and two clones (AG 27-7 and 27-9) showed an interesting isochromosome 2p rearrangement (Fig. 3). The remaining eight clones (AG 27-1, 27-3, 27-4, 27-5, 27-6, 75-1, 75-6, and 150-1) retained a near-diploid karyotype in a majority of the spreads counted but showed various chromosome rearrangements.

FISH of 12 subclones provided a more refined analysis of CAD gene copy number. Three clones (AG 27-2, 27-9, and 75-3) revealed single-copy hybridization signals to the CAD gene, but multiple copies of chromosome 2 were present, suggesting aneuploidy as a mecha-
Clones AG 27-1 to AG 27-10 were grown in 27 μM PALA, and 75-1 to 75-6 in 75 μM PALA, 150-1 in 150 μM PALA and 400-1 in 400 μM PALA. Individual subclones were isolated and expanded, and metaphases were prepared for microscopy. Particular attention was given to the status of chromosome 2 since the CAD gene is located on chromosome 2p12. Cells were analyzed for chromosome counts and chromosome 2 status using bright-field microscopy as well as FISH. The major mode represents the most often seen chromosome count, and the range of counts is also given. ND not determined.

Table 1 Chromosome Analysis of IMR-90, AGO3204, and its PALA resistant clones.

<table>
<thead>
<tr>
<th>Clones</th>
<th>Parent</th>
<th>Population doubling</th>
<th>No. of cells analyzed</th>
<th>Major modes (range)</th>
<th>Chromosome rearrangements</th>
<th>Cells with telomere association</th>
<th>Chromosome 2 status</th>
</tr>
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<tbody>
<tr>
<td>AG 27-1</td>
<td>AGO3204</td>
<td>65</td>
<td>10</td>
<td>43, 46 (41-79)</td>
<td>10/10 cells</td>
<td>10/10 cells</td>
<td>2 copies^a</td>
</tr>
<tr>
<td>AG 27-2</td>
<td>AGO3204</td>
<td>66</td>
<td>10</td>
<td>75 (66-90)</td>
<td>10/10 cells</td>
<td>10/10 cells</td>
<td>4 copies</td>
</tr>
<tr>
<td>AG 27-3</td>
<td>AGO3204</td>
<td>66</td>
<td>10</td>
<td>45 (44-47)</td>
<td>10/10 cells</td>
<td>8/10 cells</td>
<td>2 copies^a</td>
</tr>
<tr>
<td>AG 27-4</td>
<td>AGO3204</td>
<td>66</td>
<td>10</td>
<td>46 (44-85)</td>
<td>10/10 cells</td>
<td>10/10 cells</td>
<td>2 copies^a</td>
</tr>
<tr>
<td>AG 27-5</td>
<td>AGO3204</td>
<td>66</td>
<td>10</td>
<td>42, 44 (42-46)</td>
<td>10/10 cells</td>
<td>10/10 cells</td>
<td>2 copies^a</td>
</tr>
<tr>
<td>AG 27-6</td>
<td>AGO3204</td>
<td>68</td>
<td>50</td>
<td>43, 46 (41-84)</td>
<td>50/50 cells</td>
<td>50/50 cells</td>
<td>2 copies,^a 5 cells with random rearrangements</td>
</tr>
<tr>
<td>AG 27-7</td>
<td>AGO3204</td>
<td>69</td>
<td>10</td>
<td>43, 46 (42-68)</td>
<td>10/10 cells</td>
<td>10/10 cells</td>
<td>2 copies,^a 3 cells with i(2p)</td>
</tr>
<tr>
<td>AG 27-8</td>
<td>AGO3204</td>
<td>65</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>AG 27-9</td>
<td>AGO3204</td>
<td>66</td>
<td>50</td>
<td>46 (41-90)</td>
<td>43/50 cells</td>
<td>42/50 cells</td>
<td>2 copies,^a 25 cells with i(2p)</td>
</tr>
<tr>
<td>AG 27-10</td>
<td>AGO3204</td>
<td>66</td>
<td>10</td>
<td>87 (69-&gt;100)</td>
<td>10/10 cells</td>
<td>10/10 cells</td>
<td>4 copies</td>
</tr>
<tr>
<td>AG 75-1</td>
<td>AGO3204</td>
<td>67</td>
<td>10</td>
<td>43, 46 (43-46)</td>
<td>10/10 cells</td>
<td>6/10 cells</td>
<td>2 copies,^a 1 cell with 2;14 translocation</td>
</tr>
<tr>
<td>AG 75-2</td>
<td>AGO3204</td>
<td>66</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>AG 75-3</td>
<td>AGO3204</td>
<td>67</td>
<td>10</td>
<td>85, 46 (42-&gt;100)</td>
<td>10/10 cells</td>
<td>10/10 cells</td>
<td>4 to 2 copies, 1 cell with translocation</td>
</tr>
<tr>
<td>AG 75-4</td>
<td>AGO3204</td>
<td>67</td>
<td>10</td>
<td>85 (79-&gt;100)</td>
<td>10/10 cells</td>
<td>10/10 cells</td>
<td>4 copies, CAD gene amplification</td>
</tr>
<tr>
<td>AG 75-5</td>
<td>AGO3204</td>
<td>67</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>AG 75-6</td>
<td>AGO3204</td>
<td>67</td>
<td>10</td>
<td>43 (42-92)</td>
<td>10/10 cells</td>
<td>10/10 cells</td>
<td>2 copies^a</td>
</tr>
<tr>
<td>AG 150-1</td>
<td>AGO3204</td>
<td>67</td>
<td>12</td>
<td>44 (41-81)</td>
<td>9/10 cells</td>
<td>4/10 cells</td>
<td>2 copies,^a CAD gene amplification</td>
</tr>
<tr>
<td>AG 400-1</td>
<td>AGO3204</td>
<td>67</td>
<td>10</td>
<td>&gt;100 (46-&gt;100)</td>
<td>10/10 cells</td>
<td>2/10 cells</td>
<td>2 copies,^a</td>
</tr>
<tr>
<td>AGO03204-5</td>
<td>AGO3204</td>
<td>68</td>
<td>50</td>
<td>46 (42-90)</td>
<td>50/50 cells</td>
<td>44/50 cells</td>
<td>2 copies,^a 2 cells with 2q-&gt;2 copies</td>
</tr>
<tr>
<td>AGO3204-8</td>
<td>AGO3204</td>
<td>67.5</td>
<td>50</td>
<td>46 (41-&gt;100)</td>
<td>49/50 cells</td>
<td>50/50 cells</td>
<td>4 cells with random rearrangements</td>
</tr>
<tr>
<td>AGO3204-9</td>
<td>AGO3204</td>
<td>69.5</td>
<td>50</td>
<td>46 (42-90)</td>
<td>50/50 cells</td>
<td>47/50 cells</td>
<td>4 cells with random rearrangements</td>
</tr>
</tbody>
</table>

^a A subpopulation of cells (5-10%) was noted to be near-tetraploid and contained four copies of chromosome 2 per cell.
breaks (16). The drug-resistant colonies displayed many of these

cells, when placed in PALA, respond by blocking at both the G, and
4-5 orders of magnitude lower than the frequency of PALA-resistant
demonstrates that immortalization is not a prerequisite for drug resis-
tance. Although T-antigen is known to immortalize human fibroblasts,
immortalization is not a prerequisite for drug resistance. In contrast to the nor-
whole range of PALA concentrations suggests that the mechanisms generating colony formation in these SV40-
infected human fibroblasts are independent of the stringency of the drug. An identical curve was generated when the immortal derivative
AGO2804C was tested, showing that immortalization does not change the frequen-
y of drug resistance to high levels of PALA is presently unknown.

forms of genomic instability, but only rarely was gene amplification
seen. This is in contrast to the PALA-resistant colonies generated by
many other cell lines which display amplification of the endogenous

When SV40-infected human fibroblasts were examined using a
clonogenic assay, PALA-resistant colonies emerged at the very high
frequencies previously reported for highly tumorigenic cell lines (10^{-3}-10^{-2}; 10, 17). In most cell lines examined previously,
AGO3204C was tested, showing that immortalization docs not change
in precrisis AGO3204 cells is independent of the stringency of the drug.

14 senesced at or before PD70. The senescence of these colonies
demonstrates that immortalization is not a prerequisite for drug resis-
ence, although T-antigen is known to immortalize human fibroblasts,
the frequency of immortalization (3 \times 10^{-7} for IMR-90 cells; 15) is
4-5 orders of magnitude lower than the frequency of PALA-resistant
colony-forming ability of the cells by one-half; (f) the drug-
transported inside the cells. However, several pieces of information
suggest that the drug was affecting the cells: (a) 3 /MM PALA reduced
the percentage of cells in S phase and in transition from S to G2,
model system to enter the cells (data not shown). Perhaps transport
of drug inside the cells remains at a fairly low level. The mechanism
of drug resistance is that the virus is affecting the uptake of the drug so that little drug is actually
transported inside the cells. However, several pieces of information
suggest that the drug was affecting the cells: (a) 3 \mu M PALA reduced
the colony-forming ability of the cells by one-half; (b) the drug-
resistant colony isolated at the highest concentration of PALA, AG
resistant colony isolated at the highest concentration of PALA, AG

Fig. 2. AGO3204, IMR-90, AGO2804C, and NHF-pSV3neo cells were plated at ap-
propriate densities and selected at the concentrations of PALA indicated. When colony
size reached or exceeded 50 cells, plates were fixed and stained, and the colonies were
counted. Relative plating efficiency (R.P.E.) is the ratio of colonies growing in PALA to
colonies growing in the absence of drug. The curves represent an average of four deter-
cinations. AGO3204 and AGO2804C have LD_{50} values of 3 \mu M, the LD_{50} for IMR-90
cells is 1.5 \mu M, and the LD_{50} for NHF-pSV3neo cells is 5 \mu M. At 9 \times \text{LD}_{50}, AGO3204C
form colonies with a frequency of 6.5 \times 10^{-2}, and NHF-pSV3neo cells form colonies at a frequency of 2 \times 10^{-1}, while
IMR-90 cells show no colonies at 9 \times \text{LD}_{50}. Plating efficiencies for each cell line were
as follows: IMR-90, 19%; AGO3204, 15, 21, 17, and 14% at PD 41, 46, 48 and 72,
respectively. □ pSV3neo; A, AGO3204; •, IMR-90; Δ, AGO2804C.

Cell Cycle Analysis. We previously demonstrated that normal
cells, when placed in PALA, respond by blocking at both the G1 and
G2 phases of the cell cycle (11). To investigate whether these PALA-
sensitive cell cycle checkpoints were present in the SV40-infected
human fibroblasts used in this work, cells grown in the presence or
absence of PALA were labeled with BrdUrd and the nuclei stained
with propidium iodide for cell cycle analysis. The IMR-90 cells ar-
ested in G1 and G2 (Fig. 4) after drug exposure, as expected, with
only 4% of the cells in S phase (compared to 47% in S with no drug).
In contrast, AGO3204 cells continued to cycle in the presence of
PALA, with the differences being a reduction in the number of cells
in transition from S to G2, and the percentage of cells in S was also
reduced from 33-22%. Therefore, one function of T-antigen is to
relieve the PALA-sensitive cell cycle checkpoints operating in normal
cells.

Discussion

Normal and SV40-infected precrisis human fibroblasts were grown
in the presence of the drug PALA, and the resulting colonies were
examined for evidence of genomic instability. In contrast to the nor-

colony-forming ability of the cells by one-half; (f) the drug-
resistance can precede immortalization. Infection of human fibroblasts
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absence of PALA were labeled with BrdUrd and the nuclei stained
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DISCUSSION

Normal and SV40-infected precrisis human fibroblasts were grown
in the presence of the drug PALA, and the resulting colonies were
examined for evidence of genomic instability. In contrast to the nor-

Several categories of genomic instability were seen in the drug-
resistant colonies: tetraploidy, aneuploidy, and chromosomal rearr-
rangements, including a marker isochromosome 2p, CAD gene ampli-
fication, and telomeric association (Table 1). Many of the colonies
were examined by fluorescence in situ hybridization for CAD gene
amplification, but all of the colonies grown in 27 \mu M PALA showed
single-copy hybridization. In addition, Southern analysis of the 27 \mu M
colonies revealed no increase in CAD gene copy number. Since no
double minutes were seen, and because even small episomes carrying
eXtra copies of the CAD gene would be represented in the genomic
dNA examined by Southern analysis, we do not believe that extra-
chromosomal gene amplification is responsible for the PALA resis-
Fig. 3. Karyotype of AGO3204 clone 27uM-9. This clone was near-diploid but showed an isochromosome 2p in half of the spreads examined.

tance. In these colonies with no apparent increase in CAD gene copy number, the mechanism of PALA resistance remains unknown. It is interesting that the instability generating PALA-resistant colonies in SV40-infected cells is not limited to gene amplification, since previous studies with rodent and human cell lines showed that all drug-resistant colonies examined at these stringencies contained CAD gene amplification. In a parallel study using human fibroblasts infected with different viral oncoproteins, human papilloma virus E6/E7, CAD gene amplification was seen in 100% of drug-resistant colonies, indicating that different viral genes can lead to drug resistance through multiple mechanisms. This study found only two colonies (AG 75-4 and 150-1) which demonstrated a 2-fold amplification of the CAD gene in a small fraction of the cells examined. Even in these colonies, it is difficult to explain how only a 2-fold amplification of the CAD gene can result in such a high level of drug resistance (discussed in Ref. 18); therefore, other mechanisms may be responsible for the colony growth. An alternate mechanism of resistance would be increased expression of the CAD genes. Increased CAD expression through up-regulation of the CAD gene in response to PALA should be equivalent to increased amounts of CAD due to gene amplification. Since the AGO3204 colonies senesced before enough cells could be obtained for quantitative RNA analysis, RNA from cells exposed to several concentrations of PALA was analyzed, and no increased expression was seen. Another explanation for the growth of PALA-resistant colonies is that sufficient nucleosides for DNA synthesis are provided in the medium by dying cells on the same plate. Since the medium is routinely changed, this is unlikely to be the case.

The mortal nature of the parent AGO3204 cells and AGO3204 colonies isolated after growth in drug was demonstrated by careful recording of the population doublings. During routine expansion of the clones, 14 PALA-resistant colonies were noted to grow slowly and eventually senesce at or before PD70. Therefore, immortality does not precede the acquisition of genetic instability as measured by drug resistance. Furthermore, the immortalized AGO2804C cells had an identical frequency of drug resistance.

When placed in PALA, normal human fibroblasts will arrest in both the G₁ and G₂ phases of the cell cycle. When the ability of parental IMR-90 and SV40-infected AGO3204 cells to arrest in response to PALA was tested, the G₁/S and G₂/M blocks seen with IMR-90 and other normal cells were lost in their virus-containing counterpart. These experiments suggest the loss of one or more cell cycle checkpoints, mainly one at the G₁/S border but also one at G₂/M.

There has been much interest recently in the role of the p53 tumor suppressor gene product as a cell cycle checkpoint and as a negative regulator of cell growth. As postulated by Vogelstein and Kinzler (6), a tetramer of wild-type p53 molecules normally binds upstream of growth inhibitory genes and activates their expression. When p53 is mutated or inactivated (such as through binding to viral oncoproteins like SV40 T-antigen), this negative regulatory function is lost, and cells enter the cell cycle under conditions when wild-type p53-containing cells would arrest. Kuerbitz et al. (19) showed a G₁/S checkpoint function for wild-type p53, and only cells with wild-type p53

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were able to arrest in the cell cycle after exposure to γ-irradiation. The SV40-infected human fibroblasts used in this work continued to cycle in the presence of the drug, likely due to the interaction between SV40 T-antigen and p53. Kuhar and Lehman (20) examined AGO3204 cells and showed, as expected, that all detectable p53 was located in the nucleus and was bound to T-antigen. Therefore, the p53-binding function of T-antigen may relieve a PALA-sensitive checkpoint and lead to the high frequency of PALA resistance seen in AGO3204 cells. However, because T-antigen also binds several other cellular proteins including the retinoblastoma gene product, the inactivation of retinoblastoma and other cell cycle regulators may also contribute to the genetic plasticity of AGO3204 cells. In order to test this hypothesis, T-antigen mutants lacking the ability to bind various negative regulators were able to arrest in the cell cycle after exposure to γ-irradiation. The SV40-infected human fibroblasts used in this work continued to cycle in the presence of the drug, likely due to the interaction between SV40 T-antigen and p53. Kuhar and Lehman (20) examined AGO3204 cells and showed, as expected, that all detectable p53 was located in the nucleus and was bound to T-antigen. Therefore, the p53-binding function of T-antigen may relieve a PALA-sensitive checkpoint and lead to the high frequency of PALA resistance seen in AGO3204 cells. However, because T-antigen also binds several other cellular proteins including the retinoblastoma gene product, the inactivation of retinoblastoma and other cell cycle regulators may also contribute to the genetic plasticity of AGO3204 cells. In order to test this hypothesis, T-antigen mutants lacking the ability to bind various negative regulators were tested in AGO3204 cells after 4 days in PALA. Cells were labeled for 5 h with BrdUrd, fixed, and stained with anti-BrdUrd fluorescein isothiocyanate and propidium iodide. x axis, new DNA synthesis, with cells at the right having more BrdUrd incorporated. y axis, DNA content as measured by propidium iodide staining so the lower population has a G1 DNA content and the higher population has a G2 DNA content. AGO3204 cells continue to cycle in the presence of PALA, whereas IMR-90 cells show a dramatic decrease in the population in S phase. The percentage of cells in each phase of the cell cycle is: AGO3204 with no drug, G1 = 52.9%, S = 33%, G2 = 13.5%; AGO3204 + PALA, G1 = 49.2%, S = 22.3%, G2 = 28.4%; IMR-90 with no drug, G1 = 45.9%, S = 47.3%, G2 = 6.8%; IMR-90 + PALA, G1 = 66.3%, S = 4.2%, G2 = 29.5%.

REFERENCES

Multiple Mechanisms of \(N\)-(Phosphonoacetyl)-l-aspartate Drug Resistance in SV40-infected Precrisis Human Fibroblasts

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