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ORIGINAL ARTICLE

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Bcl2-negative MCF7 cells overexpress p53: implications for the cell cycle and sensitivity to cytotoxic drugs

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Abstract *Purpose*: Bcl2 is a mitochondrial protein endowed with cytostatic and antiapoptotic activities. In this work we studied the effects of the lack of Bcl2 in MCF7 cells. *Methods*: The breast cancer cell line MCF7 (Bcl2-positive) and its derivative MCF7/50B (Bcl2-negative) were compared in terms of the level of p53 expression, doubling time and distribution of cells among the cycle phases. Sensitivities to the proapoptotic drugs cisplatinum and staurosporine were measured using a clonogenic assay and the contribution of apoptosis to cytotoxicity was determined with a mitochondrial membrane potential-sensitive dye. *Results*: Relative to MCF7, MCF7/50B cells overexpressed p53 and slowly proliferated with a significant accumulation at G_0/G_1 and depletion in S phase. The cytotoxicity of the DNA-damaging agent cisplatinum was decreased, while that of the protein kinase inhibitor staurosporine was increased. The induced cytotoxicity was essentially due to apoptosis and necrosis, respectively. *Conclusions*: These results suggest that the lack of Bcl2 accompanied by p53 overexpression affects the distribution of cells among the cell cycle phases and modifies the sensitivity to cytotoxic drugs and the type of cell death.

Keywords Bcl2 · P53 · Cell cycle · Drug sensitivity · Apoptosis

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Introduction

The transient downregulation of a given gene expression is one of the strategies that can be used to study its functional role. Most of the approaches use DNA decoys [1], antisense oligonucleotides [2] or ribozymes [3] to achieve inhibition. However, the transient effect does not allow extensive studies that could give more information on the role of that gene and other genes related to it.

Bcl2 is a mitochondrial protein endowed with cytostatic [4] and antiapoptotic [5, 6] activities. When overexpressed it causes a decrease in growth rate [7] and sensitivity to cytotoxic drugs [8, 9]. In a recent study dealing with *bcl2* overexpression, we identified MCF7/50B, an MCF7-derived clone in which Bcl2 level is apparently abolished. Thus the MCF7/50B clone offers a suitable model to investigate Bcl2 function in the case of stable downregulation.

We report that Bcl2-negative cells showed significant modification of both distribution in the cell cycle phases and sensitivity to cytotoxic drugs. The possible involvement of the oncosuppressor protein p53 is discussed.

Materials and methods

Cells and culture conditions

MCF7 (IST-CBA, Genoa, Italy) is a Bcl2-positive, $p53^{+/+}$ cell line derived from a human breast carcinoma. MCF7/50B is a Bcl2-negative cell line isolated as a $G418^r$ clone from cotransfection of pMC1-*neo* and pEE12-*bcl2* plasmids into MCF7 cells. In MCF7/50B cells, although Southern blot analysis has shown the presence of both endogenous and exogenous copies of *bcl2* (data not shown), the level of Bcl2 is not higher but indeed lower than in MCF7 parental cells. We therefore considered these cells as Bcl2-negative, even if the causes of this silencing remain to be investigated.

Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS_i), 100 IU/ml penicillin and 100 mg/ml streptomycin at 37°C in a humidified atmosphere containing 6% CO_2.

Cell growth

Cells were seeded at 5×10^5 per 60-mm diameter dish (P60) and counted 48 h and 72 h later. Cell doubling (CD) was calculated using the formula $\ln(N_f–N_i)/\ln 2$ where N_f is the cell number at 72 h and N_i the cell number at 48 h [10]. Doubling time (DT) was consequently obtained by dividing the time interval (24 h) by CD.

Flow cytometry

Cells collected at 72 h were fixed in 70% ethanol for 2 h at –20°C and permeabilized with 0.25% Triton X-100 for 5 min at 4°C. The suspension was then centrifuged and cells resuspended in a solution containing 10 μg/ml propidium iodide, 0.1 mg/ml RNAse and 0.5% IGEPAL CA-630. Finally, the cell suspension was passed through a flow cytometer and cell cycle analysis was done using Cell Quest analysis software (Becton-Dickinson).

Clonogenic survival

The ability of single cells to form colonies in the presence of cytotoxic agents was determined. Cells were seeded in P60 dishes at a density of 2×10^2 cells per dish. Cisplatinum (CDDP) and staurosporine (STS) were added at specified concentrations 24 h later. After 14–21 days colonies were stained with crystal violet and counted to determine the clonogenic survival.

Mitochondrial membrane potential assay

The loss of mitochondrial membrane potential ($\Delta\psi_m$), a molecular marker of apoptosis [11], was measured using MitoCapture reagent (BioVision) which is a $\Delta\psi_m$-sensitive fluorescent dye. In healthy cells the MitoCapture reagent accumulates and aggregates within the mitochondria fluorescing red (590 nm). In apoptotic cells, in which $\Delta\psi_m$ has been lost, it instead remains in the cytoplasm in its monomer form and fluoresces green (530 nm).

Cells were seeded at 7.5×10^5 per P60 dish and 24 h later 10 μ*M* CDDP or 1 μ*M* STS was added for 2 h. The cells were then collected, centrifuged and resuspended in 1 ml incubation buffer containing 1 μl MitoCapture reagent. The suspension was incubated at 37°C and in an atmosphere containing 6% CO_2 for 20 min, then centrifuged, and the cells resuspended in 1 ml incubation buffer and finally analyzed by flow cytometry.

The green fluorescence distribution of untreated cells was subtracted from that of CDDP- or STS-treated cells. The corresponding number of counts was normalized and taken as the percentage apoptotic cells.

Results

Bcl2-negative cells overexpress p53

Figure 1 shows the levels of expression of Bcl2 and p53 proteins in MCF7 and MCF7/50B cells. The Bcl2 content of MCF7/50B cells was 16-fold lower than that of MCF7 parental cells, while the p53 content was 2-fold higher. The Bcl2/p53 ratio was 3.1 in MCF7 cells and 0.15 in MCF7/50B cells, indicating an inversion of the equilibrium between the concentrations of the proteins.

Bcl2-negative cells show a novel distribution in the cell cycle

The DT, as a functional assay of Bcl2 cytostatic activity, was longer, even if not significantly ($P=0.057$), in MCF7/50B than in MCF7 cells (36.1 ± 1.8 h versus 32.4 ± 0.9 h). We also determined the distribution of cells in the various phases of the cell cycle. MCF7/50B cells significantly ($P<0.05$) accumulated at G_0/G_1 ($66.4\pm0.7\%$ versus $56.1\pm0.1\%$) and were depleted in the S phase ($19.9\pm0.5\%$ versus $27.8\pm0.1\%$).

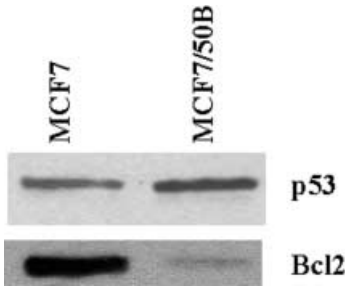

Fig. 1. Western blot analysis of Bcl2 and p53 in MCF7 and MCF7/50B cells. The radiographic signals were quantified by densitometric analysis using Gel-base software (UVP video-image recorder/analysis)

Novel sensitivity to cytotoxic drugs

Bcl2 antiapoptotic activity was evaluated by treating cells with CDDP or STS that are respectively a DNA-damaging agent and an inhibitor of protein kinases. The results are shown in Fig. 2A. CDDP-exposed MCF7/50B showed an enhanced clonogenic survival relative to CDDP-exposed MCF7 cells (Fig. 2A, left). Conversely, STS-exposed MCF7/50B cells showed a dose-dependent reduction in clonogenic survival that was stronger than that of STS-exposed MCF7 cells (Fig. 2A, right). The contribution of apoptosis to cell death was determined by 2 h treatment with 10 μ*M* CDDP and 1 μ*M* STS. The results are shown in Fig. 2B. CDDP induced apoptotic cell death in MCF7/50B cells and not in MCF7 cells (Fig. 2B, left). The level of STS-induced apoptosis was lower in MCF7/50B than in MCF7 cells (Fig. 2B, right).

Discussion

As a consequence of the lower protein level, in MCF7/50B cells we expected a decrease in Bcl2 cytostatic activity and thus a faster growth. On the contrary, they did not show a shortening of DT relative to parental MCF7 cells and even significantly accumulated at G_0/G_1. Such a remodeling was reflected by the sensitivity to CDDP. CDDP is a DNA-damaging, S phase-specific agent [12, 13], and therefore by definition it needs cells to pass through S phase to transform DNA damage into a cytotoxic lesion. This is probably why MCF7/50B, which are S phase-depleted cells, were less sensitive to CDDP than MCF7 parental cells (Fig. 2A, left).

The contribution of apoptosis to cell death was higher in MCF7/50B cells than in MCF7 cells (Fig. 2B, left). This may be due to the increased p53 protein level

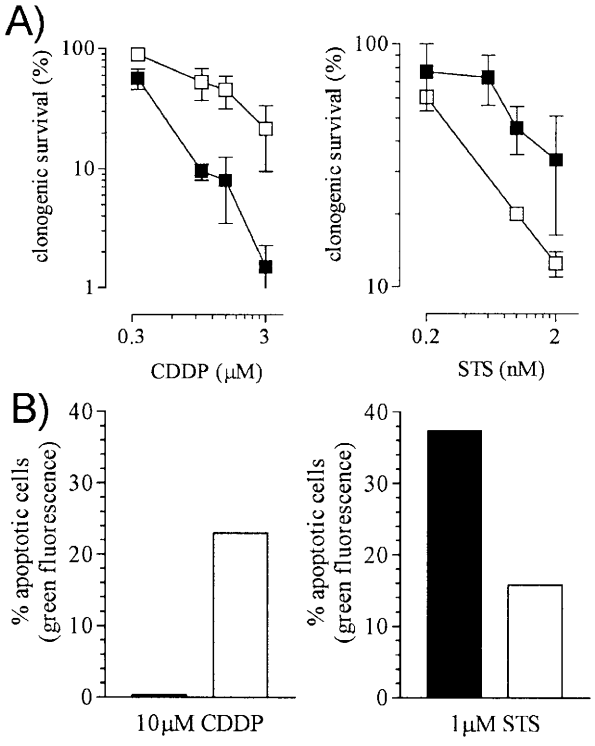

Fig. 2. **A** Clonogenic survival in the presence of CDDP and STS. MCF7 (*solid squares*) and MCF7/50B (*open squares*) cells were treated with 0.3, 1, 1.5 and 3 μ*M* CDDP (*left*) or 0.2, 0.6, 1 and 2 n*M* STS (*right*). After 14–21 days colonies were counted. Each point is the mean ± SD of three independent experiments. **B** Mitochondrial membrane potential assay. MCF7 (*solid bars*) and MCF7/50B (*open bars*) cells were treated with 10 μ*M* CDDP (*left*) or 1 μ*M* STS (*right*) for 2 h, then the percentage of green fluorescing apoptotic cells was determined using a $\Delta\psi_m$-sensitive fluorescent dye (MitoCapture reagent, BioVision)

in MCF7/50B cells. The decision of a DNA-damaged cell to proliferate or to die is taken at the G_1 checkpoint [14]. The oncosuppressor protein p53 arrests damaged cells at G_1 [15] allowing them to repair the damage [16, 17]. If this happens, cells reenter the cycle, otherwise they undergo p53-dependent apoptosis [18, 19]. P53 is a functional antagonist of Bcl2 [20]. It represses *bcl2* transcription [21, 22] and conversely Bcl2 prevents p53-dependent apoptosis [23]. In accordance with this antagonistic relationship, we found a higher p53 content in Bcl2-negative MCF7/50B cells than in MCF7 cells (Fig. 1). In p53-overexpressing MCF7/50B cells a better functioning of the G_1 checkpoint and hence a stricter control of DNA integrity would be expected. The higher contribution of apoptosis to cell death observed in MCF7/50B cells than in MCF7 cells (Fig. 2B, left) seems to confirm this hypothesis. It suggests that, among cells damaged by CDDP, only those correctly repaired are allowed by p53 to proliferate, while the others undergo p53-dependent apoptosis.

The results obtained with STS indirectly confirm that the accumulation at G_0/G_1 confers protection only against DNA-damaging agents. STS is a protein kinase inhibitor [24] which induces apoptosis [25] regardless of the phase of the cell cycle at which the cells are [26]. Bcl2 is a strong antagonist of STS-triggered apoptosis [6], so that an increase in sensitivity to STS would be expected when Bcl2 is lacking. This is what we obtained in Bcl2-negative MCF7/50B cells which were much more sensitive to STS than parental cells (Fig. 2A, right). Nevertheless, when we considered the mechanism of cell death, we found that apoptosis was only partially involved (Fig. 2B, right). This result suggests that the Bcl2 content was so low that MCF7/50B cells were completely unprotected against STS-induced damage, and necrosis instead of apoptosis was induced [27].

In conclusion, we demonstrated that the lack of Bcl2 accompanied by p53 overexpression affects the distribution of cells among the phases. The sensitivity to cytotoxic drugs and the type of cell death are consequently modified.

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