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ORIGINAL ARTICLE

Adenovirus-mediated human topoisomerase IIα gene transfer increases the sensitivity of etoposide-resistant human and mouse breast cancer cells

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Abstract

Cellular resistance to chemotherapeutic agents is attributable to several mechanisms, including alteration of topoisomerase IIα gene expression. Our previous studies have shown that transient transfection with a vector containing either *Drosophila* or human topoisomerase IIα gene into drug-resistant tumor cells enhanced their drug sensitivity. Furthermore, we constructed a recombinant adenovirus, Ad-hTopoIIα, containing the human topoisomerase IIα gene that was able to selectively increase etoposide sensitivity in drug-resistant tumor cells. We also examined Ad-hTopoIIα for therapeutic efficacy in vitro using additional etoposide-resistant cell lines, including a mouse breast cancer cell line and a human leukemia cell line. The etoposide-resistant mouse breast cancer cell line FvP, which is derived from FM3A, and etoposide-resistant human breast cancer cell line, MDA-VP, which derived from MDA-P cells showed increased sensitivity to etoposide as well as increased expression of human Topoisomerase IIα mRNA, but this was not seen in FM3A and MDA-P cells. On the other hand, the etoposide-resistant human leukemia cell line K562/MX2 and the parental cell line K562/P did not show enhanced sensitivity against etoposide or an increase in human Topoisomerase IIα mRNA. Using a recombinant adenovirus containing β-galactosidase gene (Ad-β-gal), K562 cells were not transducted by the recombinant adenovirus, while both etoposide-sensitive FM3A cells and etoposide resistant FvP cells were transducted by recombinant adenovirus. Ad-hTOP2α and etopside treatment showed reduced inoculated tumor weight in the mice. We concluded that a recombinant adenovirus containing the human Topoisomerase IIα gene might be a powerful tool for overcoming drug resistance in breast cancer cells, but not in leukemia cells.

Introduction

Drug resistance continues to be a serious problem in cancer therapy and cellular resistance has been attributed to several different mechanisms, including alteration of topoisomerase IIα [1]. The topoisomerase IIα nuclear enzyme is essential for the survival, such as DNA replication, chromosome segregation, of eukaryotic cells [2–5]. Topoisomerase II is also a target for important antineoplastic drugs, including etoposide and adriamycin. Drugs that target topoisomerase II act as poisons by stabilizing the enzyme-DNA cleavable complex and the stabilization initiates a biochemical cascade, leading to cell death. Resistance to topoisomerase II-targeting drugs involves quantitative and/or qualitative changes in topoisomerase II gene expression. Low cellular levels of topoisomerase II lead to reduced formation of drug-stabilized topoisomerase II-DNA cleavable complexes, which quantitatively correlate with cell death [6]. Therefore, elevation of the topoisomerase IIα level could theoretically result in increased drug sensitivity. Our previous studies have shown that transient transfection with a vector containing either the *Drosophila* or human topoisomerase IIα gene into drug-resistant tumor cells enhanced their drug sensitivity [7–9]. Although this vector was suitable for *in vitro* testing, highly efficient gene transfer is mandatory for *in vivo* testing. Adenovirus vectors have a number of advantages for gene delivery, including high titer production and high transfection efficiency. Adenoviruses can infect and direct high levels of protein expression in both proliferating and

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quiescent cells, an important feature for use *in vivo* [10]. Recombinant adenoviral vectors containing various genes are already being used in clinical trials [11]. We have constructed a recombinant adenovirus containing the human topoisomerase IIα gene (Ad-hTopoIIα) and transduction of this adenovirus into MDA-VP cells significantly enhanced their sensitivity to etoposide [12]. These results prompted us to investigate the effectiveness of Ad-hTopoIIα against other cancer cell lines as well as in vivo setting.

Materials and methods

Cell lines

Human embryonic kidney cells transformed with adenovirus type 5 (293 cells) were obtained from American Type Culture Collection (Manassas, MA). The etoposide (VP-16)-resistant human breast cancer cell line MDA-VP was initially derived and cloned from the MDA-MB-231 parent cell line by Dr. T. Fojo (National Cancer Institute, Bethesda, MD, Ref 13). K562/P, a parental cell line of human myelogeneous leukemia, was obtained from American Type Culture Collection (Manassas VA., USA). The MX2-resistant K562 cell line, which were generous gifts of Dr. Hirofumi Fujii (Department of Medicine, National Cancer Center Hospital East), was selected by stepwise and continuous exposure to MX2 [14,15] using the limiting dilution method and characterized by Dr. K. Nakamura, K. Hasegawa and T. Isoe (Pharmaceutical Research Laboratory, Kirin Brewery Co., Ltd. Gunma, Japan). Prior to each experiment, K562/MX2 was cultured without MX2 for two weeks. The mouse breast cancer cell line FM3A and their etoposide-resistant sub line, FvP cells [16], were obtained from RIKEN GINE BANK (Tsukuba, Japan). All cell lines were confirmed to be free from *mycoplasma* organisms using the MycoFluor^{TM} Mycoplasma detection kit (Molecular Probes, Eugene, OR, USA). All experiments were approved by the institutional ethics board.

Reagents and drugs

Adriamycin, etoposide, which was solubilized in dimethyl sulfoxide (DMSO), vincristine, indomethacin, and DMSO were purchased from Wako Pure Chemicals Co., Ltd. (Osaka, Japan). DMEM, RPMI 1640, Hanks' Balanced Salt Solution without Ca^{2+} or Mg^{2+} (HBSS), fetal calf serum (FCS), and gentamicin were purchased from Life Technologies, Inc. (Gaithersbug, MD, USA).

Construction of recombinant adenovirus vector containing human topoisomerase IIα gene

Construction of the recombinant adenovirus vector containing the human topoisomerase IIα gene was described previously [12,17]. After viral DNA purification, the presence of the human topoisomerase IIα gene was confirmed by PCR [18]. PCR was performed using the primers 5′-GTGTGG-AACTAGAAGGC-3′ and 5′-GGAGGTGGAA-GACTGAC-3′ (for the topoisomerase IIα gene) and 5′-TCGTTTCTCAGCAGCTGTTG-3′ and 5′-CATCTGAACTCAAAGCGTGG-3′ (for the adenovirus E2B fragment). PCR products were resolved in 1% agarose gel, stained with ethidium bromide, and visualized under UV light.

Northern Blot Analysis

Total RNA (20 μg) was extracted, electrophoresed, transferred, and hybridized with a human topoisomerase IIα gene probe (generous gift of Dr. L. Liu, Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey, NJ) and β-actin probe. Band intensity (densitometry) was assessed using NIH image software (http://rsb.info.nih.gov/nih-image/).

Colony formation assay

Colony formation assay was performed as described previously [7–9]. Briefly, 1000 cells (MDA-P and MDA-VP cells) were plated in 35 mm × 6 well plates under various conditions (Costar Corp., Cambridge, MA). After treatment with 100 plaque-forming units (pfu) of Ad-hTOP2α for 72 hours, cells were treated with etoposide for two hours. After drug treatment, cells were washed twice with phosphate-buffered saline and re-cultured with DMEM containing 10% fetal calf serum and gentamicine. Colonies were allowed to form for 12 days; and were then stained with 0.04% crystal violet in methanol and counted. Results were expressed as the survival fraction relative to the colony-forming efficiency of the etoposide untreated control.

Cytotoxicity assay

FM3A, FvP, K562/P and K562/MX2 cells were cultured in suspension. Therefore, we measured cytotoxicity using the previously described trypan blue dye exclusion assay [19]. Briefly, 1×10^6 cells were incubated with 100 pfu of Ad-hTOP2α or Ad-β-gal for 72 hours. After viral treatment, cultures were incubated with various concentrations of anticancer drugs, including etoposide, adriamycin, or

vincristne for 72 hours and viable cells were counted using trypan blue staining. Results were expressed as survival fraction relative to that of the untreated control.

β-galactosidase assay

100 pfu of Ad-β-gal adenovirus or a recombinant adenovirus that did not contain the β-galatosidase gene (control vector) were transducted into FM3A, FvP, K562/P, and K562/MX2 cells for 72 hours. After 72 hours of transduction, cells were harvested and β-galactosidase assay was performed using a β-Gal Assay Kit (Invitrogen) according to the manufacturer's instructions. Results were expressed as the relative β-galatosidase activity when compared with the activity from the control vector.

Animal experiments

BALB/cA Jcl mice were purchased from Saitama Experimental Animals Supply Co., Ltd. (Saitama, Japan). The mice were matched for age (5 weeks) and sex (female) and used when they were 6-8 weeks of age. Animals were maintained in facilities approved by institutional guidelines. FvP cells (5×10^6) were implanted using the intra-peritoneal injection and were simultaneously treated with 10^8 pfu of Ad-hTOP2α or Ad-β-gal recombinant adenovirus by intra-peritoneal injection. One week later, these mice were treated with 2 mg/kg of etoposide or normal saline by intra-peritoneal injection. The inoculated tumor weight was measured after one month of treatment.

Results

Characterization of MDA-P, MDA-VP, FM3A, FvP, K562/P and K562/MX2 cells

MDA and MDA-VP cells were characterized previously with respect to their sensitivity to etoposide, adriamycin, amsacrine and cisplatin (Table 1 and Ref. 7). Etoposide-resistant FvP, derived from the mouse breast cancer cell line FM3A, and the etoposide-resistant human leukemia cell line K562/MX2, derived from K562/P, showed cross resistance to adriamycin, but not vincristine (Table 1, and data from Nakamura et al.). Doubling time was longer in resistant lines when compared with in parent lines (Table 1). The most significant difference between the parent and resistant cell lines was the level of topoisomerase IIα expression (Figure 1 and data from Nakamura et al.). MDA-VP cells, FvP cells and K562/MX2 cells exhibited a marked reduction in steady-state mRNA expression levels on densitometric analysis when compared with their parent cells (Figures 1–3).

Expression of human topoisomerase IIα mRNA in MDA-P, MDA-VP, FM3A, FvP, K562/P and K562/MX2 Cells transducted with Ad-hTopoIIα

To determine whether the expression of human topoisomerase IIα mRNA in etoposide-resistant human and mouse breast cancer cells and human leukemia cells could be increased, MDA-VP, FvP, and K562/MX2 cells were transducted with Ad-hTopoIIα at a transduction multiplicity of 100 pfu/cell for 72 hours. Levels of topoisomerase IIα expression were significantly increased in MDA-VP and FvP cells after transduction with Ad-hTopoIIα,

Table 1. Characterization of tumor cell lines.

	Doubling time (hours)	Etoposide	Adriamycin	Vincristine (nM)
MDA-P	15±4	4.0±1.6 μM	0.1±0.03?μM	Nd
MDA-VP	36±7	103.0±15.8 μM*	3.0±0.5 μM*	Nd
FM3A	24±4	10±2 nM	20±3 nM	5±3
FvP	75±7	200±15 nM*	100±15 nM*	10±5
K562/P	25±4	10±4 nM	20±3 nM	2.0±2.1
K562/MX2	30±4	94±15 nM*	150±20 nM*	2.3±1.8

Cytotoxicity was assessed by colony forming assay for MDA-P and MDA-VP cells (2 hours drug exposure), and trypan blue dye exclusion assay for FM3A, FvP, K562/P and K562/MX2 cells (72 hours drug exposure).
Average±standard deviation from five independent experiments.
*: Cytotoxicity in parent cells vs. resistant cells ($p < 0.05$).
nd: not done.
Etoposide dose we used were 10, 50, 100 nM and 10 and 50 μM in these experiments.
Adriamycin dose we used were 1, 10, 50, 100, 250 and 500 nM and 0.01, 0.1, 0.5, 1.0, 5.0, and 10.0 μM in these experiments.
Vincristine dose we used were 1, 5, 10 and 50 nM in these experiments.

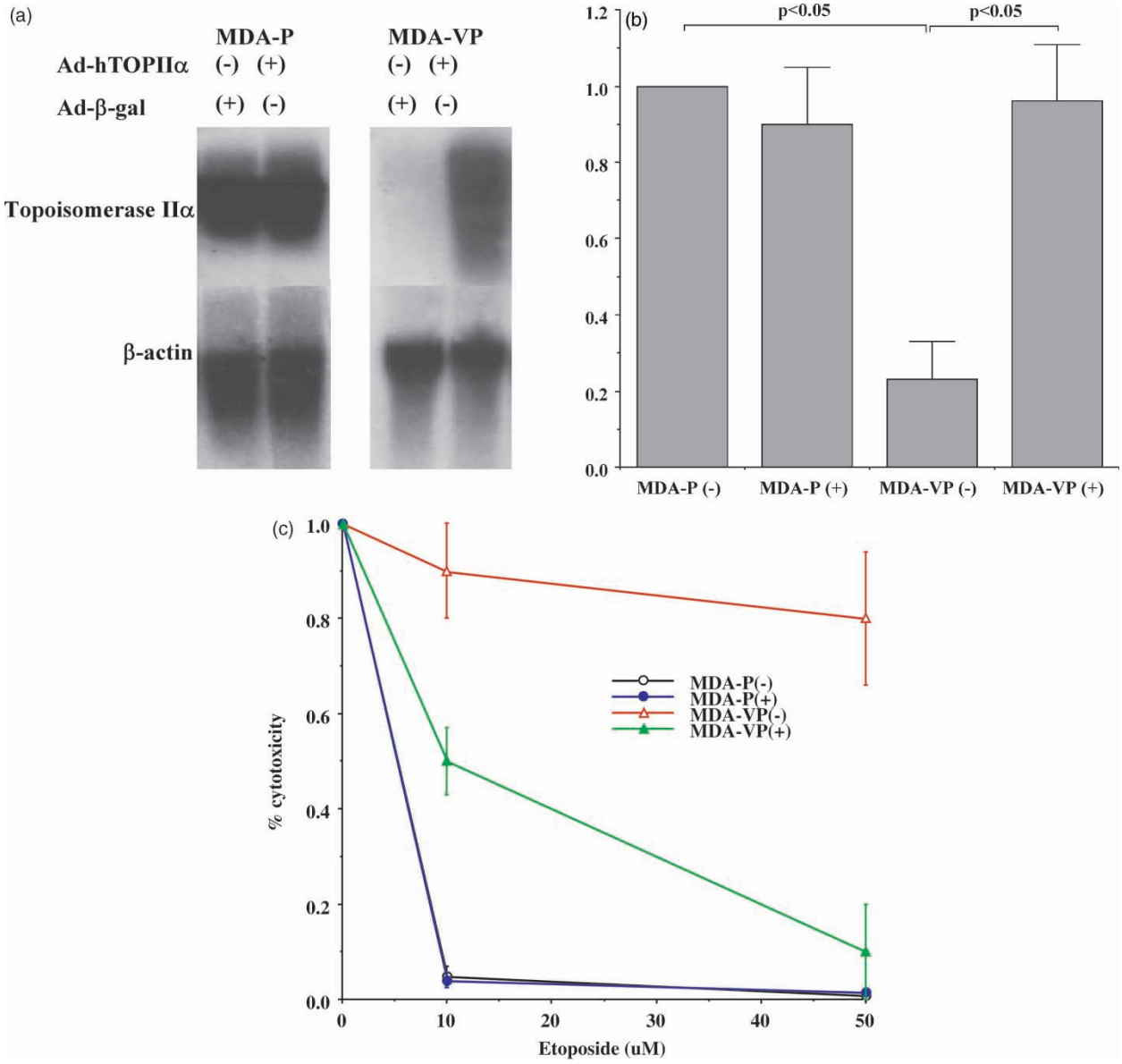

Figure 1. (a): Human topoisomerase IIα mRNA expression, (b): Densitometric analysis of human topoisomerase IIα gene, (c): Increased cytotoxicity of MDA-VP cells to etoposide after transduction with Ad-hTopoIIα.

but such increases were not seen in K562/MX2 cells (Figures 1–3 and data from Nakamura et al.). Transduction of MDA parent cells and FM3A cells with Ad-hTopoIIα resulted in no significant increase in topoisomerase IIα mRNA (Figures 1 and 2). In contrast, K562/P and K562/MX2 cells did not exhibit increased topoisomerase IIα expression after transduction with Ad-hTopIIα (Figure 3).

Enhanced sensitivity of MDA-VP and FvP cells, but not K562/MX2 cells, to etoposide after infection with Ad-hTopoIIα ..

MDA-VP, FvP and K562/MX2 cells were less sensitive to the cytotoxic actions of etoposide than their parent cells with IC_{50} values of 103 and 4 μM (MDA-VP vs. MDA-P), 200 and 10 nM (FvP vs. FM3A) and 94 and 10 nM (K562/MX2 vs. K562/P) (Table 1). As shown in Figure 3and 5, infection with Ad-hTopoIIα significantly increased the sensitivity of the MDA-VP and FvP cells to etoposide. Ad-β-gal had no effect on cell sensitivity to etoposide. There were no significant differences in cytopathic effect after exposure to Ad-hTopoIIα alone, when compared with Ad-β-gal, alone at viral doses at 100 pfu/cell (data not shown). In contrast, K562/MX2 cells did not exhibit increased cytotoxic activity against etoposide after transduction with Ad-hTopIIα (Figure 3).

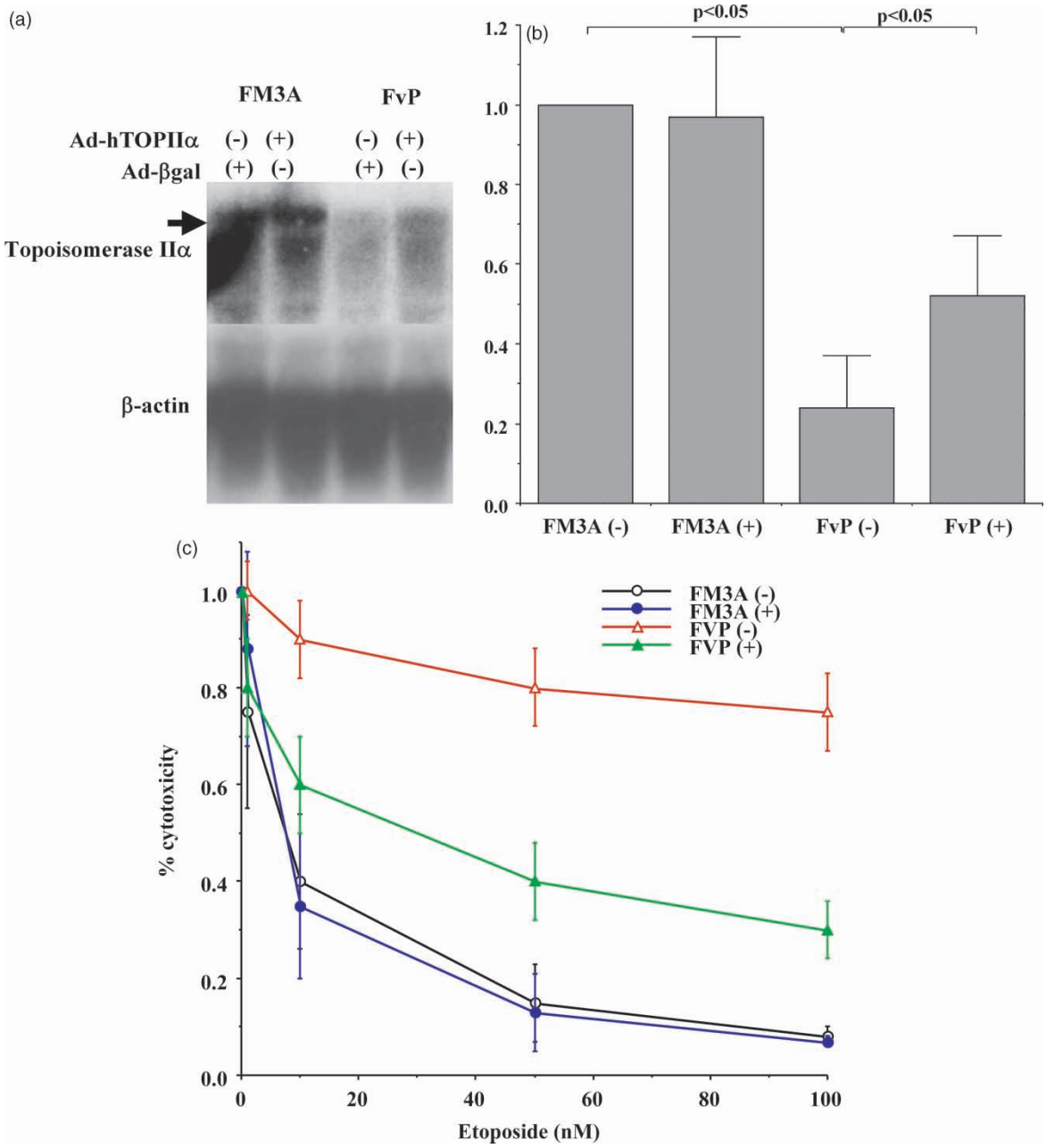

Figure 2. (a): Human topoisomerase IIα mRNA expression, (b): Densitometric analysis of human topoisomerase IIα gene, (c): Increased cytotoxicity of FvP cells to etoposide after transduction with Ad-hTopoIIα.

No enhanced sensitivity of FvP or K562/MX2 cells to adriamycin, and vincristine after infection with Ad-hTopoIIα

FvP cells and K562/MX2 cells were less sensitive to the cytotoxic actions of adriamycin than their parent cells (Table 1). As shown in Table 2, transduction with Ad-hTopoIIα did not increase the sensitivity of FvP cells or K562/MX2 cells. Ad-β-gal also had no effect on cell sensitivity to adriamycin. There was no significant difference in the cytotoxic effects after exposure to Ad-hTopoIIα alone when compared with Ad-β-gal alone at viral doses at 100 pfu/cell (data not shown).

Effects of Ad-β-galactosidase on FM3A, FvP, K562/P and K562/MX2

To elucidate the difference in the effects of Ad-hTopIIα between breast cancer cells and leukemia cells, we investigated the transduction capability with recombinant adenovirus vector. Staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside using Ad-β-gal virus showed that good transduction

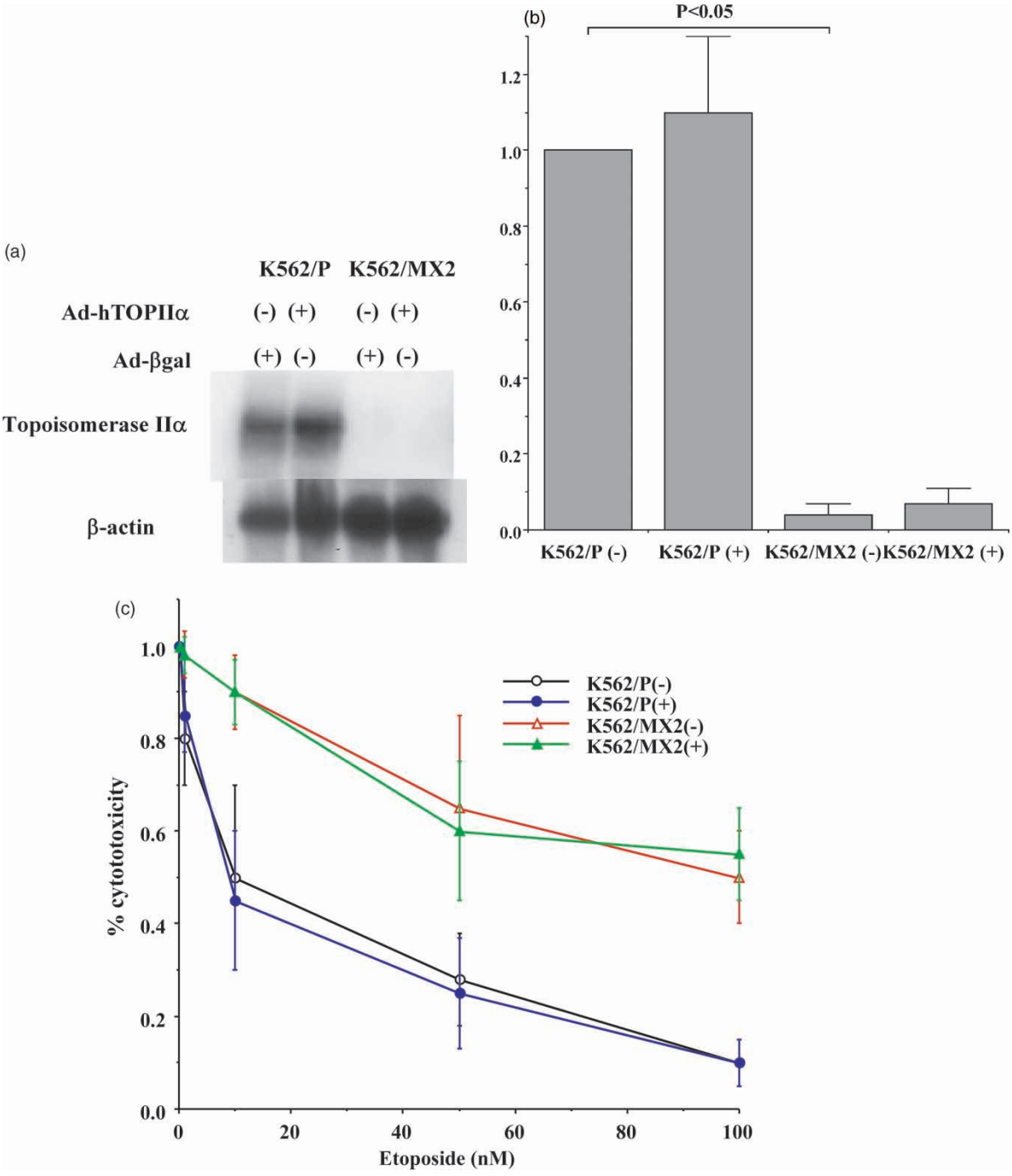

Figure 3. (a): Human topoisomerase IIα mRNA expression, (b): Densitometric analysis of human topoisomerase IIα gene, (c): Cytotoxicity of K562/P and K562/MX2 cells to etoposide after transduction with Ad-hTopoIIα.

occurred in FM3A and FvP cells, but transduction did not occur in K562/P and K562/MX2 cells (Table 3). These results confirmed adenovirus uptake in FM3A and FvP cells, but not in K562 cells.

Reduced weight of inoculated tumor with Ad-hTOP2 and etoposide treated mice.

Because FvP cells formed bulky tumors in intraperitoneal region in the mice, we measured the total tumor weight in each treated mice. As shown in Table 4, tumor weight was clearly reduced in Ad-hTOP2α and etoposide treated mice compared to Ad-β-gal with etoposide and/or saline treated, or Ad-hTOP2α and saline treated mice.

Discussion

This study demonstrated that the sensitivity of etoposide-resistant human and mouse breast cancer

Table 2. Cytotoxiciy of tumor cell lines after transduction with Ad-hTopoIIα.

	Ad-hTopoIIa transduction	Adriamycin (nM)	Vincristine (nM)
FM3A	(−)	20±3	5±3
		[100]	[100]
	(+)	29±4	3±4
		[145]	[60]
FvP	(−)	100±15*	10±5
		[100]	[100]
	(+)	95±18	12±4
		[95]	[120]
K562/P	(−)	20±3	2.0±2.1
		[100]	[100]
	(+)	18±4	1.9±15
		[90]	[95]
K562/MX2	(−)	150±20 *	2.3±1.8
		[100]	[100]
	(+)	140±25	3.0±2.0
		[93]	[130]

Cytotoxicity was assessed by trypan blue dye exclusion assay (72 hours drug treatment).
Average±standard deviation from five independent experiments.
*: Cytotoxicity in parent cells vs. resistant cells ($p < 0.05$).
[]: percent of cell number with viral treatment/without viral treatment.

cells in vitro and in vivo, but not leukemia cells, can be increased by transduction with a recombinant adenovirus expressing the human topoisomerase IIα gene. The adenoviral system provides a method of delivering a wild-type topoisomerase IIα gene to tumor cells with decreased enzyme levels. Our studies have also provided evidence that drug resistance to topoisomerase II inhibitors can be circumvented by topoisomerase IIα gene transfer.

MDA-VP cells, FvP cells and K562/MX2 cells are resistant to the cytotoxic actions of etoposide. MDA-VP, FvP, and K562/MX2 cells did not express mdr-1 or p-glycoprotein [Ref. 7–9 and data not shown]. Resistance is presumed to result from the decreased

Table 3. β-galactosidase expression ratio with/without Adeno-β-galactosidase (Ad-β-gal) virus transduction.

	β-galactosidase acitivty with Ad-β-gal transduction
K562/P	1.5±0.6
K562/MX2	1.4±0.4
FMA3A	8.0±2.0*
FvP	5.0±2.0*

100 pfu of Ad-β-gal were transducted into FM3A, FvP, K562/P and K562/MX2 cells for 72 hours. Then the cells were harvested and performed β-gal assay. Data were β-gal activity in cells transducted with Ad-β-gal/β-gal activity in cells transducted with control adenovirus vector. Average±standard deviation from three independent experiments.
*: $p < 0.05$, significantly increased expression in β-galactosidase activity with Ad-β-gal viral transduction.

Table 4. Tumor weight in the Ad-hTOP2α, Ad-β-gal recombinant adenovirus and etopoide treated mice.

	Saline treatment	Etoposide treatment
Ad-hTOP2α treatment	3.0 g, 3.2g	0.3g, 0.4g
Ad-β-gal treatment	1.8 g, 2.0 g	1.7 g, 1.9g

FvP cells (5×10^6) with 10^8 pfu of Ad-hTOP2α or Ad-β-gal recombinant adenovirus were simultaneously treated by intra-peritoneal injection to BALB/cA Jcl mice. One week later, these mice were treated with 2 mg/kg of etoposide or identical volume of normal saline by intra-peritoneal injection. The inoculated tumor weight was measured one month after the treatment.

expression of topoisomerase IIα (Figures 1, 2 and 3). We previously demonstrated that topoisomerase IIα gene transfer using a pMAM vector system increases etoposide-mediated cytotoxicity [7–9]. The pTOP2-MAMneo vector, transfected into cells by calcium phosphate co-precipitation is not, however, suitable for *in vivo* investigations. Recombinant adenoviral vectors efficiently infect cells and provide one method for successful gene transfer. Our goal was to design a recombinant adenoviral vector that would increase intracellular levels of topoisomerase IIα, the molecular target of etoposide and other topoisomerase II inhibitors [20–23]. Because human topoisomerase IIα is a large gene (5.6 kb), we selected the pBHG10 packaging vector, which has a larger capacity for gene insertion [12]. The strategy described here was successful because transduction with this adenoviral construct containing the human topoisomerase IIα gene led to the expression of topoisomerase IIα in MDA-VP cells and FvP cells.

Our data indicates that adenovirus-mediated gene transfer provides a novel way to circumvent drug resistance in cells with decreased topoisomerase IIα in vitro as well as in vivo. The topoisomerase II activity in primary breast tumors is considerably lower than that found in cervix, colon, and lung tumors [24]. In addition, large variations in individual cellular topoisomerase II expression within each tumor have been described [24], thus helping to explain the heterogeneous response to topoisomerase II-directed therapy of some patients and the emergence of resistance after previously successful drug therapy. Increasing the cellular levels of normal topoisomerase II in the tumor may offer a way to increase the sensitivity of emerging resistant cells to the cytotoxic action of topoisomerase II-reactive agents. Our data suggest that, regardless of the mechanisms, increased gene expression and cytotoxicity will be sustained only in etoposide-resistant tumor cells with decreased topoisomerase IIα. Transduction of MDA, FM3A parent cells, and normal cells with Ad-hTopoIIα resulted in no significant increase in topoisomerase IIα mRNA

(Figures 1 and 2, Ref. 12). The data presented support our hypothesis that topoisomerase IIα gene transfer may circumvent drug resistance by increasing the target for etoposide and other topoisomerase II-reactive antineoplastic agents.

Low transduction activity in hematopoietic cells, such as K562 cells, is one of the problems of adenoviral gene therapy shown in our experiments. Inefficient entry pathways by adenovirus may be responsible because K562 cells exhibit low levels of Coxsackie and adenovirus receptors and αv integrin [25]. Recently, histone deacetylase inhibitor has been found to enhance adenovirus infection of hematopoietic cells, including K562 cells [25]. Combined therapy with histone deacetylase inhibitor and Ad-hTopIIα could increase sensitivity to etoposide in etoposide-resistant hematopoietic cells.

In conclusion, a recombinant adenovirus containing the human Topoisomerase IIα gene might be a powerful tool to overcome drug resistance in breast cancer cells in vitro and in vivo.

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