Simultaneous Genetic Chemoprotection of Normal Marrow Cells and Genetic Chemosensitization of Breast Cancer Cells in a Mouse Cancer Gene Therapy Model

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ABSTRACT

Repeated exposures to high doses of chemotherapy are often required to eradicate solid tumors. The success of such high-dose therapy is often limited by the myelosuppressive and toxic effects of these drugs on bone marrow cells and by the intrinsic resistance of the cancer cells to chemotherapy. To test ways of using genetic modification of somatic cells to circumvent both of these problems, we first genetically modified normal bone marrow cells with multidrug resistance-1 (MDR-1) cDNA retroviral vectors to render these cells more resistant to p-glycoprotein-transformed agents. Experiments conducted previously in a mouse model in our laboratory (E. G. Hanania et al., Cancer Gene Ther., 2: 251–261, 1995; E. G. Hanania and A. B. Deisseroth, Cancer Gene Ther., 1: 21–25, 1994), which involve transplantation of mouse marrow cells modified with the human MDR-1 cDNA, showed that the majority of the marrow cells of these animals were resistant to repetitive administration of myelotoxic doses of Taxol, a MDR-1-transported drug. Next, to test the effects of genetically modifying marrow cells to make them resistant to chemotherapy, and genetically modifying tumor cells to make them more sensitive to chemotherapy, a mouse breast cancer cell line was transfected with a plasmid expression vector that contained a wild-type p53 chemosensitization transcription unit. Others have shown that restoration of the p53 gene can lead to decreased proliferation, reduced tumorigenicity, and increased sensitivity to chemotherapy-induced apoptosis. In this animal model, the simultaneous use of both chemoprotection and chemosensitization vectors, which provided protection of the normal cells to the chemotherapy and at the same time sensitized the tumor cells to the toxic effects of the chemotherapy, resulted in levels of in vivo tumor reduction that were not possible when either genetic chemoprotection of marrow cells or chemosensitization of tumor cells was used alone. These data should be of interest to those who are studying ways of using genetic modification to improve the outcome of established chemotherapy treatment programs for solid tumors.

INTRODUCTION

The human MDR4-1 gene codes for an ATP-dependent, transmembrane efflux pump (p-glycoprotein) of M, 170,000 that is capable of extruding many lipophilic metabolites and xenobiotics from the inside to the outside of the cell membrane (1, 2). An increase in the intracellular levels of the MDR-1 mRNA and elevated levels of p-glycoprotein have been associated with increased drug resistance to one or more chemotherapy drugs that are transported by the MDR-1 gene product (3). Studies of in vitro model systems have shown that chemotherapy resistance in many tumors and tumor cell lines was attributable to a decrease in the intracellular accumulation of chemotherapy drugs in these resistant cells (1). Such an increase in resistance constitutes a major problem in planning chemotherapy for patients with advanced stages of cancer. Although higher and repeated doses of these drugs are required to eradicate these resistant tumors, the delivery of such high doses is limited by the myelosuppressive and toxic effects that these drugs generate in the bone marrow. Studies from several groups have demonstrated that retroviral-mediated transfer of the human MDR-1 gene into murine bone marrow cells conferred on these cells the phenotype of drug resistance (4, 5–10).

Acquisition of point mutations of the p53 gene plays an important role in the process of multistep carcinogenesis of solid tumors (11–14) and in the evolution of resistance to chemotherapy. Because loss of p53 function due to these mutational changes is associated with genetic instability and resistance to DNA damaging agents, restoration of wild-type p53 expression in human cancer cell lines also establishes sensitivity of the neoplastic cells to chemotherapy-induced apoptosis (15–19).

In this study, the metallothionein promoter was used to regulate p53 transcription in vectors introduced into tumor cells for chemosensitization. In these genetically modified tumor cells, p53 expression is rapidly and transiently induced by
exposure of cells to low, nontoxic levels of heavy metals such as zinc. These modified tumor cells were then injected into the mammary glands of BALB/c mice. We also introduced the cDNA for the MDR-1 chemotherapy resistance gene into the hematopoietic cells of these same mice. Under these conditions, the administration of several cycles of high doses of Taxol to the mice may increase the resistance of the bone marrow cells at the same time that the p53 transcription unit is sensitizing the breast cancer cells to chemotherapy. The results of this comprehensive genetic approach, which is designed to improve the performance of chemotherapy, is summarized in this report.

MATERIALS AND METHODS

Establishment of the Mouse Mammary Tumor Cell Lines. The 11A1 mouse mammary cancer cell line was established by Adams et al. (20) at the Oak Ridge National Laboratory. BALB/c female mice were irradiated with 100 cGy of γ-rays. Four weeks after irradiation, the mammary glands were removed and used for establishing different cell lines.

The cell line T24, which is one such mammary carcinoma-derived cell line, was established as described by us previously (21–23). T24 is a cell line derived from breast cancer tumors induced by T24 cells in BALB/c mice. All cells are maintained in Hams F12:Dulbecco (1:1) medium supplemented with fetal bovine serum (3–10%), transferrin (5 μg/ml), and epidermal growth factor (0.8 ng/ml).

Constitution of PMVBNEO.P53. A RB suppressor gene expression vector, PMVBNEO.MRB (21), is composed of pML, a derivative of pBR322, which lacks the region of the pBR322 vector from nucleotides 1092–2485, the mouse metallothionein-I promoter sequences, SV40 DNA sequences, the Tn5 neo' gene, and the mouse RB cDNA. The latter (mouse RB cDNA) was removed by EcoRI endonuclease digestion. The resulting open linear form of the remainder of the pMVBNEO.MRB plasmid was ligated at the EcoRI site and linearized by digestion at the BamHI site, generating pMVBNEO.MRB.M. The human p53 cDNA excised from the plasmid pCMVp53 (24) was then inserted into the BamHI site of pMVBNEO.MRB.M, giving rise to pMVBNEO.P53. The correct orientation of the p53 insert was verified by a combination of endonuclease digestion: BamHI, EcoRI, and Smal. A large plasmid preparation was then made of one of the clones bearing the correct restriction map, and the purified plasmid was used for all successive transfections.

Constitution of PMVMDR-1 Vector. A retroviral vector described by us previously (5) contained the Moloney murine leukemia virus LTR driving a MDR-1 cDNA, which had been truncated previously at the 3' end by Dr. Michael Gottesman before he transferred this clone to our laboratory. The vector was packaged in the presence of 8-azaguanine, and the virus was used to transduce adherent 11A1 cells. After 48 hours, the cells that had been transduced were selected with G418, and a high degree of resistance to this drug was observed.

Transfection of p53 DNA into the Tumorigenic Cells. Cells from 11A1 [mouse mammary cancer cell line was established by Adams et al. (20) at the Oak Ridge National Laboratory] and T24.4 and 2T24.4 [cell lines established previously by us (22, 23)] were split 1:15 into 100-mm dishes 1 day before transfection. The next day, fresh medium was added to the cells. Four hours later, DNA (pMVBNEO.P53, 5–10 μg per dish) was added to the dishes. The transfection was carried out as described by us previously (21). The cells originating from 11A1 were referred to as 11A1.P53.X, those derived from T24.4 were referred to as T24P4.P53.X, and those derived from 2T24.4 were referred to as 2T24.4.P53.X, where X is the clone number.

Isolation of Clones Stably Transfected with p53. Two weeks after transfection of mammary tumor cell lines 11A1, T24.4, and 2T24.4 with p53, these cells were seeded in culture medium supplemented with G418 at a final active concentration of 800 μg/ml. Several wells developed colonies. After the expansion of 10 of these colonies in T25 culture flasks, DNA was isolated from the growing cells, and PCR was performed (see Fig. 1) using specific primers for p53 (5'-GGAGCTTCATCTGGACCTGGG and 3'-GTGACACGC'TCTCCTGGTG). One representative clone was picked from each series for subsequent experiments: 11A1.P53.2, T24.4.P53.3, and 2T24.4.P53.4.

Measurement of the Effect of Chemotherapy on Mouse Mammary Cell Lines and Their p53-transfected Clones. Mouse mammary cell lines (11A1, 11A1.P53.2, T24.4, T24.4.P53.3, 2T24.4, and 2T24.4.P53.4) were plated in 6-well plates, 5 × 10^4 cells per well in the presence of reduced serum (3%) and ZnCl₂ (33–38 μM). Chemotherapeutic agents Doxorubicin (50 nM), Etoposide (75 nM; Sigma, St. Louis, MO), or Taxol (20 ng/ml; 23.4 μM; Calbiochem, La Jolla, CA) were added at day 1. Fresh medium supplemented with Zn²⁺ and the chemotherapy agents were added at days 4 and 6. Cell counts were done on days 0, 2, 4, 6, and 8. These experiments were done in duplicate.
Measurement of the Effect of Taxol Administration and 
p53 Induction in a Mouse Animal Model with Bone Marrow
Cells Modified with a MDR-1 Retrovirus. Female BALB/c
mice were treated with 150 mg/kg 5-fluorouracil (Sigma) at 48 h
before bone marrow harvest. 5-Fluorouracil treatment eliminates
the majority of mature hematopoietic cells, stimulating the
proliferation of progenitor cells. Bone marrow cells were har-
vested from the mouse femurs and tibia and transduced with the
MDR-1 virus as described by us previously (4, 5). Upon hemato-
(5 × 10^6 in 50 μl of medium 199 per animal) were injected in
the fatpad region of mammary gland 4. The cell lines were
grouped into “parental” (before p53 modification) and “p53-
modified.” The parental cells were injected on the left flank, and
the corresponding p53-modified clone was injected on the right
flank. Three days after injection, a single dose of Taxol [15
mg/kg (17.6 nmol/kg)] was administered as a single bolus. The
Taxol was dissolved in DMSO at a concentration of 100 mg/ml
(0.12 μl), and diluted with DMSO, ethyl alcohol, and water at a
ratio of 3:3:4 before injection. At the same time, zinc treatment
was initiated (300 μl of ZnCl₂, 100 μM i.p. once every day for
2 weeks). The mice were then observed for the rate of tumor
growth at both flanks. After 2 weeks, all the mice were
sacrificed, and their tumors were excised and weighed.

Measurement of the Long-Term Effect of High-Dose
Taxol and p53 on Tumor Growth Rate. Control BALB/c
mice (without any modification) or mice that had been trans-
planted with bone marrow cells modified with MDR-1 retroviral
vector were injected with 2T24.4.P53.4 and 2T24.4 cells as
described above. Taxol in doses 0, 2.5, 5.0, 10.0, 15.0, 20.0, 25.0,
and 30.0 mg/kg (0.293, 5.85, 11.71, 17.57, 23.42, 29.27, and
35.13 mmol/kg) was administered i.p. 3 days later. The animals
were sacrificed 14 days later, and their tumors were excised and
weighed.

RESULTS

Effect of p53 on the Growth Rate of Transfected
Clones. We first transfected p53 into mammary carcinoma
clones. On the basis of direct PCR analysis of the transfected
cell lines (see Fig. 1), we chose the following cell lines for
further study: 11A1P53.2 (Fig. 1A, Lane 4), T24.4.P53.3 (Fig.
1A, Lane 9), and 2T24.4.P53.4 (Fig. 1B, Lane 8). Because the
wild-type p53 that was transfected into the mammary cell lines
is driven by the mouse metallothionein promoter, adding ZnCl₂
will induce its expression as shown by us previously (21, 25).
To determine the optimal ZnCl₂ concentration that will cause
the induction of transcription of p53 without any cytotoxic
effects on the cells, both the parental breast cancer cell lines
and their p53-derived clones were grown in different concentra-
tions of ZnCl₂ ranging from 10–100 μM. It was found that 33 μM is
ideal for the 11A1 series of the mammary cancer cell lines
established by Adams et al. (20), and 38 μM was ideal for the
T24.4 and 2T24.4.2 series of mammary cancer cell lines derived
by us previously (22, 23).

To see whether there is any effect of the inserted wild-type
p53 on the growth rate of each of these cell lines, 5 × 10^6 cells
of each were plated in 6-well plates. ZnCl₂ was added to the
medium for both the parental cell lines and the p53-transfected
clones. As seen in Fig. 2, p53 modification results in a reduction
in the growth rate of the p53-derived clones.

In Vitro Effect of Chemotherapy on Mouse Mammary
Cell Lines before and after p53 Modification. To compare
the effect of chemotherapy on the mouse mammary cell lines
before and after the introduction of a p53 transcription unit, we
cultured the cell lines (before and after transfection) in the
presence of Doxorubicin and Etoposide. This combination
chemotherapy (Doxorubicin and Etoposide) seems to have a
greater inhibitory effect on the growth of 2T24.4.P53.4 as shown
in Fig. 3A. Taxol [20 ng/ml (23.4 μM)] also inhibits the
growth of the mammary cell line 2T24.4 and has a greater
inhibitory effect after introduction of the p53 clone as shown in
Fig. 3B. With both treatments received, the p53 clones exhibited

![Graphs showing the growth of mouse mammary cell lines with and without p53 sequences.](clincancerres.aacrjournals.org)
The experiments were done in duplicate. Cells from the unmodified breast cancer cell line 2T24.4 were plated in 6-well plates, 5 x 10^4 cells per well, in the presence of reduced serum (3%) and ZnCl_2 (33-38 μM). A. Doxorubicin (50 nm) and Etoposide (75 nm) were added on day 1. Fresh medium supplemented with Zn^{2+}. Doxorubicin, and Etoposide, was added on days 4 and 6. Cell counts were done on days 0, 2, 4, 6, and 8. The experiments were done in duplicate. B. 2T24.4.p53.4 cells after p53 transfection. C. parental cell line 2T24.4 before p53 transfection. D. Taxol (20 ng/ml) was added on day 1. Fresh medium supplemented with Zn^{2+}. Taxol was added on days 4 and 6. Cell counts were done on days 0, 2, 4, 6, and 8. The experiment was done in duplicate. E. 2T24.4.p53.4 cells after p53 transfection. F. parental cell line 2T24.4 before p53 transfection.

Effect of p53 on Chemotherapy-induced Tumor Suppression in a Mouse Mammary Tumor in Vivo Model. To first determine whether the expression of p53 has an inhibitory effect on the growth of breast cancer cell lines in mice, female BALB/c mice were first injected with mouse mammary tumor cells. Cells from the unmodified breast cancer cell line 2T24.4 were injected into the fatpad region of mammary gland 4 on the right flank, whereas the fatpad region of the left flank in the same mouse was injected with the breast cancer cells transfected with the metallothionein p53 transcription units 2T24.4.p53.4 (Fig. 4). Some of the animals received daily i.p. ZnCl_2 injections (100 μM, 300 μl) starting on day 3 after the inoculation of the tumor cells to induce the expression of the exogenous wild-type p53, and others did not. Similarly, some of the animals received one low dose of Taxol [10 ng/kg (11.71 nmol/kg)]. Two weeks later, the animals were sacrificed, and the tumors were excised and weighed. The results of this experiment are summarized in Table 1. As can be seen in this table, there is a significant reduction in the growth rate of the parental cell line 2T24.4 before p53 transfection.

Table I: Effect of p53 on Chemotherapy-induced Tumor Suppression in a Mouse Mammary Tumor in Vivo Model

<table>
<thead>
<tr>
<th>Taxol Dose (mg/kg)</th>
<th>2T24.4</th>
<th>2T24.4.p53.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>450,000</td>
<td>450,000</td>
</tr>
<tr>
<td>2</td>
<td>450,000</td>
<td>450,000</td>
</tr>
<tr>
<td>5</td>
<td>450,000</td>
<td>450,000</td>
</tr>
<tr>
<td>10</td>
<td>450,000</td>
<td>450,000</td>
</tr>
<tr>
<td>20</td>
<td>450,000</td>
<td>450,000</td>
</tr>
<tr>
<td>30</td>
<td>450,000</td>
<td>450,000</td>
</tr>
</tbody>
</table>

As shown in Table 1, there was no difference in the effect of a moderate dose of Taxol [10 mg/kg (11.71 nmol/kg)] in the p53-transfected clones compared with the parental cell lines in either the presence or absence of Zn^{2+}.

We then compared the effect of increased doses of Taxol [14-30 mg/kg (17.53-35.14 nmol/kg)] on tumor growth that have been transplanted or not with wild-type p53. The mice used for these experiments have been transplanted with bone marrow cells that were modified with the MDR-1 retroviral vector, because normal mice have been shown not to tolerate Taxol doses higher than 15 mg/kg [17.57 nmol/kg; equivalent to 135 mg/m^2 (0.16 μmol/m^2) in man]. The animals were followed for 3 weeks after Taxol injection. The period of 3 weeks ensured that the tumor burden would not be very high and thereby kill the animals before an experimental result could be measured. In these long-term experiments, we used only one-half of the number of cells (2.5 x 10^6) that were used for the short-term experiments (5.0 x 10^6). The animals were then injected twice a day with ZnCl_2 to induce the expression of p53. As shown in
p53 gene proved very effective in controlling the tumorigenic cells become tumorigenic (23). This observation suggests that growth rate in culture and tumorigenicity are distinct and separate from that identified in these culture media. This might have been due to the metabolism of the zinc by endogenous metallothioneins. It is noteworthy that the zinc-unstimulated level of p53 expression was substantial, thus making the detection of additional increments of expression difficult. The metallothionein promoter is a “leaky promoter,” and the amount of zinc normally present in the regular tissue culture medium induces the promoter.

The effect of the p53 on tumor growth in vivo is greater than that identified in vitro. Some investigators argue that growth rate in culture and tumorigenicity are distinct and separate phenomena. In both conditions, expression of wild-type p53 gene proved very effective in controlling the tumorigenic characteristics of mouse mammary cancer cells, but the effect was not sufficient to completely abolish this growth. Cytogenetic analysis of different passages of these cells showed detectable chromosomal abnormalities (22). We have shown previously that the level of the RB protein is reduced when these cells become tumorigenic (23). This observation suggests that many genes, in addition to RB and p53, may have been involved in the multistep process that led to the mammary cancer in these mice. Therefore, introducing a wild-type RB or p53 transcription unit into these cells as the only genetic change may not be sufficient to overcome all the defects present in these cells.

In our experiments, the effect of p53 in regulating the proliferation of these cancerous cells is detectable, although it may not be the dominant one. If corrective genetic strategies are to be successful in the real gene therapy setting, we must identify the single mutation that, when overcome, will result in a reversal of the neoplastic phenotype for each organ site of cancer if such modification strategies are to have a clinical impact. Another problem that must be addressed is the low frequency of modification of somatic cells by any vector system. The stability of the expression of the transcription unit must also be addressed. Finally, future vectors that can be targeted to the neoplastic cells so that the normal cells are not damaged by the chemotherapy should also be developed.

Our results show that the tumor was partially responsive to Taxol and that the introduction of the wild-type p53 further sensitized the cells to the chemotherapy. As mentioned above, the tumor was not completely eradicated by the chemotherapy even in the presence of the p53 transcription unit. Usually, doses above 15 mg/kg cannot repetitively be administered to mice without life-threatening cytotoxic side effects. MDR-1 genetic modification in the present experiments may have made possible the safe administration of high doses of Taxol. As shown previously by us, MDR-1 genetic modification highly reduced the severity of the effect of Taxol on the hematopoietic tissue (4, 5), and thereby makes safe the administration of high doses of Taxol. When the tumor was developed in the presence of the wild-type p53 transcription unit, the tumors were resistant to Taxol chemotherapy. Such double genetic modification strategies may one day be of potential use in the clinic for the treatment of patients with advanced stages of epithelial neoplasm who require very high doses of chemotherapy Taxol and whose tumors are resistant to chemotherapy due to the loss of p53 function.

### Table 1 Short-term effect of p53 on tumor growth

Three days postinjection of both 2T24.4 parental cells and 2T24.4.p53.4 transfected cells into the mice (5 × 10⁶ cells in 50 μl of medium 199 per animal), the animals were divided into four groups: (a) no treatment; (b) Taxol treatment in which a single dose of Taxol (Calbiochem, 15 mg/kg), was administered as a single bolus; (c) zinc treatment in which 300 μl of ZnCl₂, 100 μl, i.p. once each day for 2 weeks; and (d) both Taxol and zinc treatments as stated in b and c. The mice were then observed for the rate of tumor growth in both flanks. After 2 weeks, all the mice were sacrificed, and their tumors were excised and weighed. A comparison is made between the tumors on both sides of each animal.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Weight of tumor induced by 2T24.4</th>
<th>Weight of tumor induced by 2T24.4-p53.4</th>
<th>Percentage of reduction in tumor weight with/without p53</th>
<th>Average reduction in tumor weight with/without p53</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.48</td>
<td>0.46</td>
<td>4.2</td>
<td>37.3 ± 16.02</td>
</tr>
<tr>
<td>None</td>
<td>1.92</td>
<td>0.61</td>
<td>68.2</td>
<td>34</td>
</tr>
<tr>
<td>None</td>
<td>2.59</td>
<td>1.57</td>
<td>39.4</td>
<td>37.3 ± 6.15</td>
</tr>
<tr>
<td>Taxol only</td>
<td>1.16</td>
<td>1.11</td>
<td>43</td>
<td>53.05 ± 26.94</td>
</tr>
<tr>
<td>ZnCl₂ only</td>
<td>0.97</td>
<td>0.64</td>
<td>34</td>
<td>46.3</td>
</tr>
<tr>
<td>ZnCl₂ only</td>
<td>2.01</td>
<td>0.56</td>
<td>72.1</td>
<td>34</td>
</tr>
<tr>
<td>Taxol and ZnCl₂</td>
<td>0.67</td>
<td>0.36</td>
<td>46.3</td>
<td>34</td>
</tr>
<tr>
<td>Taxol and ZnCl₂</td>
<td>1.09</td>
<td>1.06</td>
<td>28.8</td>
<td>24.55 ± 30.75</td>
</tr>
</tbody>
</table>

**DISCUSSION**

We introduced a wild-type functional p53 gene into a breast cancer cell line in which the endogenous p53 had a loss of function, and we studied the effect of the modification on the sensitivity of these cells to chemotherapy agents. Loss of function of p53 is found in many tumors (15, 26–30). Continuous overexpression of p53 may be cytotoxic to the cells, leading to either G₁ cell arrest or apoptosis (30). To overcome this, we have attempted to control expression of the transfected p53 DNA by using the metallothionein promoter (31, 32). Addition in some cases of Zn²⁺ (which induces the metallothionein promoter, and therefore p53) to the culture of these cells or to the animals in which these cells were growing led to p53 expression (33).

In our experiments, we found that the presence of the p53 had an effect on the growth rate of the tumor cells both in vitro and in vivo. It is interesting that the p53-modified cells retained their original growth rate for a few days after addition of zinc to these culture media. This might have been due to the metabolism of the zinc by endogenous metallothioneins. It is noteworthy mentioning that the zinc-unstimulated level of p53 expression was substantial, thus making the detection of additional increments of expression difficult. The metallothionein promoter is a “leaky promoter,” and the amount of zinc normally present in the regular tissue culture medium induces the promoter.

The effect of the p53 on tumor growth in vivo is greater than that identified in vitro. Some investigators argue that growth rate in culture and tumorigenicity are distinct and separate phenomena. In both conditions, expression of wild-type p53 gene proved very effective in controlling the tumorigenic characteristics of mouse mammary cancer cells, but the effect was not sufficient to completely abolish this growth. Cytogenetic analysis of different passages of these cells showed detectable chromosomal abnormalities (22). We have shown previously that the level of the RB protein is reduced when these cells become tumorigenic (23). This observation suggests that
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