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Bcl-2 Phosphorylation in a Human Breast Carcinoma Xenograft: A Common Event in Response to Effective DNA-Damaging Drugs

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ABSTRACT. A variety of cytotoxic agents effective as antitumor drugs are known to kill tumor cells through induction of apoptosis as the most relevant modality of cell death. A specific role for the protein Bcl-2 in the cell death pathway induced by antimicrotubule agents has been proposed, because Bcl-2 phosphorylation occurs in response to microtubule damage. In this study, we compared efficacy, apoptosis, and Bcl-2 phosphorylation in the Bcl-2-overexpressing MX-1 human breast carcinoma xenograft after treatment with cytotoxic agents characterized by different mechanisms of action. We demonstrated that, in addition to antimicrotubule agents, effective DNA-damaging agents were also able to induce Bcl-2 phosphorylation irrespective of the type of genotoxic lesion. A comparison of effects of drugs belonging to the same class but endowed with a different antitumor activity (i.e. cisplatin versus a novel multinuclear platinum complex and doxorubicin versus a disaccharide analogue) showed a correlation between drug efficacy, apoptotic response, and Bcl-2 phosphorylation. In conclusion, overexpression of Bcl-2 did not counteract the apoptotic effects of a number of cytotoxic agents and could not be regarded as a mechanism of cellular resistance. Since Bcl-2 phosphorylation is a common event in response to different types of cytotoxic damage and is not only related to microtubule dysfunction, we suggest that many cell death pathways converge on Bcl-2 and protein phosphorylation is a step of the signaling cascade activated by diverse stimuli and likely related to the onset of drug-induced apoptosis. BIOCHEM PHARMACOL **60**;1:77–82, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. Bcl-2; tumor xenografts; anthracycline; taxane; cisplatin; apoptosis

Bcl-2 is recognized to play a critical role in the regulation of apoptosis. The Bcl-2 protein is localized primarily in the mitochondrial outer membrane. The protective action of Bcl-2 against diverse cytotoxic insults has been related to its ability to prevent apoptosis [1]. In fact, in several cell systems, prolonged cell survival is observed when elevation in the level of Bcl-2 protein is achieved through gene transfer [2], whereas an antisense-mediated reduction in *bcl-2* gene expression has been shown to accelerate the rate of cell death [3, 4]. In human cancers resistant to a variety of anticancer drugs, Bcl-2 overexpression has often been reported, suggesting a protective role of the protein from drug-induced apoptosis [5]. The role of Bcl-2 in regulating sensitivity to antitumor drugs has not been conclusively defined. Bcl-2 function has been described to be regulated through phosphorylation, and loss of Bcl-2 antiapoptotic function has been observed in lymphoid cells following protein phosphorylation [6]. Several potential phosphorylation sites in the Bcl-2 molecule have been described [7].

In human cancer cells of different tumor types, Bcl-2 phosphorylation followed by cell death has been reported after treatment with taxol or taxotere [8, 9]. The ability of antimicrotubule agents to induce phosphorylation of Bcl-2 has been ascribed to their peculiar interaction with the cellular target and to mitotic arrest [9]. However, in a human mammary tumor xenograft, Bcl-2 phosphorylation and down-regulation associated with apoptosis induction have also been described after treatment with cisplatin, a DNA-interacting drug [10]. Since apoptosis induction is a common response to a variety of cellular injuries induced by most antitumor drugs [11, 12], drug capability to modulate Bcl-2 function and/or expression may represent a critical event underlying the chemosensitivity resistance status of tumor cells [11].

Thus, the aim of the present study was to investigate the relationship between Bcl-2 phosphorylation in a human tumor xenograft and tumor response to treatment with antitumor agents of diverse mechanisms of action. The MX-1 mammary carcinoma was chosen for the study because it is characterized by differential responsiveness to conventional cytotoxic agents [13] and exhibits a marked apoptotic response to cisplatin, in spite of Bcl-2 overexpression [10]. Also included in the study were established and investigational antitumor compounds that belong to different mechanistic classes (topoisomerase II inhibitors, microtubule-interacting agents, and DNA-interacting agents). Our results indicated that Bcl-2 phosphorylation was always

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associated with an effective treatment irrespective of the type of cellular injury, since both DNA-damaging and antimicrotubule agents were effective in modulation of Bcl-2.

MATERIALS AND METHODS

Drugs

Clinically established and investigational drugs of various mechanistic classes were used throughout the study: i.e. paclitaxel, docetaxel, and 13-(*N*-Boc-β-isobutylisoserinyl)-14β-hydroxybaccatin III 1,14-carbonate (IDN 5109, Indena SpA) as tubulin-interacting drugs; cisplatin (Pharmacia & Upjohn) and the new trinuclear platinum compound *trans*-{bis[*trans*-diamminechloroplatinum(μ-1,6-hexanediamine)]} diammineplatinum tetranitrate salt (BBR 3464, Boehringer Mannheim) as DNA-interacting drugs; doxorubicin and 4-demethoxy-7-*O*-[2,6-dideoxy-4-*O*-(2,3,6-trideoxy-3-amino-α-L-*lyxo*-hexopyranosyl)-α-L-*lyxo*-hexopyranosyl]-adriamycinone (MEN 10755, Menarini SpA) as topoisomerase II inhibitors. Cisplatin and BBR 3464 were dissolved in saline; doxorubicin and MEN 10755 in distilled water; paclitaxel, docetaxel, and IDN 5109 in EtOH + cremophor + saline (5:5:90). All drugs were delivered intravenously (i.v.) in a volume of 10 mL/kg of mouse body weight.

Animals

Six- to ten-week-old female athymic nude CD-1 mice were used in the study. Animals (Charles River) were kept in laminar air-flow rooms. Sterilized cages, bedding, and acidified water were used for mice care. The air was maintained at a temperature of 24–26° and 50% humidity. The experiments were approved by the Ethics Committee of our Institute, according to UKCCCR guidelines [14].

Tumor

The MX-1 human mammary tumor line, from an infiltrating duct cell breast carcinoma of an untreated patient, was established s.c. in nude mice at the National Cancer Institute (NCI, Bethesda, MD, U.S.A.). For *in vivo* line maintenance and experimental purposes, tumor specimens were grafted s.c. in both flanks of mice by a 13-gauge trocar. Growth of s.c. tumors was followed by biweekly caliper measurements of length and width. Tumor weight (TW) was calculated using the formula: TW (mg) = tumor volume (mm^3) = width × width × length/2.

Chemotherapy Studies

Mice were grafted s.c. with MX-1 tumor fragments in both flanks. Each experimental group consisted of at least 8 assessable tumors. Mice were treated i.v. according to the doses and the schedules which in our experience are considered as optimal for each drug, i.e. every 7th day for 3 times (q7dx3) for BBR 3464 and doxorubicin; every 3rd/4th day for 5 times (q3/4dx5) for MEN 10755; and every 4th day for 4 times (q4dx4) for taxanes. Cisplatin was delivered only once due to the oversensitivity of the tumor. Tumor growth curves were drawn by plotting mean TW versus day of measurement. Treatment efficacy was evaluated as TW inhibition % (TWI%*) according to the formula TWI% = 100 − (tW/cW × 100), where tW is the mean TW of treated tumors and cW that of control tumors. Reduction in body weight due to treatment never exceeded 10%.

Apoptosis Induction

Mice bearing MX-1 tumors were treated i.v. according to the best therapeutic regimen of each drug (see figures). Twenty-four hours after the last drug treatment, mice were killed and their tumors excised and fixed in 4% buffered formaldehyde. Details of tumor processing for apoptotic index assessment have already been reported [15]. Briefly, tissue sections were treated for the TUNEL (TdT-mediated dUTP nick end labeling) reaction (Boehringer Mannheim). Sections were counterstained with hematoxylin. The apoptotic index, expressed as percentage, was determined by counting the number of apoptotic nuclei/field using a light microscope at 400× magnification. Ten fields of non-necrotic areas were selected in each section and two tumors/group were examined. Mean values were used for calculation. Standard error never exceeded 20%. The mean number of cells/field was around 250.

Western Blot Analysis

Mice bearing established tumors (200–300 mg) were treated once i.v. with a dose level corresponding to the single dose used for chemotherapy studies in which multiple treatments were delivered. One and four days after the drug treatment, one mouse per group was killed by cervical dislocation; its two tumors were removed, minced, and suspended in 500 μL of Laemmli buffer [16]. Suspensions were completely disrupted using a tissue glass potter and successively sonicated for 1 min at low intensity. Protein extract aliquots of 80 μg were size-separated by SDS–PAGE (12% gel) and transferred to a nitrocellulose filter. Equal loading was routinely verified by staining the filters with Ponceau red. Immunodetection was carried out using an anti-Bcl-2 polyclonal antibody (Dako), and development was carried out as previously described [10]. The phosphorylated form of Bcl-2 protein was detected as a slower mobility form.

RESULTS

For antitumor activity studies, all drugs were delivered i.v. by repeated treatments according to the optimal schedule

* *Abbreviation:* TWI, tumor weight inhibition.

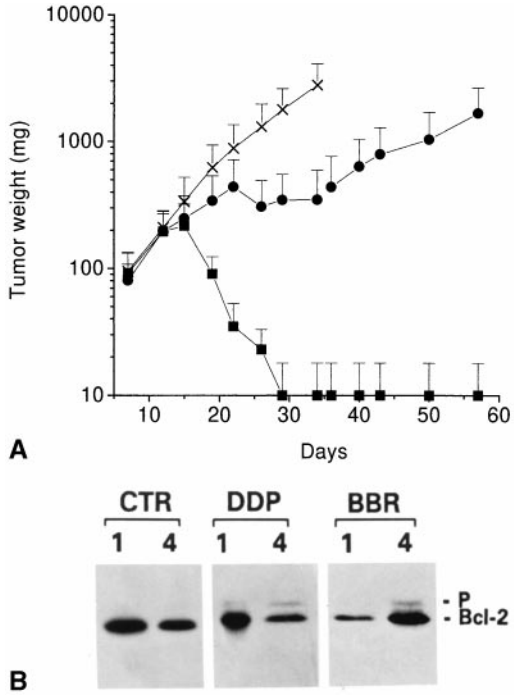

FIG. 1. (A) Growth curves of MX-1 tumor untreated (×) or treated i.v. with cisplatin (6 mg/kg, day 12) (■) or BBR 3464 (0.35 mg/kg, days 12, 19, 26) (●). Mean tumor weight and standard deviation (vertical lines) are reported. (B) Western blot analysis of Bcl-2 protein. P indicates the band corresponding to the phosphorylated protein. CTR, DDP, and BBR indicate untreated, cisplatin-, and BBR 3464-treated tumors. Lanes 1 and 4 correspond to day 1 and 4 after drug treatment.

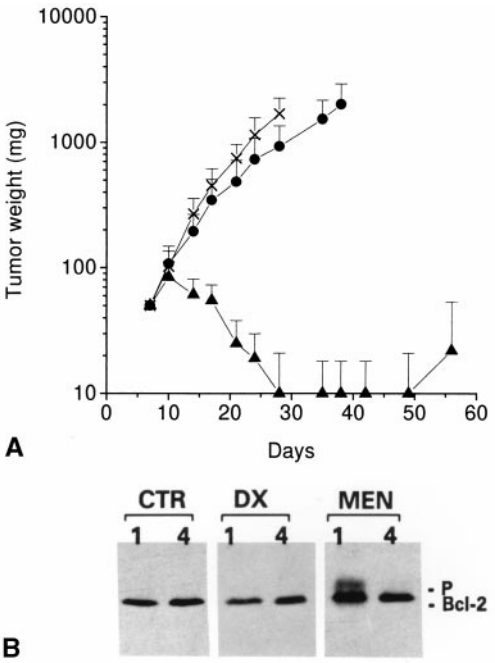

FIG. 2. (A) Growth curves of MX-1 tumor untreated (×) or treated i.v. with doxorubicin (7 mg/kg, days 7, 14, 21, q7dx3) (●) or MEN 10755 (6 mg/kg, days 7, 10, 14, 17, 21, q3–4dx5) (▲). Mean tumor weight and standard deviation (vertical lines) are reported. (B) Western blot analysis of Bcl-2 protein. P indicates the band corresponding to the phosphorylated protein. CTR, DX, and MEN indicate untreated, doxorubicin-, and MEN 10755-treated tumors. Lanes 1 and 4 correspond to day 1 and 4 after drug treatment.

for each drug (see figures). In an attempt to establish a correlation between tumor response and modulation of Bcl-2, drug-induced modification of Bcl-2 was examined 1 and 4 days after treatment with a single effective dose. A cumulative effect of multiple treatments (used in antitumor efficacy studies and apoptosis induction experiments) could generate confusion in the interpretation of the biochemical response, since an overlapping of early and delayed phosphorylation could occur in response to specific drugs (see below).

Figure 1A shows the growth curves of MX-1 tumor treated with cisplatin or BBR 3464, a trinuclear platinum compound. BBR 3464 (0.35 mg/kg) was delivered q7dx3, whereas cisplatin (6 mg/kg) was delivered only once, due to its high efficacy against the tumor [10, 13]. Differently from cisplatin, which achieves complete tumor regression, BBR 3464 only inhibited tumor growth (TWI% = 88). Western blot analysis of Bcl-2 expression showed that both platinum compounds were capable of inducing Bcl-2 phosphorylation. However, the time–course of this phenomenon was different for the two platinum complexes. A delayed effect was induced by BBR 3464, which showed Bcl-2 phosphorylation at day 4 (Fig. 1B). At this time, cisplatin treatment was also associated with a down-regulation of the protein. A different pattern of response was observed following BBR 3464 treatment, which caused down-regulation at day 1.

The growth curves of MX-1 tumors treated with doxorubicin or MEN 10755, a novel disaccharide anthracycline, are reported in Fig. 2A. Doxorubicin (7 mg/kg) was delivered q7dx3 and MEN 10755 (6 mg/kg) q3/4dx5. The new analogue was highly effective on the tumor xenograft, since it achieved a complete tumor regression and cured 63% of mice. In contrast, doxorubicin produced less than 50% TWI. Western blot analysis of Bcl-2 indicated that MEN 10755 was able to induce an early phosphorylation at day 1, whereas doxorubicin did not cause modification of the protein (Fig. 2B).

Drugs belonging to the taxane class were also investigated as reference compounds [8, 9]. The results of their antitumor efficacy against the MX-1 tumor are reported in Fig. 3A. Paclitaxel (36 mg/kg), docetaxel (18 mg/kg), and the new more soluble analogue IDN 5109 (60 mg/kg) were delivered i.v. q4dx4. All three drugs proved to be very effective against the tumor, since complete tumor regression was achieved in 100% of mice. As expected, all taxanes induced Bcl-2 phosphorylation at day 1. The effect lasted up to day 4 for docetaxel and IDN 5109 (Fig. 3B).

To better understand if the differential antitumor effect achieved by the two platinum compounds or by the two anthracyclines was related to a different drug ability to induce apoptosis, the apoptotic index was measured 24 hr after the complete therapeutic regimen of each drug had been delivered (see doses and schedules in Figs. 1–3). Cisplatin was delivered in 3 repeated injections of 2 mg/kg

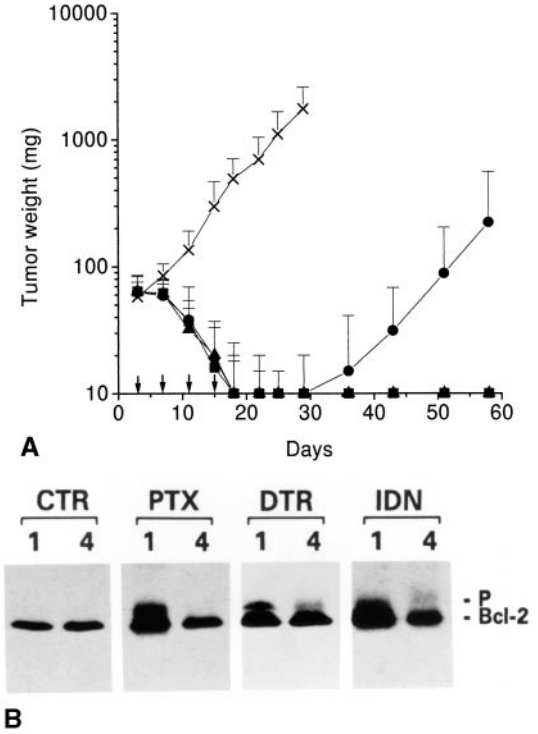

FIG. 3. (A) Growth curves of MX-1 tumor untreated (×) or treated i.v. with paclitaxel (36 mg/kg) (■), docetaxel (18 mg/kg) (▲), or IDN 5109 (60 mg/kg) (●). Days of treatment for all drugs were 3, 7, 11, 15 (q4dx4). Mean tumor weight and standard deviation (vertical lines) are reported. (B) Western blot analysis of Bcl-2 protein. P indicates the band corresponding to the phosphorylated protein. CTR, PTX, DTR, and IDN indicate untreated, paclitaxel-, docetaxel-, and IDN 5109-treated tumors. Lanes 1 and 4 correspond to day 1 and 4 after drug treatment.

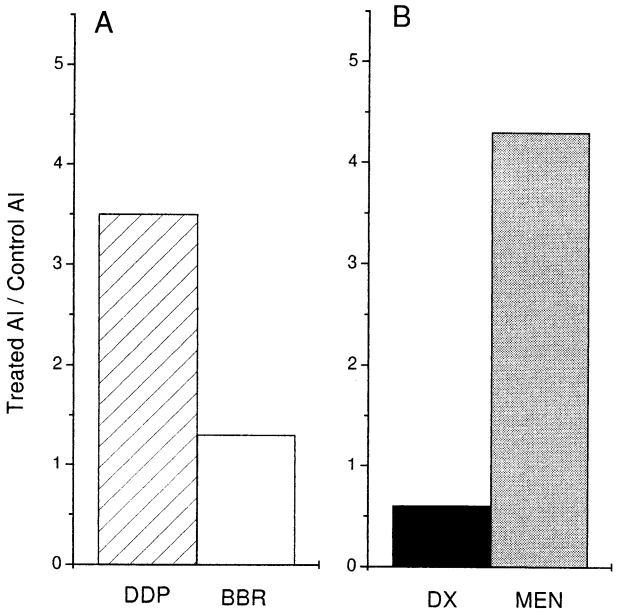

FIG. 4. Ratio between apoptotic indices (AI) of MX-1-treated versus control tumors. Treatments were delivered i.v., q7dx3 for cisplatin (DDP, 2 mg/kg), BBR 3464 (BBR, 0.35 mg/kg), and doxorubicin (DX, 7 mg/kg) and q3-4dx5 for MEN 10755 (MEN, 6 mg/kg). The apoptotic index in controls was 0.45 ± 0.07 (mean ± S.E.) for A and 1.45 ± 0.3 for B.

every 7th day, instead of a single injection of 6 mg/kg, to allow a comparison with the same schedule used for BBR 3464. The antitumor efficacy of cisplatin, 6 mg/kg, as a single dose or cumulative dose was comparable (data not shown). The ratio between apoptotic index values in treated over control tumors is reported in Fig. 4. The apoptotic effects of the most effective drugs, cisplatin and MEN 10755, were more marked than those of the less effective compounds, BBR 3464 and doxorubicin, respectively.

DISCUSSION

The basal level of Bcl-2 protein expression is not necessarily correlated with susceptibility to drug-induced apoptosis [17]. Lack of correlation between resistance and Bcl-2 expression was also found in the MX-1 mammary carcinoma which, in spite of a high basal Bcl-2 expression, was extremely sensitive to many antitumor drugs that caused complete regression of tumors. This was the case for cisplatin, MEN 10755, and taxanes. Therefore, Bcl-2 expression is not per se a determinant of tumor resistance to cytotoxic agents, but should be regarded as part of a complex interplay between regulatory proteins, eventually resulting in cell death. This interpretation is consistent with clinical observations regarding the predictive significance of Bcl-2 in the treatment of breast carcinoma [18].

Bcl-2 phosphorylation is recognized as a regulatory process, but its functional implications remain controversial. Treatment with paclitaxel in lymphoid and prostatic carcinoma cells resulted in protein phosphorylation and apoptosis induction [6, 8], and the effects were related to the peculiar mechanism of action of the taxane, i.e. alteration of microtubule function [9]. In HeLa cells treated with paclitaxel, Bcl-2 phosphorylation was reported to be a marker of M-phase arrest rather than an event associated with the cell death process [19]. The relation between prolonged phosphorylation of Bcl-2 and stimulation of apoptosis remains unclear [19, 20]. The results of our study indicate that Bcl-2 phosphorylation is a general event occurring during cell response to efficacious therapies and is not necessarily related to drug–microtubule interaction. We provide indirect evidence that the cellular pathway(s) resulting in Bcl-2 phosphorylation can be activated by cytotoxic stimuli other than disruption of microtubule dynamics. Indeed, a number of DNA-damaging agents effective against MX-1 tumor, such as platinum compounds and MEN 10755, were also able to induce Bcl-2 phosphorylation. The phosphorylation of the protein was also observed *in vitro* in the small cell lung cancer cell line POGB, 24 hr after treatment with cytotoxic concentrations of genotoxic agents causing a marked apoptotic response (data not shown). Of course, it is difficult to establish a

precise relationship between Bcl-2 phosphorylation and drug-induced apoptosis, since the time–course of the two events are expected to be different (Bcl-2 modulation is an early event preceding the onset of apoptosis). However, the use of agents with variable efficacy supports a mechanism involving Bcl-2 in the signaling cascade, resulting in cell death rather than in cellular response to microtubule damage.

The multinuclear platinum complex BBR 3464 induced a delayed protein phosphorylation as compared to cisplatin. It is likely that the somewhat different behavior of cisplatin and the multinuclear platinum complex reflects a different p53-dependent response to these complexes [21]. Indeed, in a human osteosarcoma cell line (U2-OS) carrying, like MX-1 tumor, a functional wild-type *p53*, the up-regulation of p53 following exposure to BBR 3464 was less marked than that induced by cisplatin (data not shown). The hypersensitivity of MX-1 tumor to cisplatin again reflected a marked drug ability to stimulate apoptosis after a single drug administration [10]. The novel triplatinum complex, BBR 3464, a promising compound endowed with a different spectrum of activity, exhibited a lower antitumor activity against MX-1 tumor than did cisplatin. The observation is not surprising, since the presence of a wild-type *p53* in the tumor may represent a resistance factor for BBR 3464 [21, 22]. The partial but persistent inhibition of tumor growth induced by the multinuclear platinum complex (Fig. 1A) is consistent with a delayed Bcl-2 phosphorylation.

Doxorubicin, which exhibited no efficacy against the MX-1 tumor, was unable to induce phosphorylation. The dramatic difference in tumor response to doxorubicin and its disaccharide analogue MEN 10755 has been related to an enhanced ability of the novel derivative to stimulate apoptosis [15] (Fig. 4). Thus, the protein phosphorylation induced by MEN 10755 likely reflected its peculiar feature, although the cellular and molecular basis of the different response to the same DNA lesion (topoisomerase II-mediated DNA cleavage) remains unknown.

All tested DNA-damaging agents are known to cause cell-cycle perturbations different from those of antimicrotubule agents, with accumulation in the G2 phase. In contrast to paclitaxel-induced mitotic arrest, which has been reported in different *in vivo* tumor systems [23], no increase in the mitotic index was detected following treatment with anthracyclines or platinum compounds in the MX-1 tumor (not shown). Thus, this study does not support the notion that Bcl-2 phosphorylation is related to regulation of mitotic events. Instead, our results are consistent with the involvement of Bcl-2 modulation as an indirect downstream event associated with cell death. Such an interpretation does not imply that the event is a major determinant of drug-induced apoptosis.

In conclusion, in the human MX-1 tumor xenograft, Bcl-2 overexpression could not be regarded as a marker of tumor resistance to cytotoxic drugs. Moreover, phosphorylation of the antiapoptotic protein did not occur solely in response to microtubule alterations, since a similar effect was also observed in response to DNA-damaging agents causing different types of DNA lesions. The correlation between antitumor efficacy, apoptotic response, and Bcl-2 phosphorylation supports the hypothesis that the signaling cascade involving Bcl-2 may be activated by different types of cytotoxic damage.

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