Overexpression of Cyclin D1 Enhances Gene Amplification

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Abstract

Defects in cell cycle control and increased genomic instability, including gene amplification, often occur during cancer development. Cyclin D1 plays a pivotal role in G1, and this gene is frequently amplified and overexpressed in several types of human cancer. This study demonstrates that ectopic overexpression of cyclin D1 in a rat liver epithelial cell line markedly increased the yield of cells containing amplified copies of the CAD gene. This effect was associated with a loss of G1-S checkpoint control, although the cycin D1-overexpressing cells had a normal p53 gene. The capacity of cyclin D1 to enhance gene amplification may contribute to the process of genomic instability during tumor development.

Introduction

A critical aspect of the multistage process of carcinogenesis is the apparent ability of tumor cells to develop genetic variants with an abnormally high frequency. Normal mammalian cells have checkpoints at the G1-S and G2-M stages, at which cells can delay progress through the cell cycle to permit repair of damaged DNA and thereby prevent various types of mutations (1, 2). Therefore, defects in cell cycle control and the normal function of these checkpoints might enhance genomic instability. A frequent example of genomic instability in tumors is the occurrence of gene amplification, which is often seen in cellular oncogenes and genes that play a role in drug resistance (3). Previous studies demonstrated that homozygous loss of function of the p53 gene was sufficient to increase the susceptibility of cells to gene amplification (4—7). However, other factors can also play a role since some tumor cells with wild-type p53 genes can still display a markedly increased frequency of CAD gene amplification by more than 1000-fold, and that this effect appears to be due to the loss of G1-S checkpoint control in the cyclin D1-overexpressing clones.

Materials and Methods

Cell Culture. The spontaneously immortalized rat liver epithelial cell line K22 (22) and its derivatives were grown in α-MEM (GIBCO) plus 5% dialyzed fetal bovine serum (Sigma). The neomycin-resistant clones were maintained in medium containing 50 μg/ml G418. The cultures were maintained and passaged as previously described (17). Retrovirus-mediated Transduction. The cyclin D1-containing plasmid pMV7CCD1 or the vector control plasmid pMV7pl was transfected into GPAM12 cells to generate retrovirus particles, which were then used to infect K22 cells, using methods described previously (23). After infection, transfected cells were selected in medium containing 200 μg/ml G418, and a number of individual drug-resistant clones were randomly picked and studied following clonal expansion.

Protein Extraction and Western Blotting. Proteins were extracted from exponentially growing cells and were subjected to Western blot analysis using antihuman cyclin D antibody (United Biomedical, Inc.) to detect expression of the cyclin D1, as previously described (23).

Kinase Assay with Cyclin D1 Immunoprecipitates. Assays for cyclin D1-associated in vitro kinase activity were performed as previously described (23).

Flow Cytometric Analysis. The exponentially dividing cells were collected and analyzed for the cell cycle distribution and cell size by flow cytometry, as described (17).

Drug Selection for CAD Gene Amplification. Quantification of gene amplification was done by using a previously described PALA selection assay (25). PALA specifically inhibits the aspartate transcarbamylase activity of the multifunctional CAD enzyme, and thus inhibits de novo pyrimidine biosynthesis. The major mechanism of PALA resistance is amplification of the CAD gene. The PALA concentration that reduces plating efficiency by 50% (CF50) was first determined for each clone according to the methods described before (25). The cells were then grown in medium containing PALA at a concentration of 7 × CF50. After 2—3 weeks of selection, a number of resistant colonies were randomly picked for clonal expansion, and the plates were stained and the total number of resistant colonies was counted. The frequency of amplification was expressed as the number of colonies formed in the presence of PALA relative to the plating efficiency (the number of colonies formed without PALA).

Southern Blot Analysis to Determine CAD Gene Amplification. Cells were lysed in 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 25 mM EDTA (pH 8.0), 0.5% SDS, and 0.1 mg/ml proteinase K and extracted with phenol. Five μg DNA/sample were digested with EcoRI, electrophoresed on a 0.7% agarose gel, transferred to Hybond-N membrane (Amersham), and hybridized to a 32P-labeled hamster CAD cDNA probe, as previously described (25). Autoradiographs were scanned by a densitometer.

BrdUrd Incorporation to Detect DNA Synthesis. Cells grown in 24-well plates were incubated without PALA or with PALA at a concentration of 7 × CF50 for 1, 2, or 3 days and pulse labeled with BrdUrd (1:1000 dilution; BrdUrd) for 1 h. The cells were then fixed in methanol containing 0.3% H2O2, and BrdUrd incorporation into DNA was detected by staining with an anti-BrdUrd antibody (Amersham) followed by the ABC kit (Vector). At least 4 The abbreviations used are: PALA, N-phosphonoacetyl-L-aspartate; CF50, the PALA concentration that reduces plating efficiency by 50%; BrdUrd, bromodeoxyuridine; GST, glutathione-S-transferase; Rb, retinoblastoma.
200 cells were scored to determine the percentage of BrdUrd-positive cells in each experiment.

RNA Analysis. Total RNA was extracted from cells, and 10-μg samples were used for Northern blot analysis. The blots were hybridized with a 32P-labeled human p21WAF1 cDNA probe, using previously described methods (23).

Results and Discussion

A human cyclin D1 cDNA contained within a retroviral expression vector, pMV7pl (17), was introduced by transduction (23) into the spontaneously immortalized rat liver epithelial cell line K22 (24). As shown in Fig. 1A, high levels of the M, 36,000 cyclin D1 protein were detected in the cyclin D1 plasmid-infected clones, i.e., K22D1 2, D1 11, and D1 15, whereas low levels (~7—9 times lower) of the endogenous cyclin D1 protein were seen in the parental K22 and plasmid control K22pl cells. In vitro assays for cyclin D1-associated kinase activity using a GST-Rb fusion protein as substrate indicated a 3—11-fold increase with extracts from the overexpressor clones (Fig. 1B).

The cyclin D1 overexpressors did not display any morphological evidence of transformation. Their doubling time (19 h) was similar to that of the control cells when grown in standard medium with 5% fetal bovine serum. However, when grown in medium containing 1% fetal bovine serum, the doubling times of the overexpressor clones (21 h for D1 11 clone and 23 h for D1 15 clone) were 2—4 h shorter than that of the control cells (25 h), indicating that overexpression of cyclin D1 reduces the serum dependency of K22 cells. Flow cytometry on exponentially growing cultures indicated that in the control clones 52% of the cells were in G1, and 35% in the S-phase; with the three overexpressor clones, the corresponding values were 41 and 44%, respectively. When measured by forward angle light scatter for relative cell size (17), the cyclin D1 overexpressor clones exhibited a cell size that was 20—30% smaller than that of the control cells. These results are consistent with previous findings (13, 17—19) indicating that overexpression of cyclin D1 in fibroblasts also decreases cell size, shortens G1, and elongates the S-phase.

A convenient system for quantitating gene amplification utilizes selection for clones that are resistant to the drug PALA, since this resistance is usually due to amplification of the CAD gene (25). The PALA concentration that inhibited plating efficiency by 50% (i.e., CF50), was first determined for each clone, and the frequency of occurrence of PALA-resistant clones after treating cultures with seven times this dose was quantified (Table 1). Both the parental K22 and the vector control K22pl clones failed to yield detectable PALA-resistant colonies. Both control and cyclin D1-overexpressor clones generated PALA-resistant colonies at a frequency of ~2 x 10^-4 (Table 1), i.e., more than 1000 times greater than that of the parental and vector control clones. Both control and cyclin D1-overexpressing cells showed evidence of extensive cell death within 4—5 days after exposure to PALA, and the majority of the cells came off the plates during the subsequent 30 days. 4',6-Diamidino-2-phenylindole staining following PALA treatment did not detect chromosomal condensation or the formation of apoptotic bodies, indicating that the PALA-induced death was not via the process of apoptosis.

We randomly picked 10 PALA-resistant clones obtained from each of the K22D1 2, 11, and 15 clones (designated K22D1 2P, D1 11P, and D1 15P, respectively). Southern blot analysis indicated that ~90% of the 30 resistant clones contained amplified copies of the CAD gene. Representative data shown in Fig. 2 indicate that the amplification was about 2—10-fold.

### Table 1: CAD gene amplification in control and cyclin D1-overexpressing K22 cells

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Plating (%) efficiency</th>
<th>PALA CF50 (μM)</th>
<th>PALA-resistant frequency</th>
<th>CAD gene amplification</th>
</tr>
</thead>
<tbody>
<tr>
<td>K21</td>
<td>32.3</td>
<td>18.2</td>
<td>&lt;4.4 x 10^-7</td>
<td>—</td>
</tr>
<tr>
<td>K22pl</td>
<td>39.3</td>
<td>17.8</td>
<td>&lt;3.6 x 10^-7</td>
<td>—</td>
</tr>
<tr>
<td>K22D1 12</td>
<td>31.3</td>
<td>9.8</td>
<td>3.0 x 10^-4</td>
<td>+</td>
</tr>
<tr>
<td>K22D1 11</td>
<td>42.0</td>
<td>15.0</td>
<td>3.6 x 10^-4</td>
<td>+</td>
</tr>
<tr>
<td>K22D1 15</td>
<td>35.8</td>
<td>13.2</td>
<td>2.8 x 10^-4</td>
<td>+</td>
</tr>
</tbody>
</table>

a The PALA concentration that reduces plating efficiency by 50%.
b The frequency of PALA-resistant colonies obtained after treating cells with PALA at a concentration 7 x CF50.
c CAD gene amplification was confirmed by Southern blot analysis (Fig. 2). For additional details see "Materials and Methods."
To gain insight into this marked enhancement of gene amplification, we compared DNA synthesis in the control and cyclin D1 overexpressor clones by pulse labeling the cells with BrdUrd before and after PALA treatment (Fig. 3). We found that in the absence of PALA treatment, exponentially dividing cultures of the three cyclin D1 overexpressor clones and three PALA-resistant clones derived from these cells displayed a higher fraction of cells (by ~18%) incorporating BrdUrd than the parental and vector control cells. These findings are consistent with the above described flow cytometry data indicating that with the overexpressor clones a greater percentage of the cell population was in the S-phase. When pulse labeled with BrdUrd 3 days after addition of PALA to the growth medium, only about 15% of the K22 parental and vector control clones were labeled, but this value was about 43% in the three cyclin D1 overexpressor clones (Fig. 3). As expected, BrdUrd incorporation was not inhibited by PALA in the three PALA-resistant clones (Fig. 3). Furthermore, cell counts indicated that 2 days after addition of PALA, the number of vector control cells had increased by <20%, but the number of cyclin D1 overexpressor cells had doubled. These findings are consistent with the hypothesis that cyclin D1 enhances CAD gene amplification by partially overcoming the G1 arrest that normally occurs in cells treated with PALA.

Previous studies indicated that loss of the wild-type p53 tumor suppressor is sufficient to cause CAD gene amplification in PALA-treated cells (4–7). To exclude the possibility that the enhancement of CAD gene amplification in the cyclin D1-overexpressing cells was actually due to mutations in the p53 gene, we analyzed our cells for the possible presence of point mutations in exons 5–8 of the p53 gene, utilizing single-strand conformation polymorphism analysis, since the mutations found in the p53 gene are predominantly clustered within this region (26, 27). Neither the parental K22 cells, the K22pl clone, the three cyclin D1 overexpressor clones, nor the three PALA-resistant clones showed any band shifts (data not shown), thus providing evidence that they all had the wild-type p53 gene. We also examined the abundance and cellular localization of the p53 protein before and after PALA treatment by immunostaining. In untreated cultures, both the K22pl control cells and the K22D1 11 cells displayed very weak and diffuse staining for p53. After treating the cells with PALA for 2 days, both cell lines showed more intense and predominantly nuclear staining for p53 (data not shown). The PALA-resistant cells, however, displayed only weak p53 staining both in the absence and presence of PALA (data not shown), presumably because they had overcome PALA-induced nucleotide pool depletion as the result of CAD gene amplification. Induction of the p53 protein in response to DNA damage and other stresses increases transcription of the p21WAF1 gene, whose protein product inhibits multiple cyclin-cyclin-dependent kinase complexes, thus blocking

![Figure 3: Effects of PALA on cell cycle progression.](image-url)
cell cycle progression (12). Northern blot analysis indicated that p21\textsuperscript{WAF1} mRNA was induced in the vector control K22pl, the cyclin D1 overexpressor K22D1 11, and the PALA-resistant K22D1 11P cells after exposure to 5 or 10 Gy γ-irradiation (Fig. 4A), further confirming that normal p53 functions are maintained in the cyclin D1-overexpressing cells. In addition, PALA treatment led to increased p21\textsuperscript{WAF1} expression in the K22pl and K22D1 11 clones, but not in the K22D1 11P clone (Fig. 4B), which is consistent with the results of p53 induction.

Taken together, the above results suggest that the overexpression of cyclin D1 in K22 cells partially overcomes the block to G1-S progression that normally occurs when the parental K22 cells are exposed to the drug PALA. We have found that the increased capacity of cell extracts of the cyclin D1-overexpressor cells to phosphorylate the Rb protein (Fig. 1B) persisted for at least 3 days after PALA treatment (data not shown), despite the increase in p21\textsuperscript{WAF1}. Presumably, this high level of cyclin D1-associated kinase activity blocks the inhibitory effects of Rb at the G1-S transition despite p53-mediated induction of the p21\textsuperscript{WAF1} protein. Some of the overexpressor cells that enter the S-phase during PALA treatment might then amplify the CAD gene, and possibly other genes, because of abnormalities in DNA replication resulting from the depletion of nucleotide pools induced by PALA (2). We should emphasize that the present studies do not exclude other possible mechanisms by which overexpression of cyclin D1 might enhance gene amplification. In any case, since cyclin D1 is frequently overexpressed in a variety of human tumors (20, 21), the present results suggest that overexpression of cyclin D1 may contribute to the amplification of other genes and possibly enhance other types of genomic instability, thus enhancing the process of tumor progression.

Fig. 4. Induction of p21\textsuperscript{WAF1}. A, cells were γ-irradiated with 5 or 10 Gy, and total RNA was extracted 4 h after irradiation. B, total RNA was extracted from cells incubated in the absence of PALA or in the presence of PALA for 2 days. Ten µg RNA/lane were then subjected to Northern blot analysis with a human p21\textsuperscript{WAF1} cDNA as the probe. Lower panels, ethidium bromide staining pattern of 18S rRNA.

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### References

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