E1A Specifically Enhances Sensitivity to Topoisomerase IIα Targeting Anticancer Drug by Up-Regulating the Promoter Activity

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Abstract
DNA topoisomerases I and II (topo I and II) are nuclear enzymes involved in cellular replication and are targets for several anticancer drugs. We showed previously that E1A gene transfer enhanced the sensitivity of Ewing’s sarcoma cells to the topo IIα targeting agents etoposide and Adriamycin in vitro and in vivo. To determine whether this effect was specific for topo IIα, we investigated the effect of E1A gene transfer on cell sensitivity to agents that target topo I and IIβ. Transfecting TC71 human Ewing’s sarcoma cells with an adenoviral vector containing the E1A gene enhanced their sensitivity to the topo IIα targeting agents etoposide (16-fold) and Adriamycin (8-fold). By contrast, E1A gene transfer did not affect cellular sensitivity to either amsacrine or camptothecin. Western blot analysis indicated that topo IIα protein levels increased 3.1-fold after E1A gene transfer, but topo I and IIβ protein levels did not change. A plasmid containing topo IIα gene promoter with luciferase reporter gene was constructed to determine the effects of E1A gene transfer on the activity of the topo IIα promoter. E1A increased the activity of the topo IIα gene promoter by 3.5-fold relative to that of cells transfected with Ad-β-gal. These results suggest that elevated topo IIα protein levels and enhanced sensitivity to topo IIα-targeting agents were secondary to a direct effect of E1A on the topo IIα promoter. Combining E1A gene therapy with topo IIα-targeting anticancer drugs may therefore have therapeutic benefit by increasing tumor cell sensitivity.


Introduction
Topoisomerases are nuclear enzymes that control DNA topology, cell differentiation, and cell replication. Many anticancer drugs stabilize the covalent complex formed between DNA and the topoisomerase enzymes, thereby initiating a biochemical cascade leading to cell death (1, 2). Resistance to chemotherapy has been shown to involve several different mechanisms, including alteration of topoisomerases (3-5). Topoisomerases have several different isoforms [i.e., topoisomerase I, IIα, and IIβ (topo I, IIα, and IIβ)]. Camptothecin and topotecan target topo I, etoposide (VP-16) and Adriamycin target topo IIα, and amsacrine targets topo IIβ (6, 7). We showed previously that E1A gene transfer into human Ewing’s sarcoma cells increased cellular sensitivity to VP-16 and Adriamycin in vitro and in vivo (8, 9). This increased sensitivity was shown to be secondary to an increase in topo IIα expression. To ascertain whether this effect on topo IIα was specific and to further determine the mechanism responsible for enhanced topo IIα expression, we investigated the effect of the E1A gene on cell sensitivity to topo I and IIβ-targeting agents and on the activity of the topo IIα promoter. We found that E1A gene transfer into TC71 human Ewing’s sarcoma cells enhanced their sensitivity to topo IIα-targeting drugs but not to agents that target topo I or IIβ. Topo IIα protein expression and the activity of the topo IIα promoter were also increased by E1A gene transfer. These results suggest that in addition to acting as a transcriptional repressor of HER-2/neu the E1A gene may act as a transcriptional promoter of topo IIα.

Results
Effect of E1A on the Sensitivity of TC71 Cells to Agents That Target Topo Iα, Iβ, or I
Our first step was to confirm that E1A sensitized TC71 cells to the topo IIα-targeting agents VP-16 and Adriamycin. Transfection with the E1A gene increased cellular sensitivity to VP-16 by 16-fold (IC50, 90 nmol/L versus 1.5 μmol/L), whereas transfection with Ad-β-gal did not alter cell sensitivity (IC50, 1.3 μmol/L; Fig. 1A). E1A gene transfer also enhanced the sensitivity of TC71 cells to Adriamycin (IC50, 12 versus 100 nmol/L; Fig. 1B). By contrast, E1A transfer did not affect cellular sensitivity to amsacrine (a topo IIβ-targeting agent) or camptothecin (a topo I targeting agent; Fig. 1C and D). Results for all drugs are summarized in Table 1. The IC50 for amsacrine was 2.1 versus 2.0 μmol/L with E1A and 3.0 versus 2.5 μmol/L with E1A for camptothecin.

Effect of E1A on Topo Iα, Iβ, and I Protein Expression
Western blot analyses indicated that topo IIα protein levels increased by 3.1-fold after transfection with E1A (Fig. 2, top). In contrast, transfection with Ad-β-gal did not affect topo IIα protein expression. Transfection with E1A did not affect topo IIβ (Fig. 2, middle) or topo I (Fig. 2, bottom) protein levels compared with cells transfected with Ad-β-gal (Fig. 2),
These results suggest that the E1A gene specifically increased cellular topo IIα protein levels and that this effect led to their sensitivity to the topo IIα targeting drugs VP-16 and Adriamycin.

**Effect of E1A on Topo IIα Promoter Activity**

After finding that E1A gene transfer up-regulated topo IIα expression and protein production, we next investigated the effects of the E1A gene on the topo IIα promoter by using pGL-topo IIα-557, a vector containing the topo IIα gene promoter with a luciferase reporter gene. Transfection with adenoviral vector containing the E1A gene (Ad-E1A) increased the luciferase activity by 3.5-fold compared with Ad-β-gal. Following treatment with Cdk2/cyclin inhibitory peptide II, which inactivated E2F-1 transcript factor function (10), Ad-E1A did not significantly alter the topo IIα promoter activity (Fig. 3, top). We verified the specificity of this activity by testing the effect of E1A on the Fas promoter in cells transfected with FPR-Fas-Luc. Treatment of those cells with Ad-E1A did not change Fas promoter activity. Transfer of the IL-12 gene using Ad-IL-12 served as our positive control (11) and resulted in increased Fas promoter activity (Fig. 3, bottom).

**Discussion**

In the present study, we showed that E1A gene transfer specifically enhanced the sensitivity of human Ewing’s
sarcoma cells to the topo IIα targeting drugs VP-16 and Adriamycin. E1A up-regulated both topo IIα expression and protein production but did not affect topo IIβ or I protein levels and did not alter cell sensitivity to either amsacrine (targets topo IIβ) or camptothecin (targets topo I). We further showed that E1A specifically enhanced the activity of the topo IIα promoter, indicating that E1A up-regulates topo IIα expression through a mechanism involving the promoter. The exact pathway by which E1A stimulates topo IIα promoter activity is unclear, but recent findings suggest that E1A gene expression is associated with the expression of E2F-1 (12), a transcription factor that regulates topo IIα (13). Up-regulation of topo IIα promoter activity by E1A is via the E2F-1 pathway. Following blockage of E2F-1 activity by Cdk2 inhibitor, E1A did not alter topo IIα promoter activity (Fig. 3). The Rb-binding domain of E1A is required for enhancement of sensitivity of cells to topo IIα targeting drugs (14). Sensitivity to VP-16 was not enhanced in cells with mutated E1A in the Rb-binding region or following inactivation of E2F-1 function by Cdk2 inhibitor (data not shown). These results imply that there may be a link between E1A and the up-regulation of the topo IIα promoter that involves E2F-1.

E1A is a multifunctional oncogene that has been shown by us and others to down-regulate HER-2/neu (8, 9, 15). We showed previously that the intratumoral injection of E1A in nude mice down-regulated HER-2/neu in Ewing’s sarcoma tumors in vivo and enhanced tumor sensitivity to VP-16 (9). Findings from our current study suggest that this enhanced sensitivity to VP-16 may be independent of HER-2/neu. Indeed, treatment of TC71 cells in vitro or in vivo with Herceptin (trastuzumab) led to decreased HER-2/neu expression but had no effect on either topo IIα expression or sensitivity to VP-16 (16). Therefore, HER-2/neu down-regulation does not seem to be directly linked to enhanced topo IIα expression. Whereas E1A affects both, Herceptin only affects HER-2/neu.

Cellular resistance to VP-16 has been linked to decreased cellular topo IIα protein levels (17). Restoration of topo IIα protein levels by transfection with an adenovector containing the topo IIα gene led to enhanced cellular sensitivity to VP-16 (18). E1A gene therapy is currently in clinical trials for patients with breast or ovarian cancer in which the tumors overexpress HER-2/neu (19). Our findings suggest that E1A gene therapy may prove beneficial for tumors that do not overexpress HER-2/neu by virtue of its ability to also up-regulate the topo IIα promoter, leading to increased topo IIα expression and increased topo IIα protein production, thereby increasing the drug target. Presumably, these effects will in turn enhance tumor sensitivity to topo IIα targeting agents, such as Adriamycin and VP-16.

Materials and Methods

Cell Line

TC71 human Ewing’s sarcoma cells (kindly provided by Dr. T. Triche, University of Southern California, Los Angeles, CA) were cultured in Eagle’s modified essential medium with 10% fetal bovine serum, 2 mmol/L l-glutamine, 1 mmol/L sodium pyruvate, 1× nonessential amino acid, and 2× MEM vitamin solution (Life Technologies, Inc., Grand Island, NY). This cell line was screened with a Mycoplasma Plus PCR Primer Set (Stratagene, La Jolla, CA) and found to be free of Mycoplasma.

Recombinant Adenovirus

Ad-E1A (kindly provided by Dr. M-C. Hung, Department of Molecular and Cellular Biology, The University of Texas M.D. Anderson Cancer Center, Houston, TX) is an adenovirus type 5–based vector that contains E1A but lacks E1B and E3 (15). The control adenovirus, Ad-β-gal, is an adenovirus type 5 that lacks E1A, E1B, and E3 but contains β-galactosidase. Both of these recombinant replication-deficient adenoviral vectors were propagated in human embryonic kidney 293 cells as described previously (20). The viruses were purified twice by cesium chloride gradient ultracentrifugation and then dialyzed and

Table 1. Effects of E1A on Cell Sensitivity to VP-16, Adriamycin, Amsacrine, and Camptothecin

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC50 for drug alone</th>
<th>IC50 for drug with Ad-E1A</th>
<th>Sensitivity enhancement</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP-16</td>
<td>1.5 μmol/L</td>
<td>90 nmol/L</td>
<td>16-fold</td>
</tr>
<tr>
<td>Adriamycin</td>
<td>100 nmol/L</td>
<td>12 nmol/L</td>
<td>8-fold</td>
</tr>
<tr>
<td>Amsacrine</td>
<td>2.1 μmol/L</td>
<td>2.9 μmol/L</td>
<td>1.1-fold</td>
</tr>
<tr>
<td>Camptothecin</td>
<td>3.0 μmol/L</td>
<td>2.5 μmol/L</td>
<td>1.2-fold</td>
</tr>
</tbody>
</table>

NOTE: TC71 cells were incubated with various concentrations of drugs. Cell cytostasis was quantified using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described in Materials and Methods. Drug sensitivity enhancement was calculated by dividing the IC50 for drug alone by the IC50 for drug with Ad-E1A.

FIGURE 2. E1A increased topo IIα protein expression but not that of topo IIβ or I. TC71 cells were treated with PBS (lane 1), Ad-E1A (lane 2), or Ad-β-gal (lane 3) for 48 hours. Cellular protein was extracted and subjected to electrophoresis on SDS-PAGE gel. Expression of topo IIα (top), topo IIβ (middle), and topo I (bottom) proteins was detected by Western blot using specific antibodies. Densitometric analysis of each band was normalized with a β-actin loading control. Relative expression levels were calculated in comparison with the protein levels in the TC71 control cells.
Titration of cells with various concentrations of VP-16, Adriamycin, camptothecin, or amsacrine was done at least thrice to determine the mean and SD.

Cytostasis Assay

TC71 cells were seeded onto 96-well cell culture plates (5,000 per well) and allowed to adhere overnight before being infected with Ad-\(E1A\) or Ad-\(\beta\)-gal. Cells were then transfected with PBS, Ad-\(E1A\), Ad-\(\beta\)-gal, or Ad-\(IL-12\) as indicated. Cells were then harvested, lysed, and assayed for luciferase activity. Relative luciferase activity was calculated in comparison with that of control cells. Columns, mean of three independent experiments; bars, SD.

Western Blot Analysis

TC71 cells were seeded on 100 mm dishes 24 hours (2 \(\times\) 10^5 cells per dish) before treatment. The cells were then re-fed and treated with Ad-\(E1A\) or Ad-\(\beta\)-gal at 10 plaque-forming units/cell for 48 hours. Cells were washed with cold PBS and lysed with buffer consisting of 50 mmol/L Tris-HCl (pH 8.0), 425 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mmol/L \(\beta\)-mercaptoethanol, and the protease inhibitors aprotinin (2 \(\mu\)g/mL), leupeptin (2 \(\mu\)g/mL), pepstatin A (1 \(\mu\)g/mL), and phenylmethylsulfonyl fluoride (100 \(\mu\)g/mL). Solubilized protein (50 \(\mu\)g) was run on a 7.5% SDS-PAGE gel and transferred to a Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ). Antibodies to human topo II\(\alpha\), topo I (TopoGEN, Inc., Columbus, OH), topo II\(\beta\)s (BD Bioscience Pharmingen, Inc., San Diego, CA), and \(\beta\)-actin were used for protein detection with the enhanced chemiluminescence analysis system.

Topo II\(\alpha\) Promoter Vector Construction

The topo II\(\alpha\) promoter vector pGL-topo II\(\alpha\)-557 (kindly provided by Dr. D.P. Suttle, University of Tennessee Health Science Center, Memphis, TN) was created by subcloning the PCR product containing the topo II\(\alpha\) promoter into a pGL2 basic vector linking upstream of the luciferase gene. Plasmid DNA was purified with the Qiagen plasmid purification kit (Valencia, CA) and confirmed by restriction enzyme analysis (22). The Fas promoter FPR-Fas-Luc vector (23) used to verify the specificity of the effect on the topo II\(\alpha\) promoter was kindly provided by Dr. L.B. Owen (University of California, Riverside, CA).

Luciferase Assay

TC71 cells were plated in six-well plates at 2 \(\times\) 10^5 cells per well. After 24 hours, fresh medium was replaced. Cells were treated with or without 10 \(\mu\)mol/L Cdk2/cyclin inhibitory peptide II (Calbiochem, La Jolla, CA) for 4 hours; then, cells were transfected with 1 \(\mu\)g of either the pGL-topo II\(\alpha\)-557 plasmid or the FPR-Fas-Luc vector together with 1 \(\mu\)g Renilla luciferase vector pRL-TK by using FuGene 6 transfection reagents (Roche Diagnostics Corp., Indianapolis, IN). Six hours later, the cells were treated with Ad-\(E1A\), Ad-\(\beta\)-gal, or Ad-\(IL-12\), which was used as a positive control for the Fas promoter (11) at 10 plaque-forming units/cell for 48 hours. Cells were harvested, lysed in 1 \(\times\) passive lysis buffer (Promega, Madison, WI), and gently shaken on a platform shaker for 15 minutes at room temperature. The cell lysates (20 \(\mu\)L) were then transferred into microcentrifuge tubes. Luciferase activity was quantified with a luminometer in a Dual-Luciferase Reporter Assay System (Promega). The relative luciferase activity for each sample was adjusted with an internal control for Renilla luciferase activity. Each measurement was done in triplicate. The mean and SD for each sample were calculated from three different experiments.

Acknowledgments

We thank Dr. D.P. Suttle for providing the topo II\(\alpha\) promoter vector and M-C. Hung for the Ad-\(E1A\) vector.

References


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