Reversible, p16-mediated Cell Cycle Arrest as Protection from Chemotherapy

Steven Stone, Priya Dayananth, and Alexander Kamb
Myriad Genetics, Inc., Salt Lake City, Utah 84108

Abstract

A model system has been developed to explore the relationship between cell cycle arrest and chemotherapeutic toxicity. An isopropyl-1-thio-β-d-galactopyranoside-inducible P16 construct was introduced stably into a melanoma cell line and used to promote G0/G1 arrest in the recipient cells. The state of arrest was reversible and did not compromise cell viability over a period of at least 7 days. Isopropyl-1-thio-β-d-galactopyranoside-treated, arrested cells were significantly more resistant to the chemotherapeutic agents methotrexate (~50 times), vincristine (>100 times), and cisplatin (~10 times) compared to controls. This strategy of protection from chemotherapy exploits one of the basic genotypic differences between normal cells and tumor cells: the integrity of genetic pathways that regulate growth.

Introduction

For the past several decades, radiation and chemical toxins designed to kill dividing cells have been the basis for cancer therapy. Although these anticancer agents may destroy tumor cells, they may also kill cycling nontumor cells such as intestinal epithelia, hair follicle cells, and hematopoietic precursors. These side effects have serious consequences. Side effects including cachexia (appetite loss), alopecia (hair loss), nausea, and immunosuppression limit the dose of anticancer agents. Partly for this reason, some tumor cells inevitably escape treatment. These surviving cells may give rise to tumors that are resistant to drug or radiation therapy. Despite advances in anticancer regimens, systemic toxicity and postsurgery tumor relapse remain challenging problems.

In principle, the deleterious effects of conventional cancer therapy would be diminished if nontumor cells were protected from cytotoxicity (1–4). Fewer normal cells would die and, since larger drug doses could be administered, fewer cancer cells would survive. Actively dividing normal cells might be protected from cancer therapy if they were induced deliberately to enter cell cycle arrest (1–4). In a quiescent state, cells are likely to be more resistant to the damaging effects of cancer therapy. However, for this approach to succeed, several conditions must be satisfied: (a) normal cells must arrest growth, whereas tumor cells do not; (b) the arrested cells must remain viable; and (c) the arrest must be reversible. These conditions might be achieved by taking advantage of the inherent genetic differences between normal and tumor cells. Genetic pathways that regulate cell growth in normal cells are inactivated by mutation in tumors. As a result, appropriately directed induction of these pathways should have no effect on tumor cells while causing normal cells to arrest. For instance, activation of the p16/Rb pathway by ectopic expression of p16 is sufficient to cause cell cycle arrest in a variety of Rb+ cell types but not in Rb− tumor cells (5, 6). Genetic and biochemical studies suggest that this pathway regulates growth in a wide variety of cell types and is frequently inactivated during tumor progression in vivo (7, 8). Therefore, the p16/Rb pathway is ideal for exploring the feasibility of cellular protection through reversible cell cycle arrest.

Materials and Methods

Cell Lines. H294T is a melanoma cell line in which P16 is deleted homozygously (data not shown). This cell line was used as the parent for construction of a line in which p16 expression is induced by IPTG using the lac-switch technology as described by the manufacturer (Stratagene). Briefly, the cell line HS294T was transformed with the lacI expression vector (p3′SS; Stratagene) by electroporation. Transformants were selected in medium containing 300 μg/ml hygromycin B (Boehringer Mannheim). Twelve transformed cell lines were cloned and assayed for lac repressor expression by reverse transcription-PCR. A cell line expressing relatively large amounts of the transgene was chosen for additional experiments (designated HS294T/lacI). HS294T/lacI was transformed with p16-containing expression vector (pOPRSVl-p16) by electroporation. pOPRSVl-p16 was constructed by cloning the entire p16 coding sequence into the Not I site of pOPRSVICAT (Stratagene). The p16 initiation codon was modified to match a consensus Kozak initiation sequence (9). Transformed cells were selected in media containing hygromycin B and 300 μg/ml Geneticin (Life Technologies, Inc.). Twelve independent cell lines were isolated, of which six were subsequently shown to undergo clear cell cycle arrest after treatment with IPTG. One of these cell lines, designated HS294T/P16+, was chosen for additional experiments. HS294T/P16+ was also transformed with pOPRSVICAT expression vector to create HS294T/P16+ control cell lines. Three independent cell lines of this type were isolated and used as negative controls in experiments described in this study. All cell lines were grown in DMEM media (BioWhittaker), supplemented with 10% fetal bovine serum, nonessential amino acids, and sodium pyruvate.

Western Analysis. Approximately equal amounts of total cellular protein were resolved on 15% SDS PAGE gels. After electrophoresis, proteins were transferred to Mylar membrane as described by the manufacturer (Hofer Scientific). p16 protein was detected using an anti-p16 monoclonal antibody (kindly provided by Gordon Peters, Imperial Cancer Research Fund Laboratories, London, United Kingdom) and a goat anti-mouse biotinylated secondary antibody (Vector).

Drug Resistance. Equal numbers of HS294T/P16+ cells were plated, and one-half of the samples were pulsed with IPTG at t = 0 for 12 h. At t = 24, all of the samples (IPTG-treated and untreated) were pulsed for 12 h with the indicated chemotherapeutic agent at the indicated concentration. The percentage of viability was estimated by comparing the number of viable cells present at each drug concentration to the no-drug control. Viable cells were identified by exclusion of 0.4% trypan blue. HS294T/P16− cells were treated identically in control experiments.

Results and Discussion

The melanoma-derived cell line HS294T, in which P16 is homozygously deleted, was engineered to contain P16 under the regulation of Escherichia coli lac sequences. In this construct, P16 gene expression is normally repressed but can be induced by the addition of IPTG to the growth medium. Six such cell lines were derived from the parental HS294T cell line. All six lines ceased to grow in the presence of IPTG, whereas the parental line (or lines with an IPTG-inducible promoter driving CAT expression) continued to proliferate. One of these inducible lines, termed HS294T/P16+, was chosen for subse-
quent experiments. Flow cytometry revealed that HS294T/P16+ was arrested at the G0-G1 stage of the cell cycle in the presence of IPTG (Fig. 1), and two independently derived HS294T/P16+ cell lines showed similar IPTG-induced arrest characteristics (Table 1). HS294T/P16+ is, in our model system, equivalent to P16+ normal cells; derivatives of the parental line without the p16 expression vector are analogous to P16− tumor cells.

The response of HS294T/P16+ to IPTG was examined in two additional ways: (a) different doses of IPTG were applied to the cells for 12 h. The percentage of cells in G0-G1 was measured to determine a sufficient dose to cause cell cycle arrest (data not shown). This dosages, 0.1 mm, was used for all subsequent experiments; and (b) immunoblotting using anti-p16 antibody were used to demonstrate that the addition of IPTG to HS294T/P16+ cells caused increased p16 protein expression (Fig. 2). Cell lysates were prepared from HS294T/ P16+ and P16− cells after they had been exposed to IPTG for 24 h. HS294T/P16− cells produced undetectable levels of p16 protein. In contrast, IPTG-treated HS294T/P16+ cells produced easily detectable amounts of p16 protein, approximately equal to the level of p16 expression in the Rb-negative cell line 5637.

To determine if p16-induced arrest was reversible, HS294T/P16+ cells were pulsed with IPTG for 12 h. After the IPTG was removed, the cells were monitored by flow cytometry, and the percentage of cells in G0-G1 as a function of time following IPTG removal was

Table 1 Response of P16+ cell lines to IPTG

<table>
<thead>
<tr>
<th>Cell line</th>
<th>−IPTG (%)</th>
<th>+IPTG (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS294T/P16+</td>
<td>68</td>
<td>94</td>
</tr>
<tr>
<td>HS294T/P16+(B)</td>
<td>62</td>
<td>95</td>
</tr>
<tr>
<td>HS294T/P16+(C)</td>
<td>51</td>
<td>56</td>
</tr>
<tr>
<td>HS294T/CAT (control)</td>
<td>75</td>
<td>90</td>
</tr>
</tbody>
</table>

* Response was measured by flow cytometry 36 h after the addition of IPTG to the cell culture medium.

Fig. 2. Expression of p16 protein as a result of IPTG treatment. After 24 h exposure to IPTG, roughly 10^5 cells from the indicated cell line were harvested and lysed for Western analysis. The Rb-negative (and hence p16-overexpressing) cell line 5637 served as the positive control.

Fig. 3. Reversibility of p16-induced cell cycle arrest. Graph of the percentage of cells in G0-G1 (y axis) versus time (X axis). p16−. HS294T/P16− cell line treated with IPTG. p16+. HS294T/P16+ cell line treated with IPTG. The results represent the mean of three experiments. Both cell lines were pulsed for 12 h with 0.1 mm IPTG. IPTG was added at r = 0 and removed at r = 12. Cells were harvested at 12-h time intervals, starting at r = 0. After harvesting, the cells were fixed in ethanol and analyzed by flow cytometry.
determined (Fig. 3). These results indicated that HS294T/P16+ cells were completely arrested 24 h after exposure to IPTG and remained so for another 12 h. However, by 48 h, the number of cells in G0/G1 returned to levels characteristic of the normal state of growing cells (50–60%). The number of G0/G1 cells remained relatively constant in the P16− control cell line treated identically. In addition, cells that had been arrested by p16 induction and then released could be arrested again by an additional dose of IPTG (data not shown). These data strongly suggest that p16-induced arrest is reversible in a majority of the cells. Furthermore, the reversal is rapid.

To analyze cell viability as a function of the duration of cell cycle arrest, HS294T/P16+ cells were left in IPTG for several days. The number of cells present before IPTG treatment was compared to the number of cells present at various time points after treatment with IPTG (Fig. 4). Cell viability remained high even after 7 days in the presence of IPTG. To ensure that the cells were capable of reentering the cell cycle and were not trapped in a permanent state of arrest, cells were treated with IPTG for 6 days, after which the IPTG was washed away, and the cells were monitored by flow cytometry. At the time the IPTG was removed, about 90% of the cells were in G0/G1, but after 4 days without IPTG, the percentage of G0/G1 cells had dropped to normal levels (data not shown). These results suggest that artificially induced expression of p16 is not lethal for H294T cells in the short term. Considerable numbers of cells remain viable and able to reenter the cell cycle, even after 7 days of dormancy.

Susceptibility of H294T/P16+ cells to chemotherapeutic agents was tested with three agents: methotrexate, vinblastine, and cisplatin. These compounds represent distinct classes of chemotherapeutic drugs, each with a different mode of action. Methotrexate inhibits DNA synthesis by blocking production of thymidine (10). Vinblastine is an alkaloid that destabilizes microtubules, disrupting the mitotic spindle (11). Cisplatin reacts with DNA to form platinated complexes that are repaired poorly within the cell (12).

HS294T/P16+ cells were pulsed for 12 h with IPTG to induce cell cycle arrest. After 24 h, a period sufficient for nearly complete G0/G1 arrest, the cells were exposed to either methotrexate, vinblastine, or cisplatin. Twelve h later, the drug was removed. The time at which cell viability was assessed varied depending on the chemotherapeutic agent but was always long enough after drug treatment to ensure that

Fig. 4. Viability of cells as a function of time spent in arrest. Graph of the viable cells present at the indicated day as a percentage of the number present at day 1 (Y axis) versus time spent in IPTG (X axis). Cell cultures containing the same number of HS294T/P16+ cells were treated with 0.1 mM IPTG at t = 0. At each time point, the cells from a single culture were harvested and counted on a hemocytometer. Viable cells were identified by exclusion of 0.4% trypan blue. The cells were fed with fresh medium every 4 days. The results represent the mean of three experiments; bars, SD.

Fig. 5. Resistance of arrested cells to chemotherapeutic agents. A, graph of cell viability after treatment with methotrexate (Y axis) versus drug concentration (X axis). The number of viable cells was assessed 72 h after drug addition. B, vinblastine: viable cells (Y axis) versus drug concentration (X axis). The number of viable cells was assessed 48 h after drug addition. C, cisplatin: viable cells (Y axis) versus drug concentration (X axis). The number of viable cells was assessed 60 h after drug addition. p16+, HS294T/P16+ cell line. p16−, HS294T/P16− cell line. The results represent the means of at least three experiments.
the maximum number of cells had died, and long enough after removal of IPTG to ensure that the viable cells had reentered the cell cycle. With each compound (methotrexate, vinblastine, or cisplatin), IPTG-treated HS294T/P16+ cells were resistant compared with P16− cells or HS294T/P16+ cells not exposed to IPTG (Fig. 5). For cisplatin, the difference in LD50 (defined as the drug dose at which one-half the cells are dead at the time of assay as compared to the nondrug-treated controls) between the arrested cells and the growing control cells was least impressive, about 8-fold. Resistance to methotrexate was intermediate, a difference in LD50 of about 80-fold. For vinblastine, the difference was over 100-fold. Thus, in this model system, vinblastine displayed the most dramatic specificity for killing growing cells. This may be due to the primary cytotoxic effect of vinblastine during the G2-M transition. Cells in G0-G1 arrest may be generally resistant to such activity.

The intent of these experiments was to model a therapeutic strategy based on pretreatment with a molecule that causes reversible cell cycle arrest in normal cells, followed by administration of traditional chemotherapy. In this model system, the engineered melanoma cell line enters G0-G1 arrest, withstands increased doses of chemotherapeutic agents, and reenters the cell cycle after the inducer is withdrawn. Several aspects of the strategy modeled here remain to be explored further. Will normal, nontransformed cells respond to p16 induction in a similar manner? It is possible that apoptotic programs may be activated by such treatment, or that terminal differentiation may ensue. Either of these responses could severely limit the utility of the method. How can p16 induction be achieved in vivo? In the model described here, regulation was accomplished through an artificial, IPTG-inducible construct. A specific inducer of p16 in normal cells has not been identified, although p16 expression can be increased roughly 50-fold through an unknown mechanism that may depend indirectly on Rb (13–17).

Among the strengths of the therapeutic approach modeled here is its generality. Cytotoxic effects of three anticancer agents with significantly different modes of action were ameliorated by cell cycle arrest. The most dramatic difference was observed with vinblastine, suggesting that other agents specifically directed against the G2 or M phases might be most effective. In combination with such traditional chemotherapeutic agents, reversible arrest might increase the therapeutic window, the range of drug concentrations that separate therapeutic efficacy from systemic toxicity. A second strength is that the strategy exploits the fundamental genotypic differences between normal and tumor cells, the integrity of growth control pathways. In these experiments, the p16 regulatory pathway was targeted, a pathway that is inactivated in many tumors. Tumor cells that have lost p16 function by deletion or mutation, or the activity of any elements such as Rb which lie downstream of p16 in the genetic pathway, should be incapable of arresting in response to a molecule that induces p16 expression in normal cells (5, 6). Thus, the inducer molecule need not be delivered specifically to normal cells. Other pathways for growth control, including the p53 pathway, may also be targets for this type of strategy.

Acknowledgments

We thank Gordon Peters for the gift of the p16 monoclonal antibody and Jennifer Geary for assistance with tissue culture.

References


3202
Cancer Research

Reversible, p16-mediated Cell Cycle Arrest as Protection from Chemotherapy

Steven Stone, Priya Dayananth and Alexander Kamb


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/56/14/3199

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.