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Doxorubicin-Induced Alterations of c-*myc* and c-*jun* Gene Expression in Rat Glioblastoma Cells: Role of c-*jun* in Drug Resistance and Cell Death

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ABSTRACT. We studied the effect of doxorubicin on the expression of c-*myc* and c-*jun* in the rat glioblastoma cell line C6 and its doxorubicin-resistant variant C6 0.5, at equitoxic exposures. For quantitation, the mRNA levels of these oncogenes were related to those of two domestic genes, β-actin and glyceraldehyde phosphate dehydrogenase. After a transient overexpression of the genes during the first hour of incubation, there was a selective, dose-dependent down-regulation of both genes by doxorubicin in the sensitive cells. In the resistant cell line, c-*myc* expression was also decreased in response to doxorubicin incubation, but the expression of c-*jun* remained unchanged over the whole range of concentrations. In contrast, vincristine had no effect on the amounts of c-*myc* and c-*jun* mRNAs in either line. The effect of doxorubicin on the mRNA levels of c-*jun* was also observed on the JUN proteins by immunoblotting, but the MYC protein levels remained unchanged upon doxorubicin treatment. There was a significant correlation between the levels of c-*myc* and c-*jun* gene expression and the degree of growth inhibition induced by doxorubicin. In addition, doxorubicin induced a fragmentation of DNA in sensitive cells, but not in resistant cells, thus revealing a resistance to apoptosis in this line. Doxorubicin-induced cell death did not appear to be mediated by p53 in either cell line. BIOCHEM PHARMACOL **55**;12:1963–1971, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. doxorubicin; DNA-topoisomerase II; DNA damage; nuclear oncogenes; apoptosis

DNA-topoisomerase II is a nuclear enzyme which realizes transient double-strand DNA breaks in order to facilitate the suppression of torsional constraints in the DNA molecules. Such a relaxation is required for replication and transcription of DNA [1]. Several anticancer drugs such as anthracyclines or epipodophyllotoxins are cytotoxic through their interference with DNA-topoisomerase II activity [2]. They prevent DNA strand religation by stabilization of the cleavable DNA-enzyme complex, thus creating DNA breaks leading to cell death. Resistance to topoisomerase II-interfering drugs can be mediated through P-glycoprotein overexpression (multidrug resistance) or through qualitative or quantitative alterations of DNA-topoisomerase II [3]. However, a number of observations cannot be explained by the currently identified mechanisms of resistance, and it has been suggested that, in resistant cells, the DNA breaks leading to cell death could be restricted to hypersensitive genomic sites and not evenly distributed all along the DNA molecule [4]. It has been observed in wild-type MCF-7 cells that there was a dissociation between teniposide- or amsacrine-induced bulk DNA damage and growth inhibition [5, 6], suggesting the existence of genomic sites specifically targeted by these drugs [7]. It has also been shown that the cellular oncogene c-*myc* could be the preferential target of some anticancer drugs in human tumour cell lines [6, 8, 9]. In that context, different mechanisms can be envisaged to explain resistance to topo II-interfering drugs: a lack of accessibility to those specific sites, a qualitative alteration of the sites (mutations, rearrangements, or deletions) leading to an inhibition of the formation of drug-mediated DNA-topoII complexes, or a more efficient repair of those sites as compared to other regions of the genome.

Working on a doxorubicin-resistant cell line, C6 0.5, originating from the rat glioblastoma C6, we reported that *MDR*1 gene overexpression could not explain the complete phenotype of resistance [10]. We later demonstrated that there was a dissociation between DNA breaks and cell survival in the doxorubicin-resistant cell line, resistant cells requiring approximately 2 times more DNA breaks than sensitive cells to achieve similar growth inhibition [11]. In this study, we explored the expression of two early responsive oncogenes, c-*myc* and c-*jun*, in response to topoisomerase II inhibitors, and showed that there is a specific down-regulation of both genes by doxorubicin in the

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† *Abbreviation:* GADPH, glyceraldehyde 3-phosphate dehydrogenase.

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sensitive cells, whereas only c-*myc* expression is altered in resistant cells.

MATERIALS AND METHODS

Drugs and Chemicals

Doxorubicin hydrochloride (Adriblastine®) was obtained from Pharmacia. All other chemicals were of reagent grade.

Cell Lines and Culture Conditions

The C6 rat glioblastoma cell line and its doxorubicin-resistant counterpart C6 0.5 have been described previously [10, 12]. Cultures were maintained in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum and antibiotic mixture at 37° in a 5% CO_2 humidified atmosphere. The C6 0.5 resistant subline was permanently grown in the presence of 0.5 μg/mL of doxorubicin. Before each experiment, cells were grown for one week in the absence of drug.

Growth Inhibition

Two to four days before the experiment, appropriate numbers of cells were seeded in 60 cm^2 Petri dishes (Nunc) with drug-free medium, so as to obtain 2×10^6 cells in the exponential phase of growth on the day of drug treatment. Cells were incubated with increasing concentrations of doxorubicin for 2 hr. Culture medium was then removed, the cell layers were rinsed twice, and cells were further grown for 2.5 cell-doubling times. Cell numbers were then evaluated with a Coulter ZM hemocytometer (Coultronics).

cDNA Probes

The 1.4-kb *Eco*RI-*Cla*I fragment of the human c-*myc* gene, containing the third exon [13], was a generous gift from Dr. J. F. Riou and was used as a probe recognizing a 2.4-kb rat c-*myc* mRNA. Rat c-*jun* mRNAs (2.7 and 3.2 kb) were detected using a 2.6-kb cDNA fragment removed from the mouse JAC.1 clone [14] by digestion with *Eco*RI, which was obtained from American Type Culture Collection. As controls, human β-actin and GAPDH† gene expressions were used. A 3.3-kb *Hind*III fragment was used as a probe for β-actin [15], whereas the 0.78 kb *Pst*I-*Xba*I human cDNA fragment excised from the pHcGAP clone obtained from American Type Culture Collection was used as a probe for GAPDH [16]. cDNA probes were labeled with [α-^{32}P]dCTP (Amersham) using the RPN 1601Z random priming labeling kit (Amersham) according to the manufacturer's instructions.

DNA Extraction and Southern Blot Analysis

Genomic DNA was extracted from cultured cells in phenol/chloroform as described by Sambrook *et al.* [17] and quantified by absorption spectrophotometry at 260 nm. After digestion with *Eco*RI restriction endonuclease (Eurogentec), DNA fragments (20 μg/lane) were fractionated by electrophoresis on 1% (w/v) agarose gels. Gels were treated under denaturing conditions for 20 min (0.5 M of NaOH and 1.5 M of NaCl), and neutralized in a buffer containing 1.5 M of NaCl and Tris 0.5 M of pH 8.0. They were transferred to Hybond N membranes (Amersham) in 20 × SSC (1 × SSC is 150 mM of NaCl, 15 mM of trisodium citrate) at pH 7.0. Membrane prehybridization was performed for 20 hr at 42° in 50% formamide, 5 × SSC, 10 × Denhardt solution, 50 mM of phosphate buffer pH 6.5, 1 mM of EDTA, 0.1% SDS, containing 0.1 mg/mL of Salmon sperm DNA (Sigma). Hybridization was then performed overnight in the same buffer containing the [α-^{32}P]-labeled cDNA probe, previously denatured at 100° for 10 min. After four washes at room temperature in 2 × SSC, 0.1% SDS, membranes were washed twice at 60° for 15 min in 0.1 × SSC, 0.1% SDS. Autoradiography was performed at −70° on Hyperfilm MP (Amersham) with intensifying screens. Autoradiograms were analyzed by densitometric scanning using an electronic dual transilluminator (Bioprobe) with the Densylab® software (Bioprobe).

RNA Extraction and Northern Blot Analysis

Exponentially growing cells were submitted to doxorubicin or vincristine exposures at various concentrations for a duration of 2 or 24 hr. Total cellular RNAs were isolated in guanidine isothiocyanate by cesium chloride ultracentrifugation as described by Sambrook *et al.* [17]. They were resuspended in 10 mM of Tris, pH 7.5, 1 mM of EDTA, 1% SDS and extracted with chloroform/butanol 4/1. They were quantified by absorption spectrophotometry at 260 nm. Total RNAs (20 μg) were size-fractionated by electrophoresis in a 1% agarose gel containing 7% formaldehyde, soaked for 30 min in 20 × SSC and transferred to Hybond N membranes. Transfer, prehybridization, hybridization, washings, and densitometric scanning were carried out as described above.

Nuclear Extraction and Western Blot Analysis

Preparations of nuclear extracts were realized as previously described [18]. Briefly, exponentially growing cells were submitted to 1.7 μM (C6 cells) or 550 μM (C6 0.5 cells) of doxorubicin for 2 or 6 hr, and centrifuged in a cold saline solution containing 0.02% EDTA at 1,500 *g* for 5 min at 4°. The cells were then suspended in ice-cold hypotonic 10 mM of Tris-HCl, pH 7.4, containing 10 mM of NaCl, 3 mM of $MgCl_2$, 0.5% Nonidet P40 [19], and in addition, 10 mM of phenylmethylsulfonyl fluoride, 1 mM of benzamidine, 5 μg/mL of leupeptin and 10 μg/mL of soybean trypsin inhibitor, as proteolysis inhibitors. After cell lysis, nuclei were obtained by centrifugation at 1,500 *g* for 5 min at 4°. The nuclei were resuspended in 1 mL of hypertonic extraction buffer, pH 6.5, consisting of 2 mM of K_2HPO_4, 5 mM of $MgCl_2$, 1 mM of EGTA, 0.1 mM of dithiothreitol,

1 M of NaCl, and containing the proteolysis inhibitors mentioned before. Nuclear proteins were extracted for 60 min at 4°. DNA and nuclear fragments were precipitated by centrifugation at 19,000 *g* for 45 min at 4°. The protein concentration in the supernatant was determined using the Bradford assay, with bovine serum albumin as a standard. The 1 M of NaCl extracts were fractionated after DNase I treatment for 30 min at room temperature and stored at −80° in 30% glycerol until immunoblotting.

The extracts were equalized for protein content and mixed with Laemmli loading buffer [20], loaded onto SDS-polyacrylamide gels, 0.5 mm thick, consisting of 4.5% stacking gel and 10% resolving gel. Proteins were subjected to electrophoresis at 80 V overnight, together with protein molecular weight markers, ranging from 14,300 to 200,000 kDa (Amersham). Proteins were then transferred to Immobilon-P membranes (Millipore) by electroblotting at 2.5 mA/cm^2 for 1.5 hr, using a transfer apparatus (Milliblot Graphite Electroblotter) according to the recommendations of the manufacturer. Blots were preincubated overnight at 4° in 5% nonfat dry milk in PBS containing 0.2% Tween 20. Membranes were then incubated for 2 hr at room temperature, either with a mouse anti-mouse c-JUN protein antibody (Transduction Laboratories) at a 1:500 dilution in PBS, or with a mouse anti-human c-MYC protein monoclonal antibody (Bioproducts) at a 1:10 dilution in PBS. After three 10-min washes in PBS, blots were treated for 1.5 hr at room temperature with an alkaline phosphatase-labeled rabbit antibody against mouse imunoglobulins (Dako) at a 1:200 dilution in PBS. After three 10-min and one 20-min washing in PBS, immunoreactive bands were visualized by incubation for 5–10 min in 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (BCIP/NBT tablets, Sigma) and rinsed with water. Spots were quantified by densitometric scanning as described for nucleic acids.

DNA Fragmentation Assay

Apoptosis is characterized by DNA fragmentation which was quantified by the filter elution method described by Fan *et al.* [21] with some modifications. Cells were labeled with [methyl-^{3}H]thymidine (Amersham; 0.5 μCi/mL of medium) for 1.5–2 doubling times. After a chase period of 20 hr in fresh label-free medium, 1–2 × 10^6 cells in each Petri dish were treated with different doxorubicin concentrations for 6 hr at 37°. The cell monolayers were washed immediately with prewarmed buffered saline solution and reincubated in fresh medium for 48 and 72 hr. Cells were then suspended in culture medium by gentle scraping and centrifuged at 1,500 *g* for 10 min at 4°. A 1-mL aliquot of the medium was transferred to a liquid scintillation vial for radioactivity measure. Cell pellets were resuspended in 1 mL PBS and layered, in Swinnex funnels (Millipore), onto polycarbonate membranes of 25-mm diameter and 2-μM pore size (Nucleopore). The cells were lysed *in situ* for 1 hr in 5 mL of a solution containing 2% SDS, 0.1 M of glycine, 0.025 M of EDTA and 0.5 mg/mL of proteinase K (Boehringer). Filters were then washed with 5 mL of a 0.04-M EDTA solution, pH 10. Fractions and washes were combined and an aliquot was counted for radioactivity. Filters were removed from the funnels heated for 1 hr in 0.1 M of HCl at 78°, cooled at room temperature, put in liquid scintillation vials, and 0.1 M of NaOH was added before counting. Funnels were subsequently washed with 0.5 M of NaOH, and the counts were added to those provided by the filters themselves for a complete estimation of the amount of tritium retained on the filters.

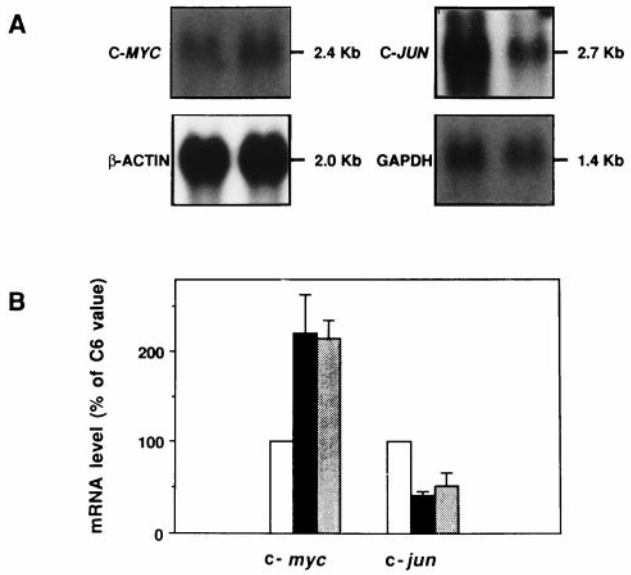

FIG. 1. (A) Basal expression of c-*myc*, c-*jun*, β-actin and GAPDH in C6 sensitive (left) and doxorubicin-resistant (right) cells in a representative experiment. RNAs were extracted and Northern blots (20 μg of total RNA) were performed as described in Materials and Methods. (B) Quantitation of c-*myc* and c-*jun* expression, in C6 sensitive cells (□) and in C6 resistant cells, relative either to the expression of β-actin (■) or to that of GAPDH (shaded box). The levels of transcripts were quantified by densitometric scanning. Values are means ± SD of three independent experiments.

Flow Cytometry

We used flow cytometry both for the quantitation of p53 in C6 and C6 0.5 cells upon doxorubicin exposure, and for a second evaluation of apoptosis in these cells. Cells were incubated with three concentrations of doxorubicin (0.1 × IC_{50}, 1 × IC_{50} and 10 × IC_{50}) for 6 hr at 37°. The cell monolayers were washed immediately with prewarmed PBS and reincubated in fresh medium for 48 hr and 72 hr. Culture medium was then collected and the cell monolayer was removed from the Petri dish with trypsin-EDTA. Cells were centrifuged and washed twice in PBS, then fixed for 30 min in PBS-1% formaldehyde (methanol-free) on ice. After two washes in PBS, cells were resuspended in 2 mL of 70% ethanol on ice and stored at −20° for at least 24 hr. The cells were then centrifuged at 800 *g* for 5 min and rehydrated in PBS for 30 min at room temperature.

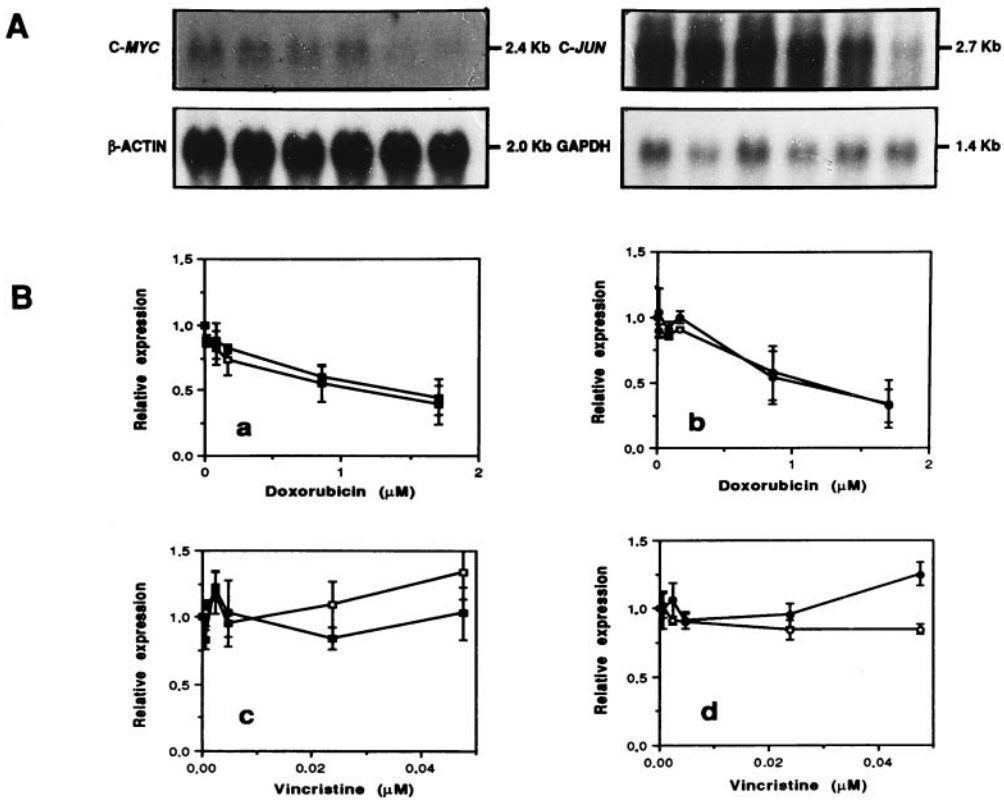

FIG. 2. A) Effects of doxorubicin on expressions of c-*myc*, c-*jun*, β-actin and/or GAPDH in C6 sensitive cells in a representative experiment. From left to right, the concentrations of doxorubicin were the following: 0; 0.017; 0.085; 0.17; 0.85; 1.7 μM, and the duration of incubation was 2 hr. RNAs were extracted and Northern blots (20 μg of total RNA) were performed as described in Materials and Methods. B) Quantitation of doxorubicin and vincristine effects, in C6 sensitive cells, on c-*myc* (a, c) and c-*jun* (b, d) expressions, relative either to β-actin (■) or to GAPDH (□). Values are means ± SD of three independent experiments.

For the evaluation of apoptosis, DNA fragments were end-labeled using the reagents of the Mebstain® Apoptosis kit (obtained from Immunotech). After two washes in PBS-0.2% BSA, each sample was separated into two test tubes. Cells were resuspended in 30 μL of a buffer solution with and without TdT enzyme containing biotine-conjugated deoxyuridine trisphosphate. After 1 hr of incubation at 37°, cells were washed twice and incubated at room temperature for 10 min in blocking reagent and for 30 min in avidine conjugated to fluorescein isothiocyanate. The cell pellet was washed twice and resuspended in 300 μL of a solution of RNase and propidium iodide.

For p53 detection, after fixation in 70% ethanol, cells were washed in PBS then in PBS-1% BSA. One hundred μL of cell suspension adjusted at 10^7 cells/mL were incubated in the presence of monoclonal antibody anti-p53 (clone pAb 122, Neomarkers) for 1 hr at room temperature. An isotypic control was run in parallel. After two washes in PBS-1% BSA, cells were incubated with a goat anti-mouse antibody conjugated to fluorescein isothiocyanate for 30 min at room temperature. After one wash in PBS, cell pellets were resuspended in 300 μL of PBS.

Flow cytometric measurements were performed on a FACScan from Becton-Dickinson using Cell Quest® software for data acquisition and analysis. DNA fragmentation and p53 labeling were measured on the green fluorescence detector, and DNA content on the red fluorescence detector. The green fluorescence signal was collected using a logarithmic scale and the red fluorescence signal using a linear scale. Data acquisition and analysis were performed on 10,000 cells for apoptosis and 5,000 cells for p53. For p53 detection, the differences in fluorescence intensity between isotypic control and test sample were often small, resulting in nondisjunct distributions. Results of p53 were expressed as a ratio of arithmetic mean fluorescence intensity of MAb/arithmetic mean fluorescence intensity of isotypic control. Before use in statistical analysis, the logarithmically amplified signals were converted to linear values. The linear value referred to the dynamic range of signal intensities (1 to 10,000). Two cell lines, HL60 and OCI, were used as negative and positive control for p53 detection, respectively. Their mean fluorescence ratios were 2.0 ± 1.5 and 37 ± 11, respectively.

RESULTS

We evaluated the changes occurring in the mRNA levels of two cellular oncogenes expressed in the C6 glioblastoma line, c-*myc* and c-*jun*, under the action of doxorubicin. Drug concentrations ranged between 0.1- and 10-fold the IC_{50} value (which amounted to 0.17 μM for C6 sensitive cells and 55 μM for C6 0.5 cells). The same was done with vincristine, which was chosen as a control drug inhibiting cell proliferation by a mechanism totally independent of topoisomerase II and DNA damage, and the same range of cytotoxic concentrations was used (from 0.1- to 10-fold the IC_{50} value). The specificity of the changes occurring in mRNA levels of c-*myc* and c-*jun* was assessed by comparison with those of two domestic genes, β-actin and GAPDH.

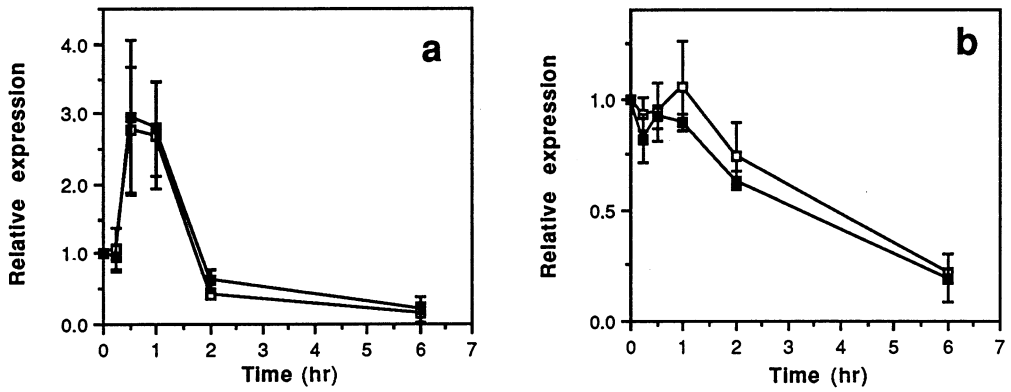

FIG. 3. Quantitation of doxorubicin effects, in C6 sensitive cells, on c-*myc* (a) and c-*jun* (b) expressions, relative either to β-actin (■) or to GAPDH (□), as a function of time. Doxorubicin concentration equaled 10 × IC_{50} (1.7 μM). Values are means ± SD of three independent experiments.

In untreated cells, the oncogenes c-*myc* and c-*jun* were differently expressed in the sensitive and the resistant cell lines (Fig. 1): c-*myc* was two-fold overexpressed in the resistant line, as compared to β-actin and GAPDH expressions, whereas c-*jun* mRNA levels were two-fold reduced. Differences in basal mRNA levels between the cell lines were not due to differences in oncogene copy number (data not shown). In C6 cells, 2-hr exposures to doxorubicin were followed by a marked reduction in c-*myc* and c-*jun* mRNA levels as compared to β-actin or GAPDH expression (Fig. 2B a–b), and this reduction was concentration-dependent: c-*myc* and c-*jun* mRNA levels reached 60% of initial values at 0.17 μM (1 × IC_{50}) ($P < 0.05$) and 30% of initial values at 1.7 μM (10 × IC_{50}) ($P < 0.001$). In contrast, no modification in c-*myc* and c-*jun* mRNA levels could be detected after 24-hr incubations with similarly cytotoxic concentrations of vincristine (Fig. 2B c–d). When studied as a function of time at the highest exposure dose (10 × IC_{50}), c-*myc* expression initially increased during the first hour of incubation (Fig. 3a) and then decreased from the hour 2 onwards, to reach 20% of initial values at 6 hr. The stimulation of c-*jun* expression by doxorubicin was not apparent in C6 cells (Fig. 3b), and the decrease was much more pronounced after 6-hr exposure than after 2-hr exposure. These effects were accompanied by an increased cellular sensitivity to doxorubicin when exposure time was prolonged from 2 to 6 hr (Table 1).

In the C6 0.5 cell line, which was selected from the C6 line by doxorubicin, we also obtained a concentration-dependent reduction in c-*myc* mRNA levels relative to β-actin or GAPDH after 2-hr doxorubicin exposure ($P < 0.001$) (Fig. 4Ba). It should be mentioned that drug concentrations were increased by a factor of 400 in order to bypass P-glycoprotein action and to compare similar intracellular drug levels. In contrast, there was only a minor effect of doxorubicin on c-*jun* mRNA levels, which only decreased by *ca.* 20% at the highest doxorubicin concentration tested ($P < 0.05$) (Fig. 4Bb). As in the sensitive line, no changes in c-*myc* and c-*jun* gene expressions were observed under the action of similarly cytotoxic concentrations of vincristine (Fig. 4B c–d). When studied as a function of exposure time to doxorubicin, there was a transient two-fold increase in c-*myc* and c-*jun* gene expressions, followed from hour 2 onwards either by a marked decrease (c-*myc*) or by a return near the baseline value (c-*jun*) (Fig. 5a–b). In resistant cells, no significant modification of sensitivity to doxorubicin was found after 6 hr vs 2 hr of exposure to the drug (Table 1).

In order to explore whether the modifications of c-*myc* and c-*jun* mRNA levels were translated at the protein level, we quantified, by immunoblotting, the alterations occurring in MYC and JUN proteins upon doxorubicin exposure of both lines, at a dose representing 10 × IC_{50}. A slight decrease in MYC protein was observed in C6 cells after 2 hr, without a significant additional decrease after 6 hr (Fig. 6a). In these cells, the JUN protein also decreased slightly after 2-hr drug exposure, and the decrease was much greater after 6 hr (50% of the protein level of untreated cells, Fig. 6b). In contrast, no modification of either MYC or JUN proteins occurred in the C6 0.5 cell line upon doxorubicin exposure (Fig. 6c–d).

The effect of doxorubicin-induced alterations in gene expression was compared to the effects of this drug on the growth of C6 and C6 0.5 cells. Figure 7 a shows that there was a highly significant correlation between the relative expression of c-*myc* and the logarithm of percent survival of either C6 or C6 0.5 cells. Such a correlation was also found for the relative expression of c-*jun* (Fig. 7b), but only for C6 cells, because the level of c-*jun* mRNAs remained almost unchanged upon doxorubicin exposure of these cells.

In order to test the physiological significance of the decrease in MYC and JUN in C6 cells as compared to C6 0.5 cells, we evaluated DNA fragmentation after 6-hr

TABLE 1. Doxorubicin IC_{50} as a function of exposure time in C6 sensitive and C6 0.5 doxorubicin-resistant cell lines

	Doxorubicin IC_{50} (μM)	
Exposure time	C6 sensitive cells	C6 0.5 resistant cells
2 hr	0.17 ± 0.03 (8)	55.0 ± 9.5 (10)
6 hr	0.041 ± 0.0005* (7)	50.5 ± 6.4 (3)

IC_{50}s (means ± SD) are the doxorubicin concentrations inhibiting cell growth by 50%, as evaluated by cell counting after 2 hr or 6 hr exposure and 2.5 cell cycle regrowth. The number of independent determinations, made in triplicate, is indicated between brackets.

*Significantly different at the $P < 0.001$ level.

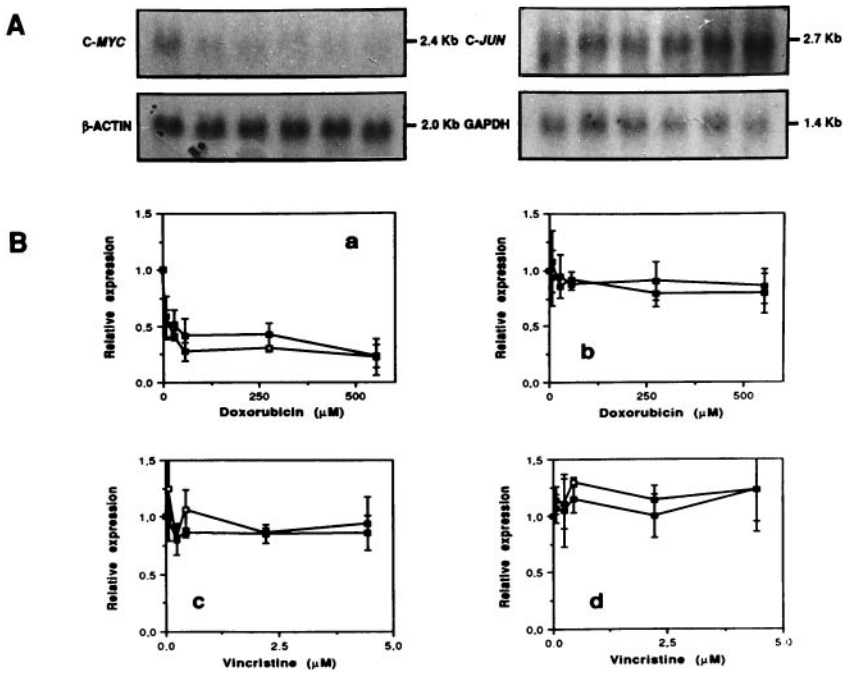

FIG. 4. Effects of doxorubicin on expressions of *c-myc*, *c-jun*, β-actin and/or GAPDH in C6 0.5 doxorubicin-resistant cells in a representative experiment. From left to right, the concentrations of doxorubicin were the following: 0; 5.5; 27.5; 55; 275; 550 μM and the duration of incubation was set at 2 hr. RNAs were extracted and Northern blots (20 μg of total RNA) were performed as described in Materials and Methods.

exposures of both cell lines to several concentrations of doxorubicin ($0.1 \times IC_{50}$, $1 \times IC_{50}$, $1 \times IC_{50}$). There was a dose-dependent DNA fragmentation in C6 cells after 48-hr reincubation in drug-free medium (Fig. 8a), which persisted 72 hr after doxorubicin treatment (data not shown). In contrast, no induction of DNA fragmentation was observed in C6 0.5 cells until at least 3 days after doxorubicin exposure. When studied by flow cytometry, apoptosis was detected in C6 sensitive cells 48 and 72 hr after 6-hr doxorubicin exposures, but to a much lower extent in C6 0.5 cells after similarly cytotoxic exposures (Fig. 8b).

Because it has been shown that p53 is a universal, although not unique, mediator of apoptosis [22, 23], we evaluated the level of expression of this protein in sensitive and resistant C6 cells upon doxorubicin treatment. Figure 9 shows that p53 was present in both cell lines at a very low basal level. Upon 6-hr exposure to doxorubicin, we observed a very low induction of p53, far below the "positivity" threshold observed in the control cell line. We concluded that the p53 of both cell lines was most likely of wild type, and that apoptosis detected in the C6 sensitive line was not mediated through p53 activation.

DISCUSSION

Resistance to topoisomerase II inhibitors has been reported to be frequently associated with quantitative or qualitative alterations of topoisomerase II [24–26], in addition to a general overexpression of P-glycoprotein. However, there

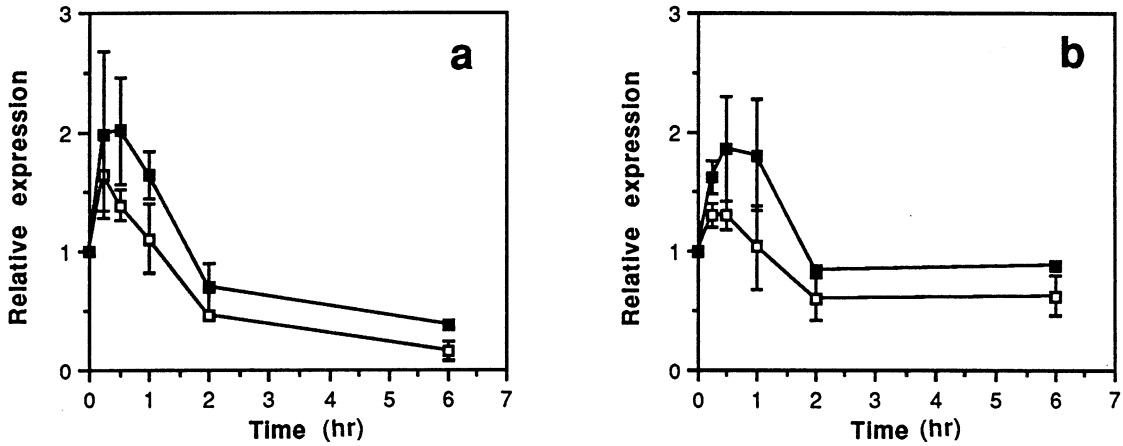

FIG. 5. Quantitation of doxorubicin effects, in C6 0.5 doxorubicin-resistant cells, on *c-myc* (a) and *c-jun* (b) expressions, relative either to β-actin (■) or to GAPDH (□), as a function of time. Doxorubicin concentration equaled $10 \times IC_{50}$ (550 μM). Values are means ± SD of three independent experiments.

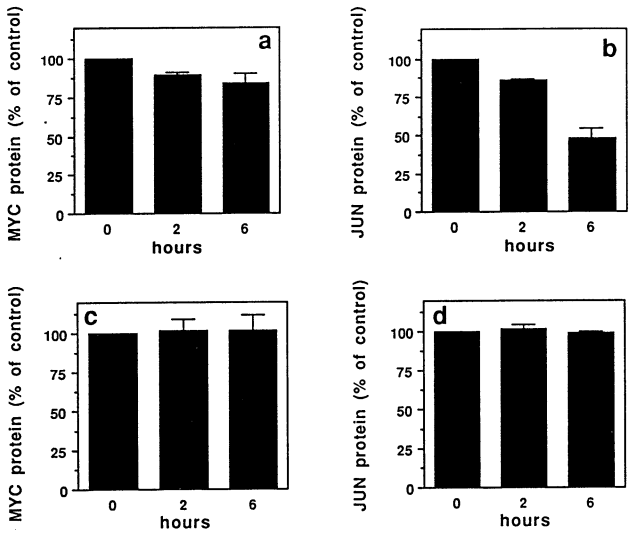

FIG. 6. Quantitation of doxorubicin effects on MYC (a, c) and JUN (b, d) proteins in C6 sensitive (a, b) and C6 0.5 (c, d) cells, as a function of exposure time to doxorubicin. Doxorubicin dose was equal to 10 × IC_{50} (1.7 μM in sensitive cells and 550 μM in resistant cells). Values are means ± SD of three independent experiments.

are many exceptions showing that the number of DNA-topoisomerase II cleavable complexes does not necessarily correlate with cell sensitivity to topoisomerase II inhibitors [27, 28], which suggests the involvement of other determinants of drug action. There are several lines of evidence showing that topoisomerase II inhibitors present some gene specificity for the generation of DNA breaks. Some of the cleavage sites that are formed by the covalent DNA-topoisomerase II complexes have been shown to affect transcription of the targeted gene [29]. Using different approaches, such specificity was shown for the action of teniposide and amsacrine on the c-*myc* gene in MCF-7 breast carcinoma cells [5, 6] and for the action of amsacrine on the c-*myc* gene in HL-60 cells [9]. Doxorubicin-induced suppression of c-*myc* expression has recently been shown to occur in MCF-7 cells, correlated to growth arrest and nonapoptotic cell death [30]. In agreement with these findings, we report in this paper that the doxorubicin-resistant rat glioblastoma cells (C6 0.5), which exhibit a reduced level of DNA topoisomerase II [18], also present changes affecting the expression of genes important for cell proliferation. The resistant line presents an increased basal level of c-*myc* mRNA, together with a decreased basal level of c-*jun* mRNA, which suggests that these two proto-oncogenes may also be involved in the determinism of cell sensitivity to anticancer drugs.

We observed a dramatic reduction in c-*myc* and c-*jun* mRNA levels in C6 cells upon doxorubicin exposures lasting 2 hr or more. This effect was dose-dependent and appeared to be specific, because it did not concern two domestic genes chosen as controls. In the case of c-*myc*, but not of c-*jun*, this down-regulation followed a transient transcriptional activation of the genes, which occurred during the first hour of incubation. Such transcriptional activation of c-*myc* and c-*jun* has been observed by several authors following induction of DNA damage [30–32] and has been suggested to generate a signal leading to programed cell death. We focused rather on the subsequent events concerning c-*myc* and c-*jun* gene expression. The reduction in c-*myc* and c-*jun* mRNA levels closely corresponded to cell survival after 2-hr doxorubicin exposure, as well as to the increase in doxorubicin sensitivity as a function of exposure time. Because vincristine had no effect on the expression of these genes, it can be concluded that the alterations we observed were not simply related to growth inhibition. In addition, C6 0.5 resistant cells presented the same reduction as the C6 sensitive cells in c-*myc* mRNA levels, occurring upon doxorubicin exposures lasting 2 hr or more, but there was no effect of this drug on c-*jun* mRNA levels. This suggests that there is in C6 0.5 cells a mechanism selectively preventing the decrease in c-*jun* expression, both at the mRNA and protein levels.

The c-*myc* and c-*jun* gene products are both DNA-binding proteins, which act as regulators of gene transcription and may be directly involved in DNA replication [33]. In C6 glioblastoma cells, there is a similar behavior of 2-hr and 6-hr doxorubicin exposures on both c-*myc* and c-*jun* gene expression. Because posttranscriptional changes affecting mRNA stability have been described [19, 32], it was

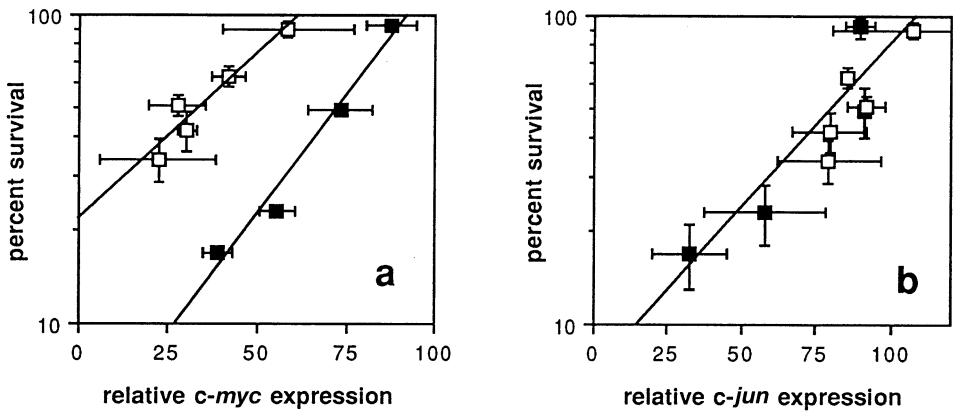

FIG. 7. Doxorubicin-induced growth inhibition plotted as a function of the relative c-*myc* (a) and c-*jun* (b) gene expressions in C6 sensitive (■) and C6 0.5 resistant (□) cells. Data are extracted from Figs. 2 and 4.

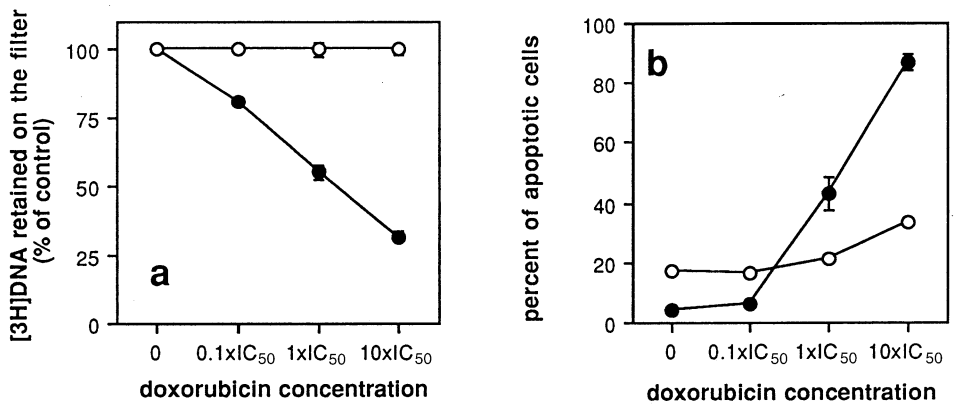

FIG. 8. Quantitation of apoptosis induced by different doxorubicin concentrations (0.1 × IC_{50}, 1 × IC_{50}, 10 × IC_{50}) in C6 sensitive (●) and C6 0.5 resistant (○) cells, as evaluated 48 hr after a 6-hr exposure to the drug, either measured by filter elution (a) or by flow cytometry (b). Values are means ± SD of three independent experiments.

of interest to determine whether reduced gene expression at the mRNA level was also to be found at the protein level. Indeed, a similar decrease in both MYC and JUN proteins occurred after 2-hr exposure to doxorubicin in the C6 cell line, but after 6-hr exposure, an additional decrease in JUN was the only one to occur. Prolongation of exposure time from 2 to 6 hr was accompanied by an increased cell sensitivity to the drug (IC_{50} decreasing from 0.17 to 0.041 μM). In contrast, there were no doxorubicin-induced modifications in MYC or JUN protein levels in the C6 resistant line, whatever the exposure time to doxorubicin. In addition, no modification of cell sensitivity to doxorubicin occurred between 2-hr and 6-hr exposure times. Taken together, these results suggest that the survival of doxorubicin-resistant cells could be dependent upon the preservation of the expression of both proteins. This was obtained with an unchanged transcription level for c-*jun*, in agreement with our hypothesis of a specific protection of c-*jun* against doxorubicin-induced DNA damage. In contrast, the preservation of MYC expression likely involved post-translational mechanisms such as an increase in mRNA and/or protein stability, as described by others [19, 32, 34].

There is increasing evidence that alterations in the expression of different proto-oncogenes are part of the apoptotic response of cells to anticancer agents [19, 35]. We evaluated the apoptotic response of our cell lines after doxorubicin exposure in order to test the physiological significance of the decrease in MYC and JUN in C6 cells as compared to C6 0.5 cells. At 48 hr and 72 hr after drug exposure, C6 sensitive cells exhibited a dose-dependent apoptotic response, whereas, at similarly cytotoxic concentrations, C6 0.5 resistant cells never entered into apoptosis.

It has been shown that the tumor suppressor p53 is a sequence-specific transcription factor mediating apoptosis [22, 36]. In view of the different behavior of C6 and C6 0.5 cells to doxorubicin-induced apoptosis, we evaluated p53 protein expression in these cells. Comparable low levels of p53 were detected in both cell lines, suggesting that the normal protein is expressed. In addition, no p53 increase compatible with induction of apoptotic response occurred upon doxorubicin exposure. We concluded that the absence of apoptotic response in resistant cells was not due to an alteration of p53 expression in this cell line.

Taken together, our results suggest that there is a mechanism preventing c-*jun* gene down-regulation in C6 0.5 cells, both at the mRNA and protein levels. We are currently investigating several hypotheses which could explain this protection of c-*jun*: (i) a mutation in the gene itself or its encompassing region, not allowing topoisomerase II recognition and binding; (ii) a concealment of the gene against topoisomerase II cleavage; and (iii) a more rapid repair of the DNA breaks occurring in the gene or its regulatory elements. In any case, this implies that the c-*jun* gene plays a crucial role in the proliferation of this cell line.

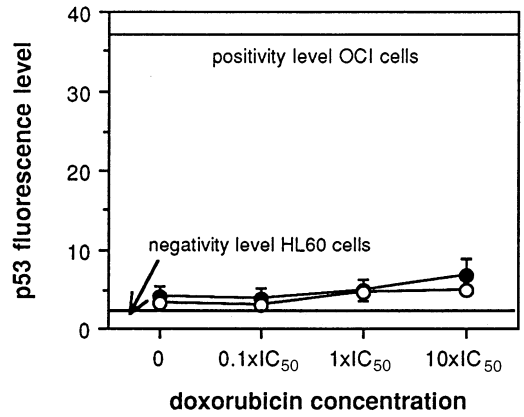

FIG. 9. Expression of p53 as detected by flow cytometry in C6 sensitive (●) and C6 0.5 resistant (○) cells, after 6-hr exposures to doxorubicin at different concentrations (0.1 × IC_{50}, 1 × IC_{50}, 10 × IC_{50}). Values are means ± SD of four independent experiments.

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