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pRb suppresses camptothecin-induced apoptosis in human osteosarcoma Saos-2 cells by inhibiting c-Jun N-terminal kinase

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Abstract This paper studies the cytotoxic effect induced by the topoisomerase I inhibitor camptothecin in human osteosarcoma Saos-2 cells, which lack p53 and contain a non-functional form of the product of the retinoblastoma gene, pRb. Cytotoxicity induced by camptothecin was dose- and time-dependent; the treatment with 100 nM camptothecin reduced cell viability by 50% at 32 h and by 75% at 72 h of exposure. The cytotoxic effect was caused by apoptosis, as ascertained by morphological evidence, acridine orange-ethidium bromide staining and flow cytometric analysis. Apoptosis was accompanied by both the activation of caspase-3 and the fragmentation of poly(ADP-ribose) polymerase. Treatment with camptothecin caused a threefold increase in the activity of c-Jun N-terminal kinase (JNK) and an eightfold increase in the level of phosphorylated c-Jun. The introduction of the RB gene into Saos-2 cells reduced the rate of cell growth. Moreover, stable clones of transfected cells were resistant to camptothecin. Exposure to 100 nM camptothecin for 72 h reduced the viability of transfected cells by only 10%; moreover, very modest effects were observed on the activity of JNK as well as on the level of phosphorylated c-Jun. The results reported in this paper support the conclusion that the expression of wild-type pRb in Saos-2 cells exerts an anti-apoptotic influence through the control of JNK activity.

Key words: pRb; Apoptosis; c-Jun N-terminal kinase

1. Introduction

The product of the retinoblastoma gene, pRb, plays a role in regulating the progression of proliferating cells through the cell cycle [1]. pRb behaves as a transcriptional repressor by binding to the transactivation domain of E2F and inhibiting its transcriptional activity, which is required for the progression of cells from G1 into S phase [1]. In recent years, much evidence has suggested that pRb is also involved in the terminal differentiation of many tissues, by inducing cell cycle exit and by potentiating the activity of tissue-specific transcription factors [2]. For example, pRb positively regulates the activity of MyoD and C/EBP, two transcription factors required for the expression of genes involved in the differentiation of respectively muscle and fat cells [3,4].

Apart from these roles in the control of the cell cycle and differentiation, an anti-apoptotic role has been suggested for pRb in a series of recent papers which correlate the loss of pRb with the induction of apoptosis. The appearance of apoptosis in specific neuronal compartments and in the developing lens has been observed in Rb−/− mouse embryos and these results have been interpreted as a response to the loss of pRb [5]. Moreover, the cleavage of pRb by caspase activities has been considered to be an early permissive step in the apoptosis-inducing pathway [6]. Apoptosis in pRb-defective cells can be induced by the wild-type form of p53 [7]. Accumulation of p53 can be promoted by E2F, a transcriptional factor which is in a free state when pRb is absent in the cells. Free E2F stimulates the expression of $p19^{ARF}$ which binds MDM2, thus inhibiting MDM2-mediated degradation of p53 and favoring the accumulation of p53 [8]. However, other apoptotic pathways independent of p53 activity have been described in different circumstances by many authors [9].

Evidence has been provided for a role in the initiation of apoptosis for c-Jun N-terminal kinase (JNK/SAPK), a member of mitogen-activated protein (MAP) kinase which can be activated by either oxidative stress or inflammatory cytokines [10,11]. Even DNA-damaging agents such as topoisomerase inhibitors or γ radiation have been demonstrated to activate the stress signalling pathway of JNK, with the consequent increase in the phosphorylation of c-Jun [12,13].

The present study was carried out to assess the potential apoptotic effect of both camptothecin and its analogue topotecan in human osteosarcoma Saos-2 cells, which are defective in both pRb and p53. Our results indicate that camptothecin induces a clear apoptotic effect in Saos-2 cells, which is independent of oxidative stress and correlates with the activation of JNK. Our results support the conclusion that the introduction of pRb into Saos-2 cells exerts an anti-apoptotic effect by inhibiting the activity of JNK.

2. Materials and methods

2.1. Cell cultures and reagents

Human osteosarcoma Saos-2 cells (obtained from the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) were grown as monolayers in McCoy's 5A medium, supplemented with 10% heat-inactivated fetal calf serum at 37°C in a humidified atmosphere containing 5% CO_2.

Unless stated otherwise, incubations were performed with cells seeded on 96-well plates (7×10^3 cells) or 100-mm tissue culture dishes (1×10^6 cells). After plating, cells were allowed to adhere overnight and then treated with chemicals or vehicle (control cells) as described in the legends of figures and in the text.

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Cell viability was determined by either a MTT colorimetric assay [14] or a standard trypan blue dye exclusion assay.

Morphological analysis of apoptosis was performed by acridine orange-ethidium bromide (AO/EB) staining, using monolayer cultures in 96-well plates. After removal of the medium, cells were rinsed and treated with a solution of 100 μg/ml AO and 100 μg/ml EB. The difference in the uptake of these two dyes allowed the identification of viable and non-viable cells by fluorescence microscopy. Viable cells display normal nuclei with a homogeneous chromatin staining pattern and bright green fluorescence whereas dead apoptotic cells show typical condensed and fragmented chromatin with bright orange fluorescence. Dead necrotic cells display a diffuse orange staining pattern.

z-VAD-fmk (benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone) was obtained from Bachem AG (Bubendorf, Switzerland). Other chemicals, except where stated otherwise, were obtained from Sigma (St. Louis, MO, USA). Stock solutions of topoisomerase inhibitors were prepared in dimethyl sulfoxide (DMSO) and diluted to their final concentrations in the culture medium. The final concentration of DMSO never exceeded 0.04%, a concentration which had no discernible effect on Saos-2 cells in comparison with the control.

2.2. Transfection of Saos-2 cells with the RB gene

Saos-2 cells were seeded on 6-well plates (1×10^5 cells/well) in 2 ml McCoy's medium, and transfected using FuGene transfection reagent (Boehringer Mannheim, Darmstadt, Germany) following the standard manufacturer's protocol. The vector used was p-CMV-Rb1-puro plasmid (1.5 μg DNA/well) (provided by Dr. M. Crescenzi, Regina Elena Cancer Institute, Rome, Italy), containing full-length RB cDNA and the puromycin resistance gene. After incubation for 48 h with FuGene reagent, resistant clones were selected for 4 weeks with puromycin (1 μg/ml) and then expanded into cell lines in medium containing 0.1 μg/ml puromycin. The three clones that showed the highest expression of pRb by means of Western blotting analysis were used for further experiments. The figures show the results obtained with clone C25.

2.3. Cell cycle analysis

The distribution of cells in the cell cycle phases was determined as previously reported [15]. Saos-2 cells were washed twice in cold phosphate-buffered saline (PBS) and stained with a hypotonic fluorochrome solution containing 50 μg/ml propidium iodide. Flow cytometric analysis of stained nuclei was performed in a FACSort instrument (Becton Dickinson) and fluorescence was analyzed as a single-parameter frequency histogram. Data were analyzed using MedFit TL software (Becton Dickinson).

2.4. Detection of intracellular H_2O_2

Production of H_2O_2 was quantified by a microassay modification of the red phenol method [16]. Saos-2 cells, seeded in 24-well plates (5×10^4 cells/well), were covered with 150 μl/well of assay solution (0.02% red phenol in red phenol-free BSS containing 6 U/ml horseradish peroxidase, type II) and incubated for 1 h at 37°C in 95% air–5% CO_2. Then 15 μl of 1 N NaOH was added to each well. The amount of oxidized red phenol was measured by an automatic microplate enzyme immunoassay photometer (Opsys MR; Dynex Technologies) at 630 nm wavelength. Mean absorbance values from 10 replicate wells were calculated and the results are expressed as nmol H_2O_2 produced per 10^6 cells.

In other experiments, intracellular peroxide levels were assessed using 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA, Molecular Probes, Leiden, The Netherlands). DCFDA (5 μM) was added to cell cultures during the last hour of treatment. Then, the dye was removed and the cells were washed twice with PBS, trypsinized and finally resuspended in 5 ml of PBS. Cells were analyzed using a flow cytometer (Becton Dickinson).

2.5. Reduced glutathione (GSH) content assay

Glutathione levels were determined using a glutathione colorimetric assay kit (Calbiochem-Novabiochem Corporation, San Diego, CA, USA). For this assay, Saos-2 cells (2×10^6) were cultured in 100-mm petri plates and treated for 48 h with 100 nM camptothecin in the conditions reported in Section 3.

2.6. JNK assay

SAPK/JNK activity was measured using a non-radioactive method (New England Biolabs, Hitchin, UK). Cells were lysed as reported in the manufacturer's instructions, sonicated in a Soniprep 150 apparatus (MSE) (four times for 5 s) and centrifuged (14 000 rpm) for 10 min. JNK activity was precipitated from whole cell lysates (250 μg of protein) with 2 μg of GST-c-Jun fusion protein/GSH-Sepharose beads overnight at 4°C. The beads were washed twice in lysis buffer, resuspended in 50 μl kinase buffer supplemented with 100 mM ATP and incubated for 30 min at 30°C. To evaluate the phosphorylation of GST-c-Jun on Ser63 equivalent amounts of protein were resolved by SDS–PAGE, electrotransferred to nitrocellulose and detected with a specific anti-phospho-c-Jun antibody. Bands were quantified by densitometric analysis.

2.7. Western blot analysis

Cells were lysed for 30 min at 4°C with a solution (15 μl/10^6 cells) composed of 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS in PBS (pH 7.4), containing inhibitors of proteases. Cells were then sonicated three times for 10 s and centrifuged at $15\,000\times g$ for 20 min at 4°C. Equivalent amounts of proteins (60 μg) were resolved by SDS–PAGE and electrotransferred to nitrocellulose for detection with specific antibodies. All antibodies employed were purchased by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Visualization was performed using nitroblue tetrazolium and bromochloro-indoyl-phosphate. Finally, bands were quantified by densitometric analysis.

3. Results

3.1. Topoisomerase inhibitors reduce the viability of human osteosarcoma Saos-2 cells

We evaluated the effect of camptothecin, an inhibitor of topoisomerase I, on the viability of human osteosarcoma

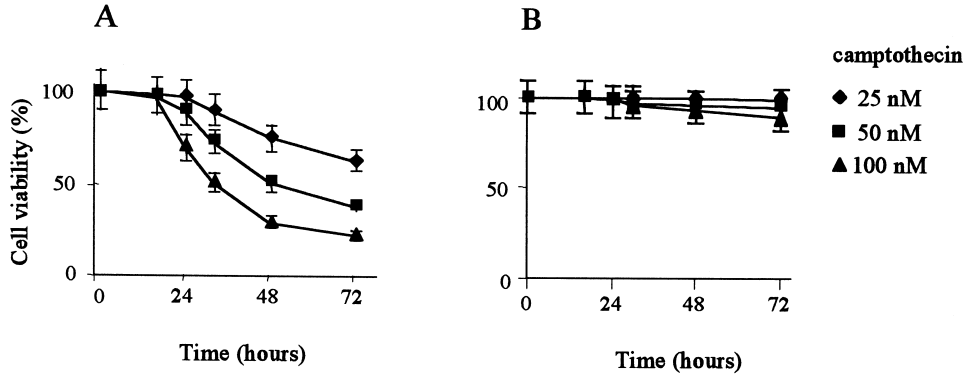

Fig. 1. Time course of the inhibitory effect induced on the viability of Saos-2 cells by camptothecin. Parental (A) or RB-transfected (B) Saos-2 cells were treated with different doses of camptothecin for 0, 16, 24, 32, 48 and 72 h. The number of viable cells was then determined using the MTT colorimetric assay and is expressed as a percentage of untreated cells. Data are the means of four separate experiments, each in triplicate.

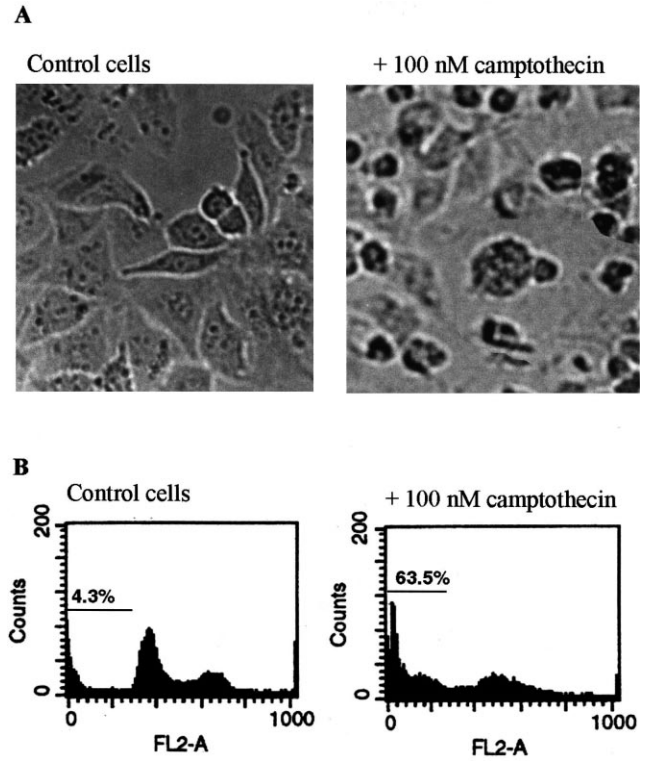

Fig. 2. Morphological (A) and flow cytometric (B) analysis of Saos-2 cells after treatment with camptothecin. Saos-2 cells were cultured for 48 h in the absence or presence of 100 nM camptothecin. Cells were then examined using conventional light microscopy and photographed (A). Flow cytometric analysis, shown in B, was performed by DNA staining with propidium iodide as reported in Section 2. The vertical axis represents the relative number of events and the horizontal axis the fluorescence intensity. The percentages indicate the subdiploid population of cells.

Saos-2 cells, as measured by the MTT assay. As shown in Fig. 1A, treatment with camptothecin reduced Saos-2 cell viability in a dose- and time-dependent manner. With 100 nM, cell viability progressively decreased after a lag phase of 16 h, reaching 50% of the control value at 32 h and 25% at 72 h of treatment.

A similar effect was obtained when the cells were treated with topotecan, an analogue of camptothecin used in cancer chemotherapy. Treatment with 100 nM topotecan reduced the viability of Saos-2 cells to 56% at 32 h and 30% at 72 h of treatment (not shown).

The inhibitors of topoisomerase II were less efficacious than camptothecin. The treatment with 1 μM etoposide reduced Saos-2 cell viability by only 10% at 32 h and 32% at 72 h of treatment (not shown).

3.2. The reduction of Saos-2 cell viability is caused by apoptotic cell death

Light microscopy demonstrated that Saos-2 cells treated with camptothecin detached themselves from the substrate and assumed a round shape (Fig. 2A). Analysis by fluorescence microscopy after staining with AO/EB permitted us to establish that camptothecin induces apoptosis in Saos-2 cells. The frequency of apoptotic cells was measured in samples treated with 100 nM camptothecin for 24 and 48 h. Comparison between the frequency of apoptotic cells and that of total dead cells (Table 1) strongly suggests that the loss of viability was primarily due to apoptosis.

The rate of apoptosis was also estimated by flow cytometry. As shown in Fig. 2B, the treatment with 100 nM camptothecin enhanced the subdiploid G0/G1 population which, after 48 h of exposure, reached 63.5% of the total cells. This value was in agreement with the percentage of non-viable cells evaluated by either AO/EB analysis or MTT assay.

Western blotting analysis was used to examine the activation of caspase-3, a protease involved in apoptotic pathways [17]. As shown in Fig. 3, the treatment with 100 nM camptothecin for 48 h induced fragmentation of procaspase-3, with the production of the active form of caspase-3. The same treatment with 100 nM camptothecin also induced the fragmentation of poly(ADP-ribose) polymerase (PARP), with the appearance of an 85-kDa apoptotic fragment (Fig. 3). Moreover, the addition of z-VAD, a general inhibitor of caspase activities, completely suppressed the effect of camptothecin on cell viability (not shown) and on PARP degradation, indicating the involvement of caspases in camptothecin-mediated apoptosis.

In some experiments we treated Saos-2 cells with combinations of camptothecin and MG132, a good inducer of apoptosis, and we observed that combined treatments gave additive effects on cell viability. For example, the viability of Saos-2 cells was reduced by 48% and 55% when the cells were separately treated with 0.5 μM MG132 and 50 nM camptothecin respectively, while combined treatment lowered the viability by 75% (not shown).

3.3. Oxidative stress is not involved in the apoptotic process induced by camptothecin

To ascertain the potential role of oxidative damage in camptothecin-induced toxicity, we studied the effect of 100 nM camptothecin on the cellular level of hydrogen peroxide.

Table 1
The loss of viability and the induction of apoptosis in Saos-2 cells treated with camptothecin

		Dead cells (%)		Apoptotic cells (%)	
		hours of treatment		hours of treatment	
		24	48	24	48
Parental cells	Control cells	2.1 ± 0.2	7.1 ± 0.7	2.2 ± 0.3	4.1 ± 0.3
	Camptothecin	32.2 ± 2.8	69.8 ± 7.1	29.3 ± 3.1	64.2 ± 6.4
	+z-VAD	4.9 ± 0.4	15 ± 1.6	5 ± 0.5	10 ± 1.1
Transfected cells	Control cells	1.5 ± 0.1	2.1 ± 0.2	1.4 ± 0.1	1.9 ± 0.2
	Camptothecin	3.1 ± 0.3	10.0 ± 0.9	2.5 ± 0.2	8.1 ± 0.7
	+z-VAD	3.2 ± 0.3	9.2 ± 0.9	2.1 ± 0.2	7.2 ± 0.6

Parental and RB-transfected cells were cultured for 24 and 48 h in the absence or presence of 100 nM camptothecin. 100 μM zVAD was added when reported. Cells were stained with trypan blue or AO/EB and the frequency of non-viable cells compared to that of apoptotic cells.

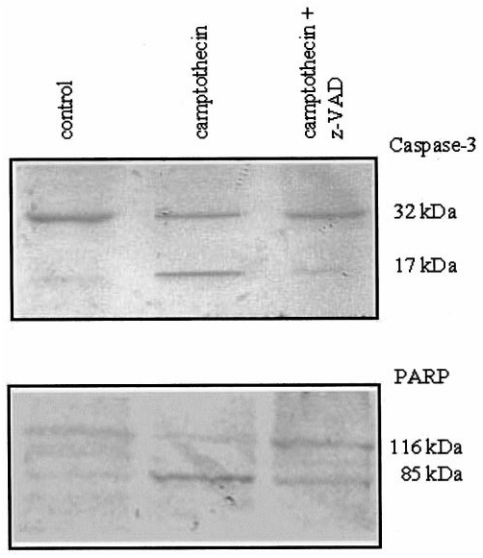

Fig. 3. Western blotting analysis of caspase-3 and PARP in Saos-2 cells. Saos-2 cells were treated for 48 h with 100 nM camptothecin in the absence or presence of 100 μM z-VAD. Cell lysates were prepared as reported in Section 2, resolved by 10% SDS–PAGE, immunoblotted and detected using specific antibodies.

During a period of time between 0.5 and 48 h of treatment only modest increases in H_2O_2 level (+10–15%) were found using the red phenol procedure (Fig. 4A). Data were confirmed by means of cytofluorimetric analysis using DCFDA as a fluoroprobe.

We also evaluated the content of GSH in cells treated for 48 h with 100 nM camptothecin. Data shown in Fig. 4B indicate that camptothecin induced only a modest decrease in the intracellular content of GSH. Moreover, the addition of GSH (400 μg/ml) (Fig. 4C) or *N*-acetylcysteine (20 mM) (not shown) to the cultures treated with camptothecin only modestly reduced the apoptotic effect caused by the drug. In line with these results, the addition of BSO, a potent specific inhibitor of γ-glutamylcysteine synthetase (the rate-limiting step in glutathione biosynthesis), was unable to increase the effect of camptothecin (not shown).

Finally, the addition of exogenous catalase (1 mg/ml), an effective cellular H_2O_2-decomposing enzyme, did not modify the apoptotic effect exerted by camptothecin (not shown).

3.4. The apoptotic effect of camptothecin is mediated by JNK activation

As it is well known that some DNA-damaging drugs activate JNK [18,19], we evaluated whether the JNK signalling pathway is functionally involved in camptothecin-induced apoptosis. JNK activity was tested using a procedure which employs the N-terminal c-Jun fusion protein. As shown in Fig. 5A, the level of JNK activity was very low in untreated Saos-2 cells. This level was enhanced with camptothecin treatment, reaching a threefold increase after 48 h exposure to 100 nM (Fig. 5C). As a consequence of JNK activation, the level of phosphorylated c-Jun was also markedly enhanced after 48 h of treatment, reaching a level of about eight times the control value (Fig. 5C). This effect was not modified by the addition of 100 μM z-VAD, indicating that caspase proteases are not involved in the activation of JNK (Fig. 5B). Similar effects on JNK activity and on phosphorylation of c-Jun were also observed on treating the cells for 48 h with 100 nM topotecan (not shown).

Using Western blotting analysis we evaluated the cellular levels of a number of apoptosis modulators such as Bcl-2, Bax, p21 and E2F. It was observed that treatment with 100 nM camptothecin did not significantly modify the levels of these proteins (not shown).

3.5. Transfection of Saos-2 cells with the RB gene reduced the effect exerted by camptothecin on both apoptosis and JNK activation

To evaluate the role of pRb in camptothecin-induced apoptosis, the wild-type RB gene was introduced by transfection into Saos-2 cells, and clones of transfected cells expressing

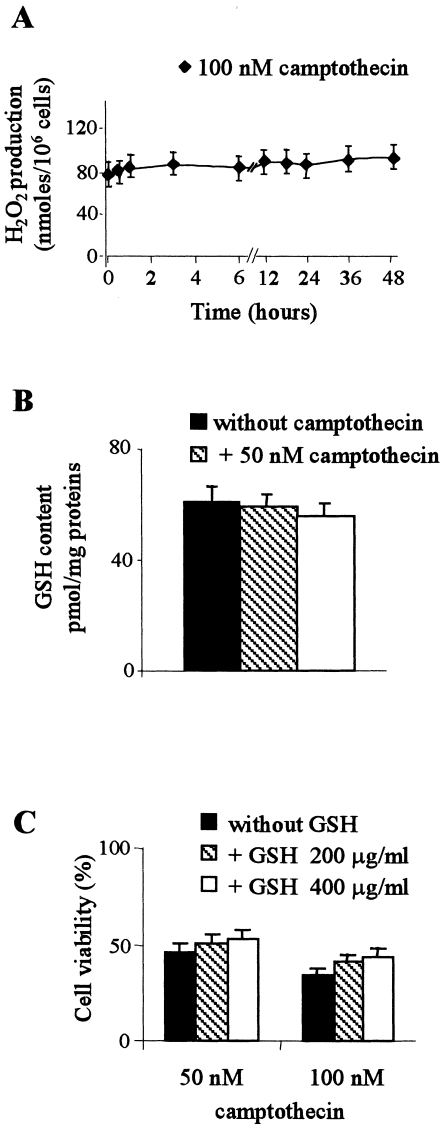

Fig. 4. Detection of hydrogen peroxide (A) and GSH (B) in Saos-2 cells treated with camptothecin. The influence of exogenous GSH on the cytotoxic effect exerted by camptothecin (C). Saos-2 cells were treated for various times with camptothecin with or without GSH, as indicated in the figure. The number of viable cells was assessed using the MTT procedure and is expressed as a percentage of untreated cells. H_2O_2 was evaluated by means of the red phenol method and GSH content by means of a colorimetric assay as reported in Section 2. Data are the means of four separate experiments, each in triplicate.

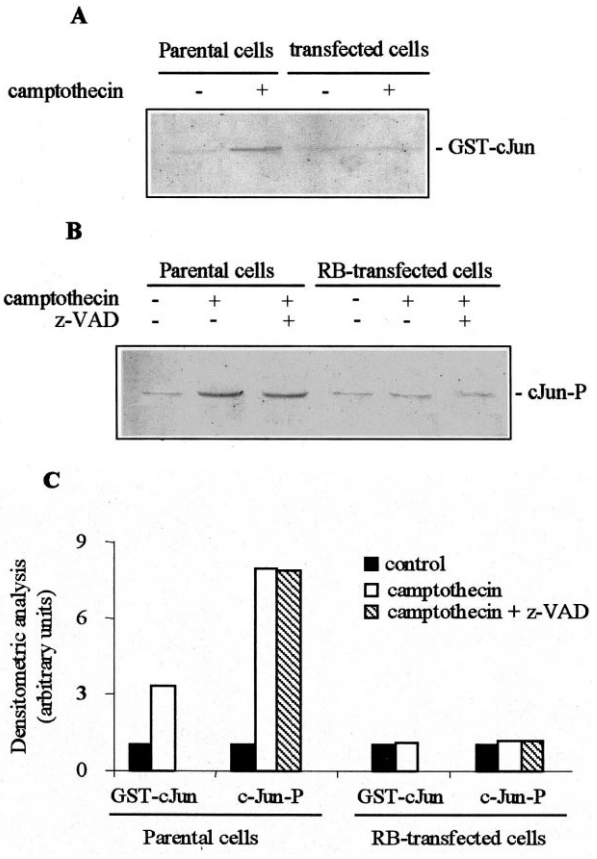

Fig. 5. The activation of JNK induced by camptothecin in Saos-2 cells. Parental and RB-transfected cells were cultured for 48 h with or without 100 nM camptothecin. 100 μM z-VAD was added as reported in the figure. At the end cell lysates were prepared and employed to estimate the activity of JNK (A), using a GST c-Jun fusion protein as described in Section 2, or the extent of phosphorylated c-Jun (B). Western blotting analysis was performed using a specific antibody which recognizes phosphorylated c-Jun. The levels of activated JNK and phosphorylated c-Jun were estimated by means of densitometric analysis (C).

wild-type pRb were selected and expanded into cell lines. The presence of pRb in transfected cells was analyzed by means of Western blotting analysis using a monoclonal antibody that recognizes the central portion of pRb. As shown in Fig. 6, only a 95-kDa band, related to a truncated form of pRb, was detected in parental Saos-2 cell line, while in transfected Saos-2 cells the presence of the normal-size Rb protein (105 kDa) was ascertained in addition to the 95-kDa truncated form. All transfected clones exhibited a slower rate of cell growth compared to parental Saos-2 cells. Moreover, RB-transfected cells appeared enlarged and flattened with many dendritic-like protrusions (not shown).

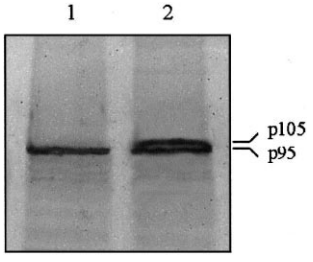

Fig. 6. Expression of pRb in RB-transfected Saos-2 cells. Protein extracts (about 50 μg) were prepared either from parental (lane 1) or RB-transfected (lane 2) Saos-2 cells and analyzed by Western blotting for pRb expression. Proteins were resolved in SDS–7.5% polyacrylamide gel and proteins were immunoblotted and detected using a specific antibody. Only clone C25 is shown, all other transfectants showed similar expression of pRb.

RB-transfected clones exhibited clear resistance to the effect of camptothecin or topotecan. In fact, with both compounds, the viability of RB-transfected Saos-2 cells decreased much more slowly than that of the parental cells. As shown in Fig. 1B, after 72 h of treatment with 100 nM camptothecin, cell viability decreased by only 10% in RB-transfected clones, while the reduction was about 70% in parental cells. Accordingly, also the frequency of apoptotic cells was much less (8.1%) in RB-transfected clones than in parental cells (64.2%) (Table 1), suggesting a suppressive influence of pRb on the apoptotic pathway. Furthermore, the effects of camptothecin on JNK activation and on c-Jun phosphorylation were also much lower in RB-transfected than in parental cells. For example, the treatment with 100 nM camptothecin over 48 h increased the extent of phosphorylated c-Jun by only 20% in transfected cells, in comparison with an eightfold increase in parental cells (Fig. 5).

4. Discussion

The retinoblastoma protein plays an important role in regulating the progression of the cell cycle in normal mammal cells [20]. Inactivation or loss of pRb impedes cell cycle exit and induces deregulation in DNA proliferation, favoring tumorigenesis in a number of cases [21–23].

We have undertaken research on the mechanisms which induce apoptosis in tumor cells defective in pRb function and we have documented that human retinoblastoma Y79 cells, which lack pRb and express functional p53, are susceptible to apoptosis after treatment with several compounds [24–27]. Particular effectiveness was observed using camptothecin or its analogue topotecan, which behave as inhibitors of topoisomerase I by stabilizing enzyme–DNA cleavable complexes [28]. Their apoptotic effect seems to be correlated with a remarkable increase in p53 level which is most likely a consequence of the conversion of p53 from a latent form into an active one [24].

This paper describes the cytotoxic effect determined by the inhibitors of topoisomerases on human osteosarcoma Saos-2 cells, which contain a non-functional form of pRb. The results indicate that camptothecin was the most active compound among the different inhibitors tested, although its efficacy in Saos-2 cells was lower than in retinoblastoma Y79 cells [24]. The camptothecin effect on the viability of Saos-2 cells was a consequence of the induction of apoptosis, as evidenced by light and fluorescent microscopy and flow cytometry. Because Saos-2 cells are lacking in p53, our finding indicates that apoptosis can occur in Rb-deficient cells also by p53-independent pathways. The apoptotic effect was accompanied in Saos-2 cells by an increase both in the activity of JNK/SAPK and in the extent of phosphorylation of c-Jun, a transcriptional factor which is phosphorylated by JNK at Ser63 and Ser73 within its N-terminal transactivation domain [29]. In addition, camptothecin induced the activation of caspases, the proteases involved in the execution phase of apoptosis [17]. Our results suggest that the activation of JNK precedes the activation of

caspases, because z-VAD, a general inhibitor of caspases, did not modify the effect of camptothecin on c-Jun phosphorylation.

JNK, a member of the MAP kinase family, seems to exert a central role in the induction of apoptosis in Saos-2 cells by either camptothecin or MG132. The activation of JNK is mediated by dual phosphorylation on specific Thr and Tyr residues, catalyzed by two MAP kinase kinases, MKK4 and MKK7 [30]. Numerous protein kinases (such as MEKK1, MEKK4, ASK1 and MLK3) function as MAP kinase kinase kinases in the JNK pathway, phosphorylating MKK4 and MKK7 [30].

It is well known that JNK can be activated in response to environmental oxidative stress [31]. Studies recently performed by us have demonstrated that treatment of Saos-2 cells with MG132, an inhibitor of the 26S proteasome, enhanced JNK activity as well as the level of phosphorylated c-Jun, although to a lesser extent than camptothecin did. In the case of MG132, our results show that the activation of JNK may be considered a consequence of the induction of oxidative stress (submitted). Instead, camptothecin-induced apoptosis seems to be independent of oxidative stress. This conclusion is based on the following: (i) treatment with camptothecin did not modify the endogenous GSH content; (ii) only a modest increase in the production of H_2O_2 was observed after the addition of the drug; (iii) the effect of camptothecin was counteracted neither by the addition of exogenous catalase nor by GSH; and finally (iv) the camptothecin effect was not increased by BSO, an inhibitor of GSH synthesis. Thus it seems that the increasing effect on JNK level induced by camptothecin is not correlated with oxidative stress.

However, many authors have provided evidence that the activation of JNK can be determined by DNA-damaging agents, such as alkylating agents (mitomycin and cisplatin), ionizing radiation and adriamycin, without the induction of oxidative stress [18,19]. Even camptothecin and etoposide, which are inhibitors of topoisomerase I and II respectively, have been shown to stimulate both apoptosis and the activity of JNK [31]. The mechanism by which genotoxic agents induce activation of JNK is unknown at the moment. However, Kharbanda et al. [32], studying etoposide-induced apoptosis, have demonstrated that the activation of JNK is correlated with the activation of MEK kinase I, the first enzyme in the JNK pathway.

Although the involvement of JNK in apoptosis has been clearly established, the mechanism of its apoptotic action is not currently well understood. c-Jun represents the primary target of JNK. The phosphorylation of c-Jun leads to the activation of the transcription factor AP-1. However, a role of AP-1 in the expression of genes which are involved in apoptosis has not yet been described. A possible relationship between JNK and apoptosis is suggested by the observation that the anti-apoptotic factor Bcl-2 is a target of JNK and that as a response to DNA damage JNK may translocate into the mitochondria, thereby inducing the phosphorylation and the inactivation of Bcl-2 [33].

This paper compares the effects exerted by the genotoxic drug camptothecin on parental Saos-2 cells and on stable clones of RB-transfected cells. We demonstrate that the introduction of RB renders Saos-2 cells not susceptible to the apoptotic action of camptothecin. This finding represents the first proof of the anti-apoptotic action exerted by pRb in tumor cells against genotoxic drugs.

Recently Shim et al. [34] observed that apoptotic cell death induced by ultraviolet radiation is abrogated in HEK293 cells transiently transfected with a plasmid expressing pRb. This fact has been interpreted as a result of the interaction of pRb with JNK and the consequent inhibition of the intracellular signal induced by JNK. In line with this finding our paper provides evidence that the introduction of pRb suppresses the effect induced by camptothecin both on the activity of JNK and on the phosphorylation of c-Jun. Therefore, it seems that pRb can exert a general anti-apoptotic effect through the control of the activity of JNK. Consequently, tumor cells which are lacking in pRb seem to be particularly susceptible to undergoing cell death when exposed to apoptotic inducers.

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