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$\alpha_v\beta_3$ Integrin-Mediated Drug Resistance in Human Laryngeal Carcinoma Cells Is Caused by Glutathione-Dependent Elimination of Drug-Induced Reactive Oxidative Species

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ABSTRACT

As a model for determination of the role of integrins in drug resistance, we used $\alpha_v\beta_3$ integrin-negative human laryngeal carcinoma cell line (HEp2) and three HEp2-derived cell clones with a gradual increase of $\alpha_v\beta_3$ integrin expression. The $\alpha_v\beta_3$ integrin expression protects cells from cisplatin, mitomycin C, and doxorubicin. In HEp2-$\alpha_v\beta_3$ integrin-expressing cells, the constitutive expression of Bcl-2 protein and the level of glutathione (GSH) were increased compared with HEp2 cells. Pretreatment of HEp2-$\alpha_v\beta_3$ integrin-expressing cells with an inhibitor of GSH synthesis, buthionine sulfoximine (BSO), decreased the level of GSH and partially reverted drug resistance to all above-mentioned drugs, but it did not influence the expression of Bcl-2. Sensitivity to selected anticancer drugs did not change with overexpression of Bcl-2 in HEp2 cells, nor with silencing of Bcl-2 in HEp2-$\alpha_v\beta_3$ integrin-expressing cells, indicating that Bcl-2 is not involved in resistance mechanism. There was no difference in DNA platination between HEp2 and HEp2-$\alpha_v\beta_3$ integrin-expressing cells, indicating that the mechanism of drug resistance is independent of cisplatin detoxification by GSH. A strong increase of reactive oxidative species (ROS) formation during cisplatin or doxorubicin treatment in HEp2 cells was reduced in HEp2-$\alpha_v\beta_3$ integrin-expressing cells. Since this increased elimination of ROS could be reverted by GSH depletion, we concluded that multidrug resistance is the consequence of GSH-dependent increased ability of $\alpha_v\beta_3$-expressing cells to eliminate drug-induced ROS.

Chemotherapy often results in the development of drug resistance. Several molecular mechanisms have been recognized as a cause of resistance, such as reduced drug accumulation, increased drug inactivation, increased ability to repair and/or tolerate DNA lesions, and inhibition of apoptosis. Recent findings show that integrin-mediated adhesion to the extracellular matrix can modify cellular response to chemotherapeutic drugs through mechanisms such as inhibition of apoptosis, decreased cellular proliferation, and alterations in a drug target (Damiano, 2002; Ambriović-Ristov and Osmak, 2006). Integrins are cell surface adhesion molecules that connect cells to components of the extracellular matrix. They are assembled from a set of diverse α-(18) and β-(8) subunits that associate to form 24 differentially composed heterodimers exhibiting specific but also redundant ligand binding and expression patterns. Integrins modulate many signaling pathways, supporting cell survival and proliferation and influencing expression of differentiation-regulated genes (Danen, 2005).

The most extensively used model for investigation of molecular mechanisms of drug resistance is the development of resistant cells by selected anticancer drug treatment and then comparing the biological and biochemical characteristics of those cells, allowing for the causes of drug resistance to be determined. Despite increased expression of several integrin subunits and/or heterodimers found in different drug-resistant cells, such as β_1, $\alpha_5\beta_1$, $\alpha_6\beta_1$, α_4, α_2, β_4, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ (Ambriović-Ristov and Osmak, 2006), complexity of integrin signaling and their importance in drug-resistant mechanisms are not resolved.

Integrin signaling modulates numerous downstream effectors of apoptosis. One of the most frequently involved is the antiapoptotic protein Bcl-2 that belongs to family of proteins that regulate apoptosis. It includes both proapoptotic and

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ABBREVIATIONS: GSH, glutathione; BSO, buthionine sulfoximine; HEp2, human laryngeal carcinoma cells; ROS, reactive oxidative species; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; ERK, extracellular signal-regulated kinase; siRNA, small interfering RNA; NAC, *N*-acetyl-L-cysteine; Ad5, adenovirus type 5; cDDP, cisplatin; DOX, doxorubicin.

antiapoptotic members that tightly control the cell death program by regulating permeabilization of the mitochondrial outer membrane and hence the release of cytochrome *c* and other proapoptotic factors (Thomadaki and Scorilas, 2006). Bcl-2 also participates in the regulation of cellular redox state, and its antioxidant function may partially explain cell protection (Hockenbery et al., 1993). In most cases, overexpression of Bcl-2 in tumor cells in vitro conferred resistance to different chemotherapeutic drugs (Thomadaki and Scorilas, 2006). However, in a panel of human ovarian carcinoma cell lines, high Bcl-2 levels confer a trend toward sensitivity, rather than resistance, to platinum drugs (Beale et al., 2000).

Currently, there is no data on implication of glutathione (GSH) in integrin-mediated drug resistance. GSH is the most prevalent nonprotein thiol in human cells that was first recognized for its antioxidant role. It plays a very important role in determining sensitivity of tumor cells to different anticancer drugs. Generally, high GSH intracellular levels prevent, whereas low GSH levels enhance cell death (Estrela et al., 2006). Depending on cell type, GSH may be involved at different levels in both apoptotic pathways: at the level of the CD95 death-inducing signaling complex (extrinsic) (Hentze et al., 2002) or at the level of mitochondria (intrinsic) (Mirkovic et al., 1997; Meredith et al., 1998).

The relationship between Bcl-2 and GSH is not clearly understood. It has been reported that overexpression of Bcl-2 in stably transfected cells increases the intracellular GSH levels (Mirkovic et al., 1997; Rudin et al., 2003). Bcl-2 overexpression causes redistribution of GSH to the nucleus, thereby altering nuclear redox potential (Voehringer et al., 1998) and by raising cellular antioxidant defense status decreases formation of some oxidative reactive nitrogen species (Lee et al., 2001). However, U937 monocytic tumor cells treated with buthionine sulfoximine (BSO), an inhibitor of GSH synthesis, up-regulated Bcl-2 expression that supported their survival (D'Alessio et al., 2004). In contrast, BSO caused GSH depletion down-regulated Bcl-2 by reducing protein half-life and induced cholangiocyte apoptosis (Celli et al., 1998).

Recently, we found the up-regulation of $\alpha_v\beta_3$ integrin in HEp2 cells resistant to cisplatin (Ambriović-Ristov et al., 2004). We show here, on the model of HEp2-derived $\alpha_v\beta_3$ integrin-expressing cells, the appearance of resistance to several anticancer drugs (cisplatin, mitomycin C, and doxorubicin). Although these drugs act through different mechanisms of action, they share one mechanism, i.e., induction of reactive oxidative species (ROS). The overexpression of $\alpha_v\beta_3$ resulted in increased constitutive expression of Bcl-2 and the total amount of GSH. Resistance to all above-mentioned drugs was independent of Bcl-2 but was at least partly the consequence of increased level of GSH. The mechanism by which GSH supports survival of $\alpha_v\beta_3$ integrin-expressing cells was independent of GSH detoxification role of cisplatin; GSH contributed to cell survival through increased ability to eliminate ROS after drug exposure. Our results describe a novel mechanism mediated by $\alpha_v\beta_3$ integrin that regulates sensitivity to anticancer drugs that kill cells by generating ROS.

Materials and Methods

Cell Lines. Human laryngeal carcinoma (HEp2) cells were obtained from cell culture bank (Invitrogen, Carlsbad, CA). The $\alpha_v\beta_3$ integrin-expressing cells HEp2-K4, HEp2-K16, and HEp2-K1 were obtained and characterized previously (Ambriović-Ristov et al., 2004). Cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% bovine serum (Invitrogen) at 37°C with 5% CO_2 in humid atmosphere.

Drugs. *Cis*-diamminedichloroplatinum (cisplatin), mitomycin C, and doxorubicin (Sigma-Aldrich, Taufkirchen, Germany) were dissolved in water and stored at −20°C. BSO (Sigma-Aldrich) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) (Millipore Bioscience Research Reagents, Temecula, CA) were dissolved in phosphate-buffered saline (PBS) and stored at −20°C (BSO) and 4°C (MTT).

Isolation of Bcl-2-Expressing Clones. The Bcl-2 expression plasmid pCHC6Bcl2 was kindly provided by T. McDonnell (M. D. Anderson Cancer Center, Houston, TX). For isolation of Bcl-2-expressing clones, HEp2 cells were transfected with pCHC6Bcl2 using Lipofectamine (Invitrogen). Cell clones were selected in the presence of 300 μg/ml hygromicin (Sigma-Aldrich) and screened by Western blot.

Determination of Cell Survival. The sensitivity of HEp2 and HEp2-derived $\alpha_v\beta_3$ integrin-expressing clones to anticancer drugs was determined using MTT assay. Cells were seeded in 96-well tissue culture plate (2.5×10^3 cells/well). Twenty-four hours later, cells were treated with different concentrations of anticancer drugs. Seventy-two hours later the absorbance of MTT-formazan product was measured with a microplate reader (Awareness Technology Inc., Palm City, FL) at 545 nm. The IC_{50} (concentration of a drug that reduces the cytotoxicity by 50%) was calculated from a log-linear plot of multiple doses of each cytotoxic drug and cell survival for each HEp2-$\alpha_v\beta_3$ integrin-expressing cell clone. The difference in resistance was calculated as the ratio of IC_{50} values for each HEp2-derived $\alpha_v\beta_3$ integrin-expressing clone and HEp2 cells.

Western Blot. The expression of proteins in HEp2 and HEp2-derived $\alpha_v\beta_3$ integrin-expressing clones was measured in total cell extracts prepared by sonication in S buffer (20 mM Tris-HCl, pH 8.5, 1 mM EDTA, 5% glycerol, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride). After sonication, cell debris was removed by centrifugation, and protein concentrations were determined. Thirty micrograms of total cell proteins was boiled for 3 min in Laemmli buffer, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and the membrane was blocked for 1 h at room temperature in PBS containing 0.01% Tween 20 and 5% dried milk. The membranes were incubated overnight at 4°C with mouse monoclonal antibody against Bcl-2 (dilution 1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or rabbit polyclonal antibody against Bax (dilution 1:1500; Santa Cruz Biotechnology, Inc.) and Bad (dilution 1:1000; Santa Cruz Biotechnology, Inc.). After washing with 0.01% Tween 20 in PBS, membranes were developed with horseradish peroxidase-coupled secondary antibody (dilution 1:4000; GE Healthcare, Piscataway, NJ) for 2 h at room temperature. Finally, proteins were visualized by enhanced chemiluminescence reagents (GE Healthcare). As loading control, all membranes were incubated with rabbit polyclonal ERK2 antibody (dilution 1:3000; Santa Cruz Biotechnology, Inc.).

Measurement of GSH Content. The intracellular GSH content in HEp2, HEp2-derived $\alpha_v\beta_3$ integrin-expressing clones as well as in HEp2-derived Bcl-2-expressing clones was measured as described by Tietze (1969). Briefly, cells were grown to 80% confluence, collected, counted, lysed by freezing and sonication, centrifuged at 13,000*g* for 15 min at 4°C. GSH was determined in supernatants after reduction of GSH with GSH reductase and reaction with 5,5′-dithio-bis-(2-nitrobenzoic acid) (Boehringer Ingelheim GmbH, Ingelheim, Germany). The kinetics of formation of 2-nitro-5-thiobenzoic acid, which absorbs at 412 nm, was monitored by a UV-Vis spectrophotometer

Camspec N330 (Camspec Ltd., Cambridge, UK) at 412 nm. GSH was expressed in moles of GSH per microgram of total proteins.

Determination of DNA Platination. HEp2 and HEp2-derived $\alpha_v\beta_3$ integrin-expressing clones were treated 4 h with 100 μM cisplatin. DNA was isolated using QIAamp DNA Blood Mini kit (QIAGEN, Germantown, NY) according to manufacturer's protocol. The amount of platinum atoms bound to DNA was measured by an inductively coupled plasma high-resolution mass spectrometer (Element 2; Thermo Fisher Scientific, Bremen, Germany). The platinum-nucleotide content was calculated using the relative molar masses of platinum and nucleotides obtained from DNA concentration (Kloft et al., 1999). Calibration standards were prepared from platinum standard solution 1 g/l in 2 M HCl (Fluka Riedel-de Häen, Seelze, Germany).

Determination of ROS Formation. ROS levels were determined by using 2,7-dichlorodihydrofluorescein diacetate (Invitrogen). The 52 mM stock solution was prepared in dimethyl sulfoxide and was kept at −20°C until use. HEp2 and HEp2-derived $\alpha_v\beta_3$ integrin-expressing clones were seeded in black 96-well plates. Following overnight incubation, the cells were loaded with 10 μM 2,7-dichlorodihydrofluorescein diacetate in serum-free medium, incubated during 60 min at 37°C, and then washed two times with PBS. Drug dilutions were prepared in fresh medium without phenol red and added in each well. The fluorescence was measured every 15 min with a microplate fluorescence reader FLUOstar OPTIMA (BMG LABTECH GmbH, Offenburg, Germany), using excitation filter at 485 nm and emission filter at 530 nm. The influence of GSH depletion on ROS production was measured after addition of 0.01 mM BSO 8 h after the seeding. Following overnight incubation, ROS levels were measured as described above. The treatment of cells with 0.1% H_2O_2 was used as a positive control.

Gene Silencing. For silencing of Bcl-2, the predesigned Bcl-2-specific siRNA sequences (Silencer Select Predesigned siRNA; Ambion, Austin, TX; locus ID 596) and control nonspecific siRNA for human (Silencer Select Predesigned siRNA Negative Control #1 siRNA; Ambion) were used. The transfection of siRNA was performed using Lipofectamine RNAiMAX Reagent (Invitrogen) according to the manufacturer's instructions. The transfected cells were first tested for Bcl-2 expression by Western blot, 48 h after transfection when cells were plated for assessment of cell survival. To confirm that measurement of cell survival was performed in conditions of permanently silenced expression of Bcl-2, 72 h later, i.e., when the survival test was completed, transfected cells were again tested for Bcl-2 expression.

Results

Overexpression of $\alpha_v\beta_3$ Integrin in HEp2 Cells Confers Resistance to Several Anticancer Drugs. Because HEp2 cells do not express $\alpha_v\beta_3$ integrin, they were used to generate several cell lines with gradually increased expression of this integrin. They were produced by stable transfection of HEp2 cells with a plasmid expressing the β_3 subunit. Since β_3 subunit can bind to α_v only, all three cell lines HEp2-K4, HEp2-K16, and HEp2-K1 possess gradually increased (HEp2-K4 < HEp2-K16 < HEp2-K1) expression of $\alpha_v\beta_3$ integrin. We measured the expression of $\alpha_v\beta_5$ in these cell lines to verify whether the expression of $\alpha_v\beta_3$ decreased the expression of other α_v integrins due to the competition of β_3 for available α_v. In all stable transfectants, the expression of $\alpha_v\beta_5$ was identical (Ambriović-Ristov et al., 2004). To determine whether the expression of $\alpha_v\beta_3$ integrin induced drug resistance, we assessed their survival following the treatment with anticancer drugs using the MTT assay. It has to be emphasized that all $\alpha_v\beta_3$ integrin-expressing cell lines had the same growth rate as parental HEp2 cells (data not shown).

Our results showed that all three HEp2-derived $\alpha_v\beta_3$ integrin-expressing cells HEp2-K4, HEp2-K16, and HEp2-K1 were 2- to 2.8-fold (based on the ratio in IC_{50} values) resistant to cisplatin (Fig. 1A) and 2- to 2.3-fold resistant to mitomycin C (Fig. 1B) compared with the parental HEp2 cell line. Cisplatin and mitomycin C are primarily described as DNA cross-linking agents generating ROS as well (Pagano, 2002). The HEp2-$\alpha_v\beta_3$ integrin-expressing clones were also resistant 2- to 2.5-fold to doxorubicin (Fig. 1C). This drug has a complex mechanism of action: it interacts with DNA by intercalation, inhibits the activity of DNA topoisomerase II, and induces formation of ROS (Müller et al., 1998). To exclude the possibility that this resistance is caused by changes in DNA topoisomerase II activity, we treated cells with another DNA topoisomerase II inhibitor, etoposide. HEp2-derived $\alpha_v\beta_3$ integrin-expressing clones exhibited equal sensitivity to this drug as HEp2 cells (data not shown). Drugs that act through other mechanisms, such as methylmethanosulfonate and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (alkylating agents) and vincristine (microtubule-damaging agent), were similarly cytotoxic for HEp2 and HEp2-$\alpha_v\beta_3$ integrin-expressing clones (data not shown).

HEp2-Derived $\alpha_v\beta_3$ Integrin-Expressing Cells Have Increased Expression of Bcl-2 and the Level of GSH.

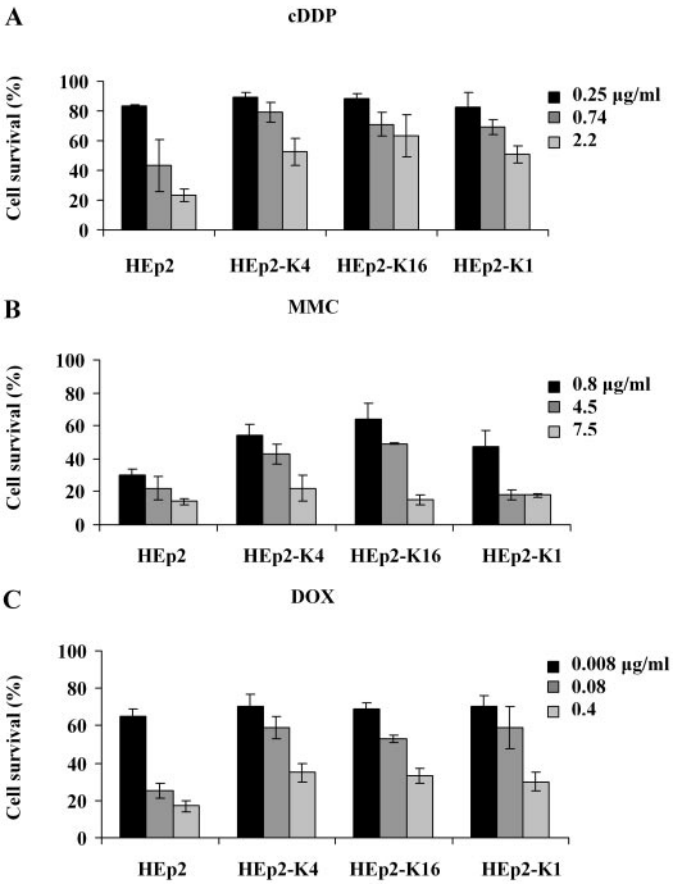

Fig. 1. Sensitivity of HEp2 and HEp2-derived $\alpha_v\beta_3$ integrin-expressing cells HEp2-K4, HEp2-K16, and HEp2-K1 to cisplatin (cDDP) (A), mitomycin C (MMC) (B), and doxorubicin (DOX) (C). Cytotoxicity was measured by MTT assay 72 h after drug treatment. Pooled data from three experiments (the mean point ± S.D.).

Although Matter and Ruoslahti (2001) have shown that Bcl-2 is up-regulated in Chinese hamster ovary cells attached to fibronectin through $\alpha_v\beta_1$ or to vitronectin through $\alpha_v\beta_3$, we examined whether increased expression of $\alpha_v\beta_3$ in HEp2 cells would increase Bcl-2. The levels of Bcl-2 were increased in all three HEp2-derived $\alpha_v\beta_3$-expressing cells (HEp2-K4, HEp2-K16, and HEp2-K1) in a dose-dependent manner as shown by immunoblotting and densitometric analysis (Fig. 2). In contrast, levels of Bax and Bad were comparable with those in HEp2 cells (Fig. 2).

Since a link between Bcl-2 and GSH has been shown in literature (Mirkovic et al., 1997; Voehringer et al., 1998; Rudin et al., 2003), we examined possible changes in the intracellular GSH content. As shown in Fig. 3, all three HEp2-derived $\alpha_v\beta_3$-expressing cells have increased levels of GSH in a dose-dependent manner relative to HEp2 cells.

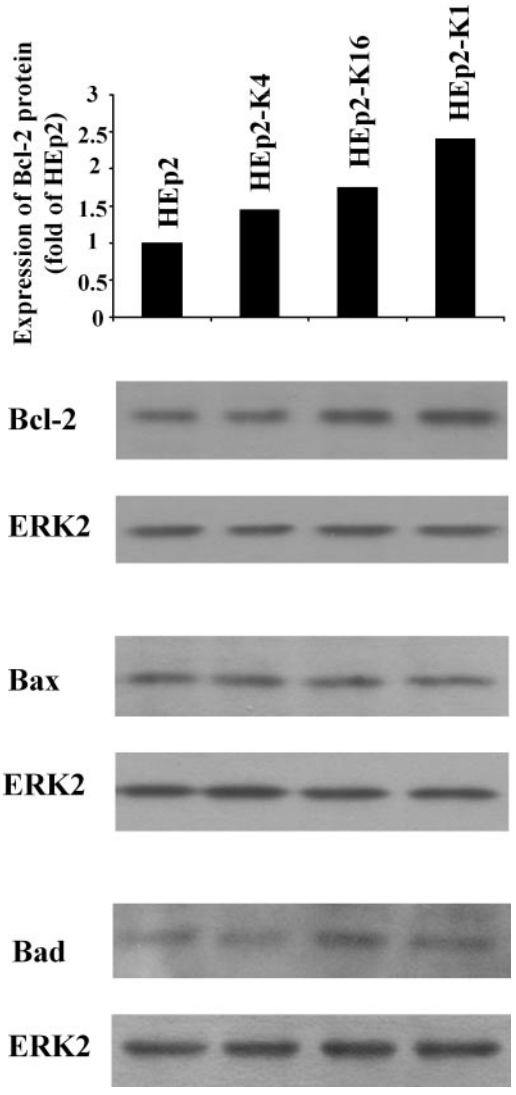

Fig. 2. Constitutive expression of Bcl-2, Bax, and Bad in HEp2 and HEp2-derived $\alpha_v\beta_3$ integrin-expressing cells HEp2-K4, HEp2-K16, and HEp2-K1. Total cell lysates were analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting. ERK2 was used as equal loading control. Representative blots of three independent experiments. Results for Bcl-2 in HEp2-derived $\alpha_v\beta_3$ integrin-expressing cells are also given as expression relative to HEp2 cell line based on densitometric analysis.

$\alpha_v\beta_3$ Integrin-Mediated Multidrug Resistance Is Dependent on GSH. The HEp2-derived $\alpha_v\beta_3$ integrin-expressing cell line HEp2-K1 was chosen for further experiments to study the contribution of $\alpha_v\beta_3$ integrin-mediated increase of GSH to multidrug resistance. To assess the nontoxic, but still effective dose of BSO that could deplete GSH stores, HEp2 and HEp2-K1 cell lines were treated with different concentrations of BSO and tested for cell survival using the MTT assay. In both HEp2 and HEp2-K1, BSO treatment (0.01 mM) reduced the total amount of GSH to a similar basal level but did not affect cell viability (data not shown). GSH depletion did not change sensitivity of HEp2 cells to cisplatin and mitomycin C but slightly decreased resistance to doxorubicin. The resistance of HEp2-K1 to all three examined drugs was reverted by BSO (Fig. 4). The different extent of abrogation of drug resistance after GSH depletion can be explained by differential importance of GSH in the mechanism of action of these drugs. Namely, these drugs act through multiple mechanisms of action; it is very likely that only one of them can be abrogated by depletion of GSH. However, this result suggests that multidrug resistance conferred by $\alpha_v\beta_3$ overexpression in HEp2 cells is at least partly dependent on the total amount of GSH. It is very important to point out that BSO treatment did not alter the Bcl-2 expression in $\alpha_v\beta_3$ integrin-expressing cells (data not shown).

HEp2-Bcl-2-Overexpressing Cells Have Similar GSH Level and Similar Sensitivity to Anticancer Drugs as HEp2 Cells. To determine whether the increased GSH content of integrin-expressing cells was caused by increased Bcl-2 expression, HEp2 cells were transfected with a plasmid expressing the human *bcl-2*. Three stable cell clones expressing Bcl-2 [HEp2-Bcl-2(18), HEp2-Bcl-2(9), and HEp2-Bcl-2(3)] were selected according to Bcl-2 protein expression in Western blot (Fig. 5A), and the total amount of GSH was measured. The expression of Bcl-2 did not affect the GSH level (Fig. 5B) nor confer resistance to cisplatin (Fig. 5C). In addition, we tested resistance of cell clone HEp2-Bcl-2(3) to mitomycin C and doxorubicin, but its survival did not differ from that of HEp2 cells (Fig. 5D). Therefore, we conclude that the up-regulation of Bcl-2 in HEp2-derived $\alpha_v\beta_3$ integrin-expressing cells is independent of increased GSH content and unrelated to drug resistance.

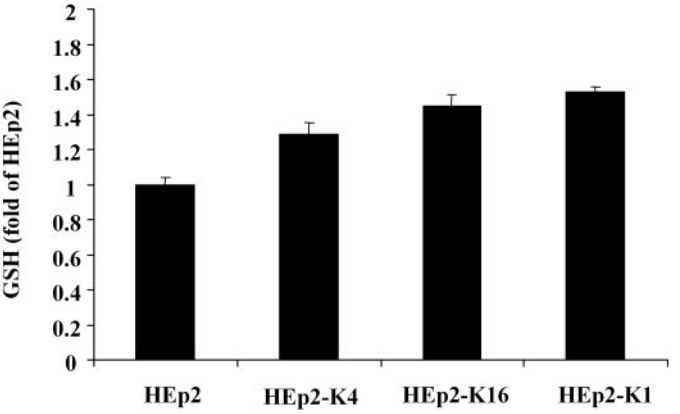

Fig. 3. GSH levels in HEp2 and HEp2-derived $\alpha_v\beta_3$ integrin-expressing cells HEp2-K4, HEp2-K16, and HEp2-K1. Total cellular GSH was measured as described by Tietze (1969). Pooled data from three experiments (the mean point ± S.D.).

The Silencing of Bcl-2 in HEp2-Derived αvβ3 Integrin-Expressing Cells Does Not Abrogate Resistance to Anticancer Drugs. To additionally confirm that increased expression of Bcl-2 does not affect sensitivity to different anticancer drugs, we investigated the impact of specific inhibition of Bcl-2 expression in HEp2-derived $\alpha_v\beta_3$ integrin-expressing cell line HEp2-K1, using siRNA-mediated gene silencing. Lysates from transfected HEp2-K1 cells underwent Western blot with antibodies to Bcl-2 and loading control ERK2 (Fig. 5E). The Western blot presented on left suggests a complete knockdown of Bcl-2 protein in the transfected cells, 48 h after transfection i.e., in the moment of plating cells for cell survival analysis. The Western blot presented on the right suggests a complete knockdown of Bcl-2 even 120 h after transfection, i.e., at the moment of analyzing cell survival by 72-h cytotoxicity test. As can be seen in Fig. 5F, the knockdown of Bcl-2 in HEp2-K1 cells does not affect the cell sensitivity to cisplatin. The survival curves corresponding to nonspecific siRNA and Bcl-2 specific siRNA, respectively, show small decrease of survival that is obviously the consequence of transfection itself. However, HEp2-K1 cells still show resistance to cisplatin in comparison with HEp2 cells. Similar results of unchanged cytotoxicity of HEp2-K1 were also obtained with doxorubicin and mitomycin C (data not shown).

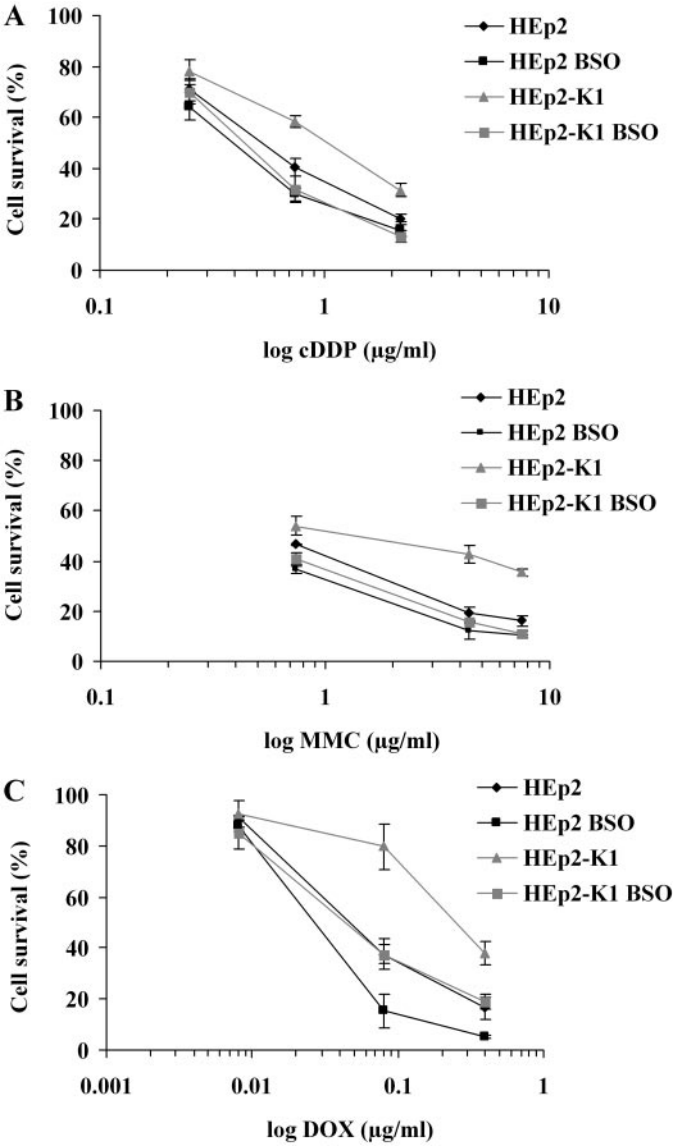

Fig. 4. BSO treatment of HEp2-derived $\alpha_v\beta_3$ integrin-expressing cell line HEp2-K1 reverted partially the resistance against cDDP (A), mitomycin C (MMC; B), and DOX (C). Sensitivity of HEp2 and HEp2-K1 to anticancer drugs was performed by MTT assay in the absence or presence of nontoxic dose of BSO (0.01 mM). Pooled data from three experiments (mean ± S.D.).

Cisplatin Resistance in HEp2-Derived $\alpha_v\beta_3$ Integrin-Expressing Cells Is Not Related to DNA Platination but to Increased Capacity to Eliminate ROS after Drug Exposure. The observation that treatment of HEp2-derived $\alpha_v\beta_3$ integrin-expressing cells with BSO partly abrogates multidrug resistance indicates the role of GSH in drug resistance. As regards cisplatin, there are two possible mechanisms that could explain how increased GSH level may inhibit cell death. Increased GSH content could increase detoxification of cisplatin or GSH could reduce damaging effects of ROS induced by the cisplatin.

In an attempt to differentiate between these two hypotheses, we measured DNA platination. Similar levels of cisplatin bound to DNA were found in HEp2 and all HEp2-derived $\alpha_v\beta_3$ integrin-expressing cells HEp2-K4, HEp2-K16, and HEp2-K1 (Fig. 6), indicating that direct detoxification of cisplatin is not responsible for cisplatin resistance of HEp2-$\alpha_v\beta_3$ integrin-expressing cells.

Since we did not find a difference in DNA platination between parental HEp2 and HEp2-derived $\alpha_v\beta_3$ integrin-expressing cell lines, we presumed that the increased total level of GSH could influence resistance through increased elimination of ROS immediately after an addition of cisplatin. The amount of intracellular ROS was measured by incubating the cells with 2,7-dichlorodihydrofluorescein diacetate that is readily oxidized by cellular ROS to fluorescent product. As shown in Fig. 7A, HEp2 and $\alpha_v\beta_3$ integrin-expressing cell line HEp2-K1 have a similar basal amount of ROS. However, 30 min after addition of cisplatin or doxorubicine to HEp2 cells, a strong increase in ROS was detected. This effect was absent in HEp2-K1, suggesting that HEp2-K1 cells have a higher capacity to eliminate drug-induced ROS. To confirm this hypothesis, we measured ROS formation in GSH-depleted cells during the 30-min treatment with cisplatin or doxorubicin. Pretreatment of HEp2-K1 cells with BSO increased the ability of these drugs to induce ROS to the level observed in HEp2 cells (Fig. 7B).

Discussion

We have previously found that human laryngeal carcinoma $CA3_{ST}$ cells resistant to cisplatin (Osmak et al., 1993) express small amounts of $\alpha_v\beta_3$ on the cell surface, whereas their parental HEp2 cells (from which $CA3_{ST}$ cells were derived) do not express this integrin (Ambriović-Ristov et al., 2004). The β_3 subunit was up-regulated in $CA3_{ST}$ cells (on protein and mRNA level), whereas expression of α_v gene was not altered (Ambriović-Ristov et al., 2004). We hypothesized that expression of $\alpha_v\beta_3$ integrin could influence the sensitivity of $CA3_{ST}$ to cisplatin. In this study, we show that the expression of $\alpha_v\beta_3$ integrin indeed protects HEp2 cells from several anticancer drugs (cisplatin, mitomycin C, and doxorubicin).

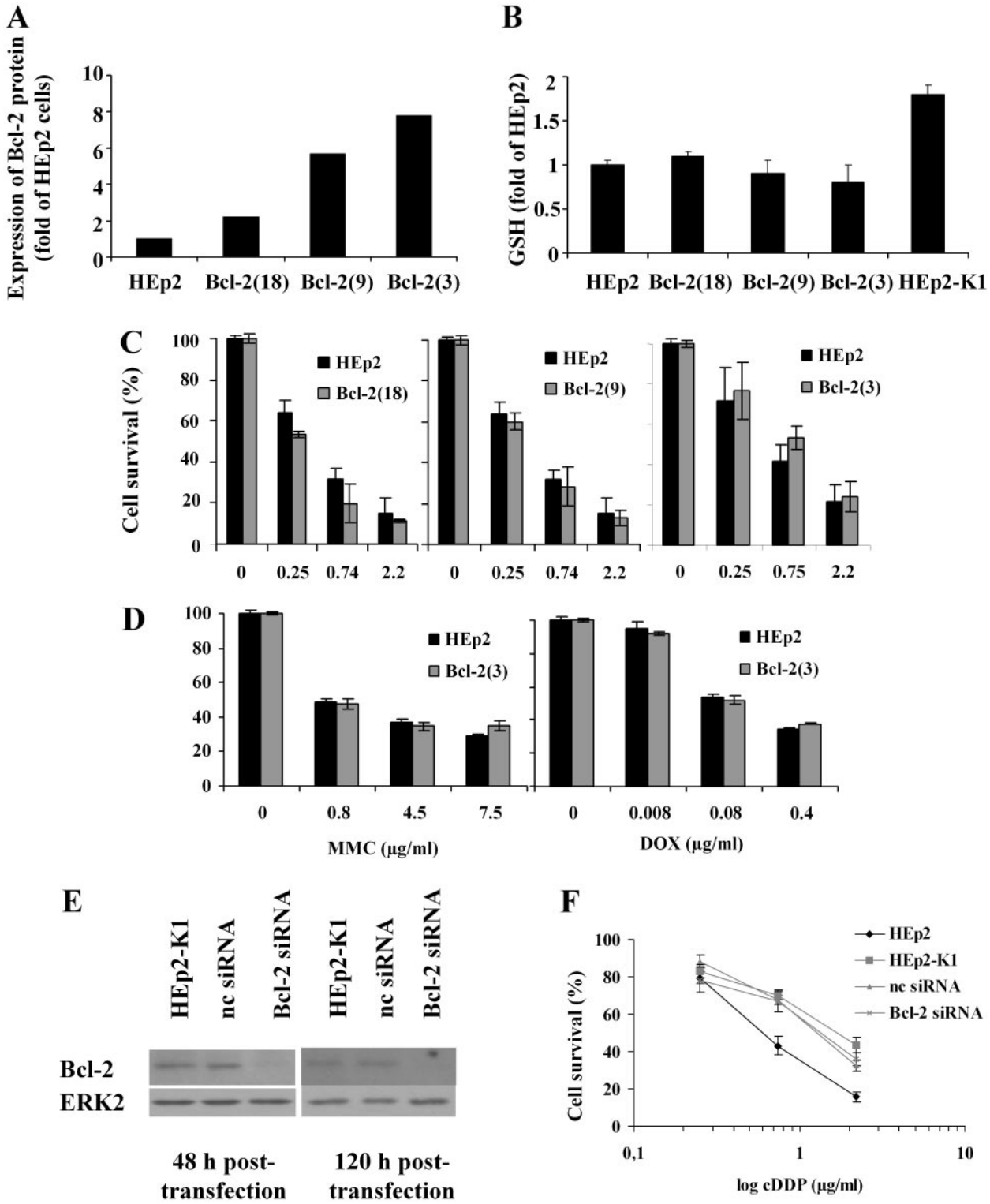

Fig. 5. Characterization of HEp2-derived Bcl-2 overexpressing cells and gene silencing of Bcl-2 in HEp2-derived $\alpha_v\beta_3$ integrin-expressing cell line HEp2-K1. A, detection of Bcl-2 expression in HEp2 and HEp2-derived Bcl-2 transfectants. Total cell lysates were analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting. Results are given as expression relative to HEp2 cell line. B, GSH concentration in HEp2, HEp2-derived Bcl-2 transfectants Bcl-2(18), Bcl-2(9) and Bcl-2(3). As a control, GSH content was measured in HEp2-derived $\alpha_v\beta_3$ integrin-expressing cell clone HEp2-K1. Total cellular GSH was measured as described by Tietze (1969). Pooled data from three experiments (the mean point ± S.D.). C, sensitivity of HEp2 and HEp2-derived Bcl-2 transfectants to cisplatin. Cytotoxicity was measured by MTT assay 72 h after drug treatment. Representative of three independently performed experiments ± S.D. D, sensitivity of HEp2 and HEp2-derived Bcl-2 transfectant HEp2-Bcl-2(3) to mitomycin and doxorubicin. Cytotoxicity was measured by MTT assay 72 h after drug treatment. Representative of three independently performed experiments ± S.D. E, gene silencing of Bcl-2 in HEp2-derived $\alpha_v\beta_3$ integrin-expressing cells HEp2-K1. HEp2-K1 cells were transfected with negative control siRNA (nc siRNA) or with Bcl-2-specific siRNA (Bcl-2 siRNA). Total cell lysates were analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting. ERK2 was used as equal loading control. The blot on the left represents Bcl-2 expression 48 h after transfection, whereas the blot on the right represents Bcl-2 expression 120 h after transfection, i.e., in the moment of determination of cell survival presented in Fig. 5F. Representative blots of two independent experiments. F, sensitivity to cisplatin of HEp2 and HEp2-derived $\alpha_v\beta_3$ integrin-expressing cell clone HEp2-K1 after Bcl-2 gene silencing. HEp2-K1 cells were transfected with nc siRNA or with Bcl-2 siRNA. Forty-eight hours after transfection, the cells were seeded for 72-h MTT assay. Data are representative of two independently performed experiments ± S.D.

It increases the level of GSH that eliminates drug-induced ROS, thus inhibiting cell death.

The role of $\alpha_v\beta_3$ integrin in cell survival was confirmed in different cell systems. Matter and Ruoslahti (2001) described protection from apoptosis under serum-free conditions in Chinese hamster ovary cells that attach to the substrate through integrin $\alpha_v\beta_3$. The expression of $\alpha_v\beta_3$ in human melanoma cells in a three-dimensional collagen gel prevented apoptosis as well (Montgomery et al., 1994). In kidney embryonic cells, $\alpha_v\beta_3$ expression provided a survival signal to protect the cells from low-level serum-induced apoptosis (Brassard et al., 1999). Finally, signaling from integrin $\alpha_v\beta_3$ promoted survival in endothelial cells (Brooks et al., 1994). Conversely, Kozlova et al. (2001) provided evidence that reduced integrin $\alpha_v\beta_3$ expression can generate an apoptosis-inhibiting signal upon disruption of the cell-matrix contact on a specific group of anoikis negative human intestinal carcinoma Caco-2 cells. The down-regulation of $\alpha_v\beta_3$ integrin was also found recently in the hamster fibroblast cell line HET-SR-2SC-LNM resistant to colchicine (and cross-resistance to vinblastine and farmorubicin) (Kozlova et al., 2004).

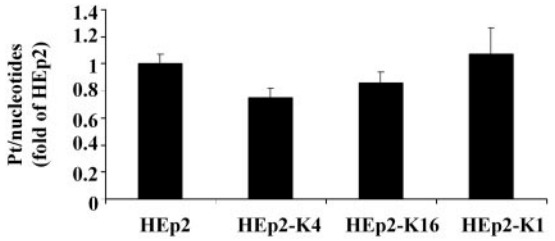

Fig. 6. DNA platination in HEp2 and HEp2-derived $\alpha_v\beta_3$ integrin transfectants HEp2-K4, HEp2-K16, and HEp2-K1. Equal number of cells were treated with 100 μM cisplatin for 4 h. Total genomic DNA was extracted and the concentration of DNA was assessed by spectrophotometry. Quantitative measurement of DNA platination was performed by inductively coupled plasma high-resolution mass spectrometer. Results are given as DNA platination relative to HEp2 cell line. Data were determined in duplicate from three separate experiments.

Thus, it seems that the role of integrin $\alpha_v\beta_3$ in apoptosis is determined by the type of cells and the nature of the signal that triggers apoptosis.

We show here that $\alpha_v\beta_3$ integrin protects cells against different drugs through GSH-mediated elimination of ROS induced by drug. The role of ROS in cisplatin cytotoxicity was reported by Miyajima et al. (1997) on bladder cancer cells. They showed that GSH depletors augment cisplatin cytotoxicity through enhancement of ROS generation. Similarly, a perturbation of ROS and intracellular GSH levels associated with enhancement of cell death was observed in cisplatin- and doxorubicin-sensitive breast cancer MCF-7 cells (Osbild et al., 2006). Reduced effect of oxidative stress in doxorubicin-resistant mouse P388/S leukemia cells toward resistant cells may be related to an increase in intracellular GSH level as well (Furusawa et al., 2001). Our conclusion that $\alpha_v\beta_3$ integrin-expressing HEp2 cells are resistant to several anticancer drugs due to the increased elimination of drug-induced ROS is further supported by the fact that these cells show resistance to the nitric oxide donor sodium nitroprusside dihydrate that acts also through induction of ROS. Similarly as observed for cisplatin and doxorubicin, we observed reversion of resistance to nitric oxide donor after BSO treatment (data not shown).

In the present study, we found increased expression of Bcl-2 protein and increased amount of GSH in HEp2-derived $\alpha_v\beta_3$ integrin-expressing cells in comparison with parental HEp2 cells. Inhibition of GSH synthesis by BSO treatment abrogated partly the resistance to cisplatin, mitomycin C, and doxorubicin, without influencing expression of Bcl-2. The ability of GSH to protect HEp2 cells against cisplatin was also supported by pretreatment of HEp2 cells with *N*-acetyl-L-cysteine (NAC, a compound that is intracellularly converted to the rate-limiting substrate for GSH synthesis). Pretreatment with NAC caused resistance to cisplatin, with no influence on Bcl-2 expression (data not shown), indicating that increased expression of GSH is sufficient for conferring

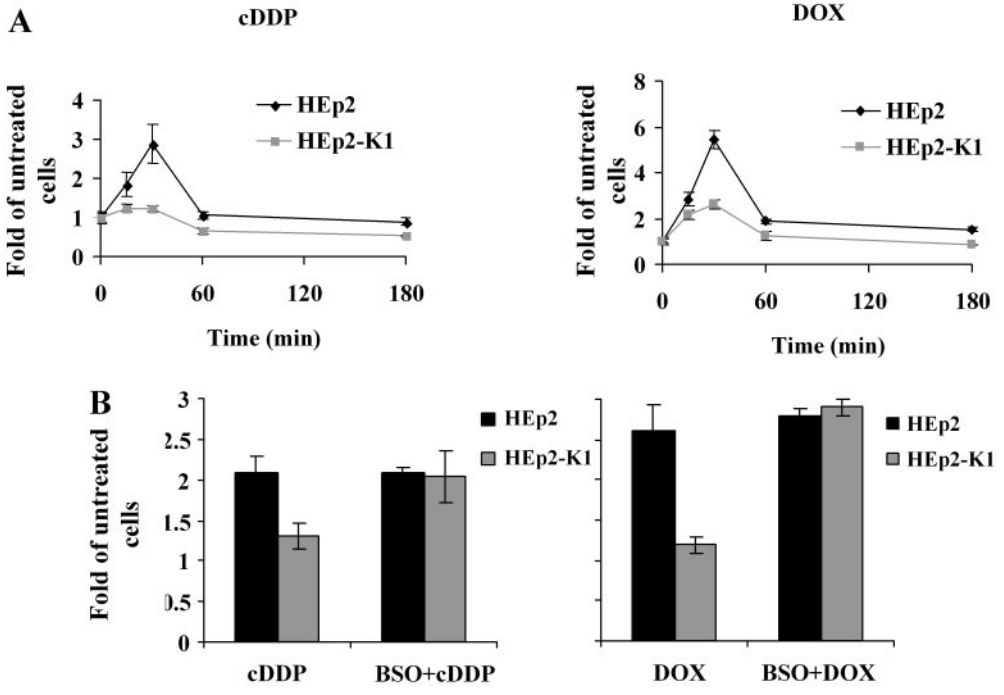

Fig. 7. The suppression of ROS formation after cisplatin and doxorubicin treatment in HEp2-derived $\alpha_v\beta_3$ integrin-expressing cell line HEp2-K1 can be abrogated by GSH depletion. A, measurement of ROS in HEp2 and HEp2-K1 after 2.2 μg/ml cisplatin and 0.4 μg/ml doxorubicin treatment. Data are shown as relative to the untreated HEp2 cells. B, measurement of ROS formation in HEp2 and HEp2-K1 pretreated with 0.01 mM BSO and treated with 2.2 μg/ml cDDP and 0.4 μg/ml DOX. Data are shown as relative to the untreated HEp2 cells.

resistance and that increased expression of GSH does not directly influence expression of Bcl-2. Finally, results obtained by HEp2 stable transfection of a plasmid encoding Bcl-2 and Bcl-2 gene silencing experiments in HEp2-derived $\alpha_v\beta_3$ integrin-expressing cell clone HEp2-K1 further reinforce the conclusion that in $\alpha_v\beta_3$ integrin-expressing HEp2 cells GSH is involved in a resistance mechanism, whereas Bcl-2 is not involved. HEp2-derived Bcl-2 transfectants had a similar level of GSH and similar sensitivity to selected anticancer drugs as HEp2 cells. The silencing of Bcl-2 in HEp2-derived $\alpha_v\beta_3$ integrin-expressing cells HEp2-K1 did not abrogate resistance nor decrease the total amount of GSH in the cell (data not shown). However, it is possible that other changes induced by $\alpha_v\beta_3$ signaling are also involved in multidrug resistance of HEp2-$\alpha_v\beta_3$ integrin-expressing cells. Controversial data exist about the role of Bcl-2 in response of tumor cells to chemotherapeutic drugs: the protective role (Mese et al., 2000) or role toward sensitivity (Beale et al., 2000), suggesting the importance of cell type and cellular context.

In an attempt to determine whether increased detoxification of cisplatin by GSH is involved in cisplatin resistance of HEp2-derived $\alpha_v\beta_3$ integrin-expressing cells, we determined levels of cisplatin bound to DNA. Similar levels of DNA platination found in HEp2 and HEp2-$\alpha_v\beta_3$ integrin-expressing cells suggest that direct detoxification of cisplatin by GSH has no role in resistance. These results are in accordance with those obtained on breast cancer MCF-7 cells, in which overexpression of Bcl-2 and increase in GSH levels were not associated with a change in cisplatin adduct formation (Rudin et al., 2003).

We have found that endogenous levels of ROS are similar in HEp2 and HEp2-$\alpha_v\beta_3$ integrin-expressing cells. Cisplatin and doxorubicin treatment stimulated ROS production in parental HEp2 cell line, whereas in $\alpha_v\beta_3$ integrin-expressing cells we observed increased elimination of ROS that could be abrogated by BSO. Similarly, in doxorubicin resistant mouse leukemic cells (P388) reduced effect of oxidative stress was related to an increase in intracellular GSH level (Furusawa et al., 2001).

Although the involvement of DNA repair mechanisms cannot be completely excluded since we measured platination 4 h after addition of cisplatin, it is more likely that the effect of increased GSH in $\alpha_v\beta_3$ integrin-expressing cells is important for elimination of ROS formed before binding to DNA. Namely, in $\alpha_v\beta_3$ integrin-expressing cells we observed elimination of drug-induced ROS very early after cisplatin or doxorubicin treatment (within 15 to 30 min) that can be abrogated by BSO. Thus, it is more likely that ROS formed through early plasma membrane events triggers cell death (Scheel-Toellner et al., 2004; Dimanche-Boitrel et al., 2005). However, because $\alpha_v\beta_3$ integrin triggers numerous signaling pathways that could result in multidrug resistance, it remains to be determined the exact mechanism.

The relationship between Bcl-2 and GSH is not clearly understood. Voehringer et al. (1998) showed that Bcl-2 overexpression causes redistribution of GSH to the nucleus. Furthermore, overexpression of Bcl-2 may increase GSH levels by binding and inhibition of membrane GSH transporters, thereby increasing net intracellular GSH levels (Meredith et al., 1998). Rudin et al. (2003) reported recently on MCF-7 breast cancer cells that Bcl-2-mediated cisplatin resistance is associated with up-regulation of GSH concentration. In contrast, Schor et al. (2000) showed that Bcl-2-overproducing MCF-7 breast cancer cells demonstrate neither altered GSH processing nor increased detoxification of chemotherapeutic drugs. In cholangiocytes, GSH depletion down-regulated Bcl-2 by reducing its half-life (Celli et al., 1998), whereas in human colon cancer cells, NAC-induced elevation in GSH level was accompanied with increased Bcl-2 expression (Ho et al., 1997). D'Alessio et al. (2004) isolated two tumor cell lines (from U937 and HEpG2 cells) resistant to BSO treatment that up-regulate Bcl-2 in response to BSO. We did not observe difference in Bcl-2 expression after GSH depletion or after increase in GSH levels by NAC treatment in human laryngeal carcinoma cells (data not shown), suggesting no interaction between Bcl-2 and GSH in HEp2 cells and presence of at least two signaling pathways triggered by $\alpha_v\beta_3$ integrin.

We have to emphasize that $\alpha_v\beta_3$ integrin is activated during our experiments performed in standard cell culture conditions. We performed the measurement of drug resistance on vitronectin-coated plates, but we did not observe any difference (data not shown). It is very likely that HEp2 cells synthesize vitronectin, the natural ligand of $\alpha_v\beta_3$. Therefore, there is always enough vitronectin to activate signal transduction pathway triggered by $\alpha_v\beta_3$ expressed on the cell surface.

We have observed the dose-response effect of $\alpha_v\beta_3$ integrin expression at the level of downstream molecules: 1) Bcl-2 whose up-regulation is the consequence of $\alpha_v\beta_3$ expression but is not implicated in resistance and 2) the total amount of GSH that is directly implicated in drug resistance. We did not observe dose response between $\alpha_v\beta_3$ integrin expression and cell survival. There are two possible explanations to this effect: 1) There might be a threshold level of integrin expression required for drug resistance, and all of HEp2-derived $\alpha_v\beta_3$ integrin clones, although expressing different integrin levels, could have this critical $\alpha_v\beta_3$ integrin threshold expression. Apart from the threshold aspect, integrin-mediated resistance might be characterized by a kinetic that very quickly enters saturation, meaning with higher expression there is no increase in cell resistance. 2) We have found that cell clones with very high amount of $\alpha_v\beta_3$ (clones HEp2-K9, HEp2-K24, and HEp2-K19 described in Ambriović-Ristov et al., 2004) are not drug-resistant. The $\alpha_v\beta_3$ integrin-expressing cells were obtained by the transfection with plasmid expressing β_3 subunit that leads to competition of β_3 with β_5 for available α_v (simultaneously, we did not find $\alpha v\beta 1$ in HEp2 cells; our unpublished data). It is possible that, by applied method, undetectable small down-regulation of $\alpha_v\beta_5$ integrin in HEp2-K16 and HEp2-K1 nevertheless occurred that limits the increase of cell survival due to increase in $\alpha_v\beta_3$ expression. Namely, we observed strong down-regulation of $\alpha_v\beta_5$ in cell clones with very high expression of $\alpha_v\beta_3$ integrin (Ambriović-Ristov et al., 2004). Thus, because even small changes in $\alpha_v\beta_3$ integrin expression may influence cell survival, as observed for HEp2-K4, it is possible that also very small changes in $\alpha_v\beta_5$ expression are an important determinant of cell sensitivity to anticancer drugs.

Increased expression of $\alpha_v\beta_3$ integrin has been shown to be a marker for metastatic potential (Felding-Habermann, 2003), also its expression is very important in cancer gene therapy using adenovirus vectors (Majhen and Ambriović-

Ristov, 2006). Namely, $\alpha_v\beta_3$ integrin is the internalization receptor for human adenovirus type 5 (Ad5) that can augment Ad5 transduction efficacy (Ambriović-Ristov et al., 2004). Therefore, cells with increased metastatic potential or cells resistant to anticancer drugs, due to the up-regulation of $\alpha_v\beta_3$ integrin, could be transduced with Ad5 more efficiently than primary tumors. Moreover, Ling et al. (2002) have shown that human embryonic kidney 293 cells expressing higher amounts of $\alpha_v\beta_3$ integrin give higher yields of Ad5, very likely because of increased infectivity and $\alpha_v\beta_3$ integrin-mediated survival signaling pathway that prolongs adenoviral production during infection. This is particularly important for oncolytic adenoviruses, which are currently considered as very promising tools for cancer gene therapy.

In conclusion, several anticancer drugs currently used for cancer treatment have been shown to cause increased cellular ROS generation. Our findings that resistance in $\alpha_v\beta_3$ integrin-expressing cells to cisplatin, doxorubicin, and mitomycin C is caused by increased levels of GSH and consequently increased elimination of drug-induced ROS may have important therapeutic implications. Since we have previously identified increased expression of $\alpha_v\beta_3$ integrin in cisplatin-resistant laryngeal carcinoma cells, it remains to be determined whether a similar mechanism of integrin-mediated drug resistance exists in vivo.

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References

Ambriović-Ristov A and Osmak M (2006) Integrin-mediated drug resistance. *Curr Signal Transduct Ther* **1:**227–237.
Ambriović-Ristov A, Gabrilovac J, Cimbora-Zovko T, and Osmak M (2004) Increased adenoviral transduction efficacy in human laryngeal carcinoma cells resistant to cisplatin is associated with increased expression of integrin alphavbeta3 and coxsackie adenovirus receptor. *Int J Cancer* **110:**660–667.
Beale PJ, Rogers P, Boxall F, Sharp SY, and Kelland LR (2000) BCL-2 family protein expression and platinum drug resistance in ovarian carcinoma. *Br J Cancer* **82:**436–440.
Brassard DL, Maxwell E, Malkowski M, Nagabhushan TL, Kumar CC, and Armstrong L (1999) Integrin alpha(v)beta(3)-mediated activation of apoptosis. *Exp Cell Res* **251:**33–45.
Brooks PC, Montgomery AM, Rosenfeld M, Reisfeld RA, Hu T, Klier G, and Cheresh DA (1994) Integrin alpha v beta 3 antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. *Cell* **79:**1157–1164.
Celli A, Que FG, Gores GJ, and LaRusso NF (1998) Glutathione depletion is associated with decreased Bcl-2 expression and increased apoptosis in cholangiocytes. *Am J Physiol* **275:**G749–G757.
D'Alessio M, Cerella C, Amici C, Pesce C, Coppola S, Fanelli C, De Nicola M, Cristofanon S, Clavarino G, Bergamaschi A, et al. (2004) Glutathione depletion up-regulates Bcl-2 in BSO-resistant cells. *FASEB J* **18:**1609–1611.
Damiano JS (2002) Integrins as novel drug targets for overcoming innate drug resistance. *Curr Cancer Drug Targets* **2:**37–43.
Danen EH (2005) Integrins: regulators of tissue function and cancer progression. *Curr Pharm Des* **11:**881–891.
Dimanche-Boitrel MT, Meurette O, Rebillard A, and Lacour S (2005) Role of early plasma membrane events in chemotherapy-induced cell death. *Drug Resist Updat* **8:**5–14.
Estrela JM, Ortega A, and Obrador E (2006) Glutathione in cancer biology and therapy. *Crit Rev Clin Lab Sci* **43:**143–181.
Felding-Habermann B (2003) Integrin adhesion receptors in tumor metastasis. *Clin Exp Metastasis* **20:**203–213.
Furusawa S, Kimura E, Kisara S, Nakano S, Murata R, Tanaka Y, Sakaguchi S, Takayanagi M, Takayanagi Y, and Sasaki K (2001) Mechanism of resistance to oxidative stress in doxorubicin resistant cells. *Biol Pharm Bull* **24:**474–479.
Hentze H, Schmitz I, Latta M, Krueger A, Krammer PH, and Wendel A (2002) Glutathione dependence of caspase-8 activation at the death-inducing signaling complex. *J Biol Chem* **277:**5588–5595.
Ho YS, Lee HM, Mou TC, Wang YJ, and Lin JK (1997) Suppression of nitric oxide-induced apoptosis by N-acetyl-L-cysteine through modulation of glutathione, bcl-2, and bax protein levels. *Mol Carcinog* **19:**101–113.
Hockenbery DM, Oltvai ZN, Yin XM, Milliman CL, and Korsmeyer SJ (1993) Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell* **75:**241–251.
Kloft C, Appelius H, Siegert W, Schunack W, and Jaehde U (1999) Determination of platinum complexes in clinical samples by a rapid flameless atomic absorption spectrometry assay. *Ther Drug Monit* **21:**631–637.
Kozlova NI, Morozevich GE, Chubukina AN, and Berman AE (2001) Integrin alphavbeta3 promotes anchorage-dependent apoptosis in human intestinal carcinoma cells. *Oncogene* **20:**4710–4717.
Kozlova NI, Morozevich GE, Shtil AA, and Berman AE (2004) Multidrug-resistant tumor cells with decreased malignancy: a role for integrin alphavbeta3. *Biochem Biophys Res Commun* **316:**1173–1177.
Lee M, Hyun DH, Marshall KA, Ellerby LM, Bredesen DE, Jenner P, and Halliwell B (2001) Effect of overexpression of BCL-2 on cellular oxidative damage, nitric oxide production, antioxidant defenses, and the proteasome. *Free Radic Biol Med* **31:**1550–1559.
Ling WL, Longley RL, Brassard DL, Armstrong L, and Schaefer EJ (2002) Role of integrin alphaVbeta3 in the production of recombinant adenoviruses in HEK-293 cells. *Gene Ther* **9:**907–914.
Majhen D and Ambriović-Ristov A (2006) Adenoviral vectors–how to use them in cancer gene therapy? *Virus Res* **119:**121–133.
Matter ML and Ruoslahti E (2001) A signaling pathway from the alpha5beta1 and alpha (v) beta3 integrins that elevates bcl-2 transcription. *J Biol Chem* **276:**27757–27763.
Meredith MJ, Cusick CL, Soltaninassab S, Sekhar KS, Lu S, and Freeman ML (1998) Expression of Bcl-2 increases intracellular glutathione by inhibiting methionine-dependent GSH efflux. *Biochem Biophys Res Commun* **248:**458–463.
Mese H, Sasaki A, Alcalde RE, Nakayama S, and Matsumura T (2000) Regulation of apoptosis reduction in the cisplatin-resistant A431 cell line by Bcl-2 and CPP32. *Chemotherapy* **46:**69–76.
Mirkovic N, Voehringer DW, Story MD, McConkey DJ, McDonnell TJ, and Meyn RE (1997) Resistance to radiation-induced apoptosis in Bcl-2-expressing cells is reversed by depleting cellular thiols. *Oncogene* **15:**1461–1470.
Miyajima A, Nakashima J, Yoshioka K, Tachibana M, Tazaki H, and Murai M (1997) Role of reactive oxygen species in cis-dichlorodiammineplatinum-induced cytotoxicity on bladder cancer cells. *Br J Cancer* **76:**206–210.
Montgomery AM, Reisfeld RA, and Cheresh DA (1994) Integrin alpha v beta 3 rescues melanoma cells from apoptosis in three-dimensional dermal collagen. *Proc Natl Acad Sci U S A* **91:**8856–8860.
Müller I, Niethammer D, and Bruchelt G (1998) Anthracycline-derived chemotherapeutics in apoptosis and free radical cytotoxicity. *Int J Mol Med* **1:**491–494.
Osbild S, Brault L, Battaglia E, and Bagrel D (2006) Resistance to cisplatin and adriamycin is associated with the inhibition of glutathione efflux in MCF-7-derived cells. *Anticancer Res* **26:**3595–3600.
Osmak M, Beketic-Oreskovic L, Matulic M, and Soric J (1993) Resistance of human larynx carcinoma cells to cisplatin, gamma-irradiation and methotrexate does not involve overexpression of c-myc or c-Ki-ras oncogenes. *Mutat Res* **303:**113–120.
Pagano G (2002) Redox-modulated xenobiotic action and ROS formation: a mirror or a window? *Hum Exp Toxicol* **21:**77–81.
Rudin CM, Yang Z, Schumaker LM, VanderWeele DJ, Newkirk K, Egorin MJ, Zuhowski EG, and Cullen KJ (2003) Inhibition of glutathione synthesis reverses Bcl-2-mediated cisplatin resistance. *Cancer Res* **63:**312–318.
Scheel-Toellner D, Wang K, Craddock R, Webb PR, McGettrick HM, Assi LK, Parkes N, Clough LE, Gulbins E, Salmon M, et al. (2004) Reactive oxygen species limit neutrophil life span by activating death receptor signaling. *Blood* **104:**2557–2564.
Schor NF, Rudin CM, Hartman AR, Thompson CB, Tyurina YY, and Kagan VE (2000) Cell line dependence of Bcl-2-induced alteration of glutathione handling. *Oncogene* **19:**472–476.
Thomadaki H and Scorilas A (2006) BCL2 family of apoptosis-related genes: functions and clinical implications in cancer. *Crit Rev Clin Lab Sci* **43:**1–67.
Tietze F (1969) Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal Biochem* **27:**502–522.
Voehringer DW, McConkey DJ, McDonnell TJ, Brisbay S, and Meyn RE (1998) Bcl-2 expression causes redistribution of glutathione to the nucleus. *Proc Natl Acad Sci U S A* **95:**2956–2960.

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