The cisplatin–irradiation combination suggests that apoptosis is not a major determinant of clonogenic death

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It is commonly believed that tumor cells treated with anticancer agents, chemotherapy and/or radiation, die by apoptosis and that tumors which do not undergo apoptosis are resistant to treatment. In this study, we investigated the molecular basis underlying cisplatin cytotoxicity in the murine teratocarcinoma F9 cell line to see whether irradiation enhances cisplatin-induced cytotoxicity. We compared the apoptosis induced by chemo and/or radiotherapy with other cellular effects such as cell survival, clonogenic capability, cell cycle perturbation, expression of p53 and p53-related mRNAs, and necrosis. When combined with radiation, a clear additive cytotoxic effect of cisplatin was demonstrated. We found that both cisplatin and radiation induced cell death, but the level of induced apoptosis was low and there was no correlation with the results of the clonogenic assays: we noted a difference between cytotoxic effects in the clonogenic assay and the extent of apoptosis by fluorescence-activated cell sorter analysis, suggesting that cell killing reflected not only apoptosis but also cell cycle arrest, and that apoptosis, cell kinetics and clonogenicity suppression were independent processes.
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Introduction

Apoptosis and necrosis are two morphologically distinct forms of cell death that are important for maintaining cellular homeostasis. Almost all cytotoxic agents can provoke cellular responses [1]; however, the duration of treatment and the dose of the agents employed determine which type of death is affected. The response of tumors to chemo, radio and hormone therapy or to treatment with biologically active agents may depend, at least in part, on the propensity of these tumors to undergo cell death. Necrosis is a passive process in which cells undergo modifications such as loss of membrane integrity, swelling, cytoplasmic vesicles and random degradation of DNA. Apoptosis is a well described active process of programmed death characterized by nuclear DNA fragmentation, condensed chromatin, nucleus fragmentation and poorly stained cytoplasm [2,3].

The use of chemotherapeutic drugs in combination with radiotherapy is a common strategy for the treatment of many advanced cancers, such as head and neck [head and neck squamous cell carcinoma (HNSCC)], cervix, esophagus, rectum and others [4]. The introduction of combined modality treatment regimens has resulted in improved local control and overall survival rates in HNSCC [5–8]. Treatment-related toxicity, however, is substantial and treatment failure owing to either primary or acquired resistance to DNA-damaging agents remains a poorly understood problem. Despite improved outcome, optimal protocols for administration of combined modality treatments have not been definitively identified. A better knowledge of the biological mechanisms of interaction between chemotherapy and radiotherapy could help to improve tumor control and survival benefits. Potentially effective treatment strategies may target biological mechanisms of interaction between radiation and chemotherapy, such as cell repair, cell cycle distribution and tumor cell proliferation.

Cisplatin (*cis*-DDP) is an effective agent against several tumors [9] and it is the chemotherapeutic drug most commonly combined with radiation in clinical practice. It causes DNA damage through intrastrand cross-link formation and inhibition of DNA synthesis: slowed DNA synthesis occurs during progression to the G_2 phase, and cells are arrested here before dying or bypassing the block and returning to normal cycling [10]. This G_2 arrest can be critical when *cis*-DDP is combined with radiation as it is well known that G_2 is a radiosensitive phase of the cell cycle. Radiation induces

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DNA damage (directly by ionization or indirectly by the generation of free radicals), which is manifested by single- and double-strand breaks in the DNA molecule. Cells treated with radiation undergo arrest in both G_1 and G_2 phases with a consequent decrease in the fraction of cells present in the S phase: G_2/M block is the predominant form of arrest induced by radiation in many cell types and provides time for DNA repair [11–13]. If damage is irreparable, the cells die [14]. In addition, the nonpaired or misrepaired DNA lesions are thought to be responsible for chromosomal aberrations, which lead to cell death [15].

A number of p53 target genes with proapoptotic activity have been identified. They are classified in three groups on the basis of their subcellular location [16]. The first group concerns genes which encode proteins localized to the cell membrane, e.g. p53 Apoptosis effector related to PMP-22 (PERP); the second encodes cytoplasmic proteins, including p53 induced genes (PIG); and the last group of genes encodes proteins that localize on the mitochondria, e.g. p53- upregulated modulator of apoptosis (PUMA) [17,18]. In response to DNA damage, p53 also mediates growth arrest through the p53 target gene p21, which has been suggested as an instigator of G_1 arrest [19] and also upregulating the transcription of 14-3-3σ, which inhibits G_2 progression [20]. If repair has been successful, cells can resume proliferation.

The nature of the radiation–chemotherapy interaction is dependent on dose, tumor type and many cellular factors, with the efficiency of DNA repair and the cell cycle control mechanism probably being the most important [15,21,22]. In this study we evaluated the clonogenic capability, cell survival, perturbation of the cell cycle and induction of apoptosis, p53 expression, and the p53 induction of target genes *PUMA*, *PERP* and *PIG8*, as well as p21 and β-galactosidase, which are involved in cell cycle arrest and in cellular senescence.

The murine teratocarcinoma stem cell line F9 has been widely used as a model for the analysis of molecular mechanisms associated with early embryonic development, and retinoic acid regulation and differentiation; as embryonal carcinoma cells, they have served as a model to study the relationship between cancer and cellular differentiation given their potential to produce tumors, and, to varying degrees, participate in embryonic development [23]. Moreover, F9 cells have been widely used to study cellular injury response to damage caused by a cytostatic drug. In fact F9 cell line expresses wild-type p53 protein, and is sensitive to γ-irradiation and cytotoxic agents [24–28]. We are aware that this cell line represent only a model of what happens *in vivo* and that the use of a mouse model could be a limitation to translate results into human cancer; anyway, F9 cells represent an appropriate system in which it is possible to analyze events occurring through the p53 signaling cascade following various cycles of γ-irradiation and cytotoxic drug administration that recapitulate multimodal regimens. F9 cells were cultured and exposed to *cis*-DDP and to radiation as independent treatments or in association.

Methods

Cell lines

The F9 murine teratocarcinoma cell line was used for the study; this cell line is p53 wild-type with a cell cycle time of 12 h [29]. Cells were grown on culture flasks in Dulbecco modified Eagle medium supplemented with 5 mmol/l glutamine, 1 mmol/l sodium pyruvate and 10% fetal calf serum at 37°C in a 5% CO_2 atmosphere. For the analysis, cell cultures were treated with trypsin ethylenediaminetetraacetic acid in phosphate-buffered saline and cells were counted by a microscope so that the appropriate concentration was acquired for each test.

Exposure to radiation and/or cisplatin and harvesting times

Growing cells were irradiated using a linac Varian 600 C (Varian Medical Systems, Palo Alto, California, USA) with a photon beam of 6 MV with 2, 4, 6, 8 and 10 Gy. The absorbed dose was 2 Gy/day at a dose rate of 200 Umol/l/min. Alternatively, or in association, cells were exposed to *cis*-DDP in growth medium for 1 h at 37°C (see Table 1). The *cis*-DDP concentration ranged from 1 to 5 μg/ml. These concentrations are representative of the clinical dose calculated according to Tisman *et al.* [30]: 1 μg/ml represents the equivalent of 20 mg/m^2 used in a daily regimen [31] and 5 μg/ml is the equivalent of 100 mg/m^2 in the conventional regimen. When *cis*-DDP was applied in association with radiation only the dose of 1 μg/ml was used. The medium was then replaced with fresh medium

Table 1 Exposure to treatments and harvesting times

Treatment	Dose	Time of exposure	Harvesting times after treatment	Analyses
Radiation	2, 4, 6, 8, 10 Gy	Increments of 2 Gy daily, up to 10 Gy	24 h after each dose	Clonogenic test, dye exclusion, cell cycle, apoptosis, molecular profile
cis-DDP	1 and 5 μg/ml	1 h at 37°C	24, 48, 72, 96, 120 h	Clonogenic test, dye exclusion, cell cycle, apoptosis, molecular profile
Combination	*cis*-DDP 1 μg/ml; radiotherapy: 2, 4, 6, 8, 10 Gy	1 h at 37°C radiation administrated 96 h after *cis*-DDP	24 h after each dose of radiation	Clonogenic test, dye exclusion, cell cycle, apoptosis, molecular profile

cis-DDP, cisplatin.

and cells were incubated until harvest. In the combined treatment, radiation was administrated 96 h after *cis*-DDP exposure.

Clonogenicity assay

A concentration of 25×10^3 cells was seeded into 6-cm culture dishes in 5 ml of growth medium. Dishes were incubated at 37°C in humidified air with 5% CO_2 for colony formation for a period of 8–16 days. On day 8, 2 ml of fresh medium was added to each dish. At the end of the incubation, colonies were fixed with ethanol, washed with distilled water and stained with hematoxylin–eosin for counting. Each assay was made in triplicate and only colonies containing at least 30 cells were counted on an inverted microscope. Cells that remained attached to the plate but failed to form a colony were considered to be reproductively dead if both nucleus and cytoplasm were present. A minimum of at least 20 cell colonies in the control dish was required for an experiment to be valid. Results were expressed as a percentage of inhibition of colony formation (ICF) in treated cells compared with untreated control dishes. Dishes were counted and scored by two independent observers. Each experiment was repeated at least three times.

Dye exclusion test

The assay was performed according to the procedure described by Weisenthal *et al.* [32]. Cells were trypsinized and stained for 10 min with 1% Fast Green dye, sedimented onto microscope slides using a cytospin centrifuge (1200 r.p.m., 7 min) and counterstained with a modified hematoxylin–eosin technique. The slides were stained, without prefixing, in Harris hematoxylin solution to which glacial acetic acid had been added to a final concentration of 1.9%. After 90 s the slides were dipped quickly two times in a solution of 7.5% ethyl alcohol, and then placed in eosin–phloxine solution for 30 s. The slides were then dehydrated by dipping quickly twice in 95% alcohol, once in 100% alcohol and then in xylene. Living cells stained pink with hematoxylin–eosin and dead cells stained green. The ratio of living cells to living and dead cells, percent cell survival (PCS) was determined for each cytocentrifuge slide. This result was then expressed as a percentage of untreated control cells. Each assay was made in triplicate and each experiment was repeated at least three times.

Cell cycle analysis

Cell cycle kinetics were analyzed by flow cytometry. Bromodeoxyuridine (10 μmol/l) was added to the culture medium for 30 min at 37°C in humidified air and 5% CO_2. Cells were then rinsed with phosphate-buffered saline, trypsinized and fixed in 70% ethanol for 30 min at 4°C. After centrifugation cells were incubated in 2N HCl/Tween 20 (0.5% v/v) for 30 min at room temperature and then neutralized in 0.1 mol/l $Na_2B_4O_7$. Cells were washed in Tween 20 and anti-bromodeoxyuridine–fluorescein isothiocyanate conjugate was added for 30 min. Propidium iodide (PI, 5 μg/ml) was used for counterstaining just before running flow cytometry. Two-parameter flow cytometry was performed on a FACScan cytometer (Becton Dickinson, San Josè, California, USA) with approximately 5×10^4 cells analyzed for each sample. Data were analyzed using CELLQuest software (Becton Dickinson) and each experiment was repeated at least three times.

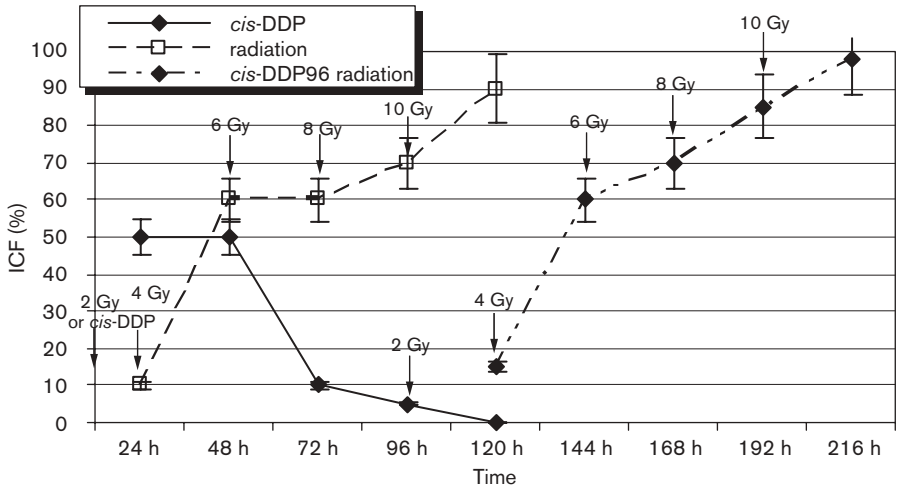
Apoptosis evaluation

Floating and adherent cells were collected and labeled fluorescently for the detection of apoptotic and necrotic cells with 5 μl of Annexin V–fluorescein isothiocyanate (Bender System, San Bruno, California, USA) in 195 μl of binding buffer. Samples were incubated at room temperature in the dark for 10 min. Immediately before analysis by flow cytometry, 10 μl of PI (1 μg/ml) was added to each sample and a minimum of 10^4 cells was analyzed. Data were represented by contour plots of PI versus Annexin V intensity, using the CELLQuest Software (Becton Dickinson) and the WinMDI2.8 Software (http://facs.scripps.edu/software.html). A graphical representation of two-parameter data was produced in which contour lines show the distribution of events. Data were analyzed by selecting the 50% log density: the inner-most contour represents 50% of the peak height, the next contour represents 50% of the first contour (or 25% of peak height) and so on (Fig. 2). To determine DNA laddering, total DNA was extracted according to the standard procedures [33] from supernatant and adherent cells, and electrophoresed on a 2% agarose gel.

Real-time quantitative reverse transcription-polymerase chain reaction

Total RNA was isolated using the Quigen RNA isolation kit (Quigen, Valencia, California, USA), and the two-step GeneAmp RT-PCR kit (Applied Biosystem, Foster City, California, USA; *https://products.appliedbiosystems.com/*) was used for RT-PCR, according to the manufacturers' protocol. For real-time reverse transcription-polymerase chain reaction (RT-PCR), triplicates of 25-μl PCR reactions were performed on 2 μl of cDNA obtained by the retrotranscription of 2 μg of RNA in a 100 μl reaction. Amplification and analysis were performed, according to manufacturer instructions, using ABI Prism 7000 Sequence Detection System (Applied Biosystems) with the following assays (Applera assay-on-demand): p53, p21, PUMA, PERP, PIG8 and β-galactosidase. Quantification of target gene transcripts was performed in comparison to the reference gene transcript Eukaryotic 18S rRNA, implementing the '$\Delta\Delta C_t$ method for comparing relative expression results between treatments in real-time PCR' as outlined by PE Applied Biosystems (Forster City, California, USA).

Fig. 1



Clonogenicity in F9 treated cells. Growing cells were irradiated with 2, 4, 6, 8 and 10 Gy; the delivered dose was 2 Gy/day at a dose rate of 200 Umol/l/min. Alternatively, or in association, cells were exposed to *cis*-DDP (cisplatin) in growth medium for 1 h at 37°C. The *cis*-DDP concentration was 1 μg/ml. In the combined treatment, radiation was administrated 96 h after *cis*-DDP exposure. Cells treated with *cis*-DDP alone were harvested after 24, 48, 72, 96 and 120 h after exposure. Cells treated with radiation alone and with the combined regimens were harvested 24 h after the administration of each dose of radiation, from 2 to 10 Gy. Radiation doses are indicated by plain arrows when applied as single treatment, as bold arrows when applied 96 h after *cis*-DDP administration. Indication of hours on the *x*-axis refers to the time past after the *cis*-DDP administration or after the first dose of radiation (when only radiation was given). Results were expressed as a percentage of inhibition of colony formation (ICF) in treated cells compared with untreated control dishes. Bars indicate standard deviation of three independent experiments.

Table 2 Treatment-induced changes in the F9 cell line cycle

	G_1 phase					$S–G_2$ late phase				
Control	16	20	24	26	25	4	3	3	4	4
Cis-DDP	24 h	48 h	72 h	96 h	120 h	24 h	48 h	72 h	96 h	120 h
	11	15	15	27	48	27	13	13	4	4
cis-DDP96 radiation	2 Gy	4 Gy	6 Gy	8 Gy	10 Gy	2 Gy	4Gy	6 Gy	8 Gy	10 Gy
	13	18	20	20	28	10	11	13	13	13

Cytometric analysis was performed 24, 48, 72, 96 and 120 h after *cis*-DDP treatment or, in the combined treatment, after 1 h with 1 μg/ml *cis*-DDP followed, starting 96 h later, by 2, 4, 6, 8 and 10 Gy radiation dose.
Data shown (expressed as percentage of cells) are representative of three independent experiments.

Results

Effect of treatment on cell survival

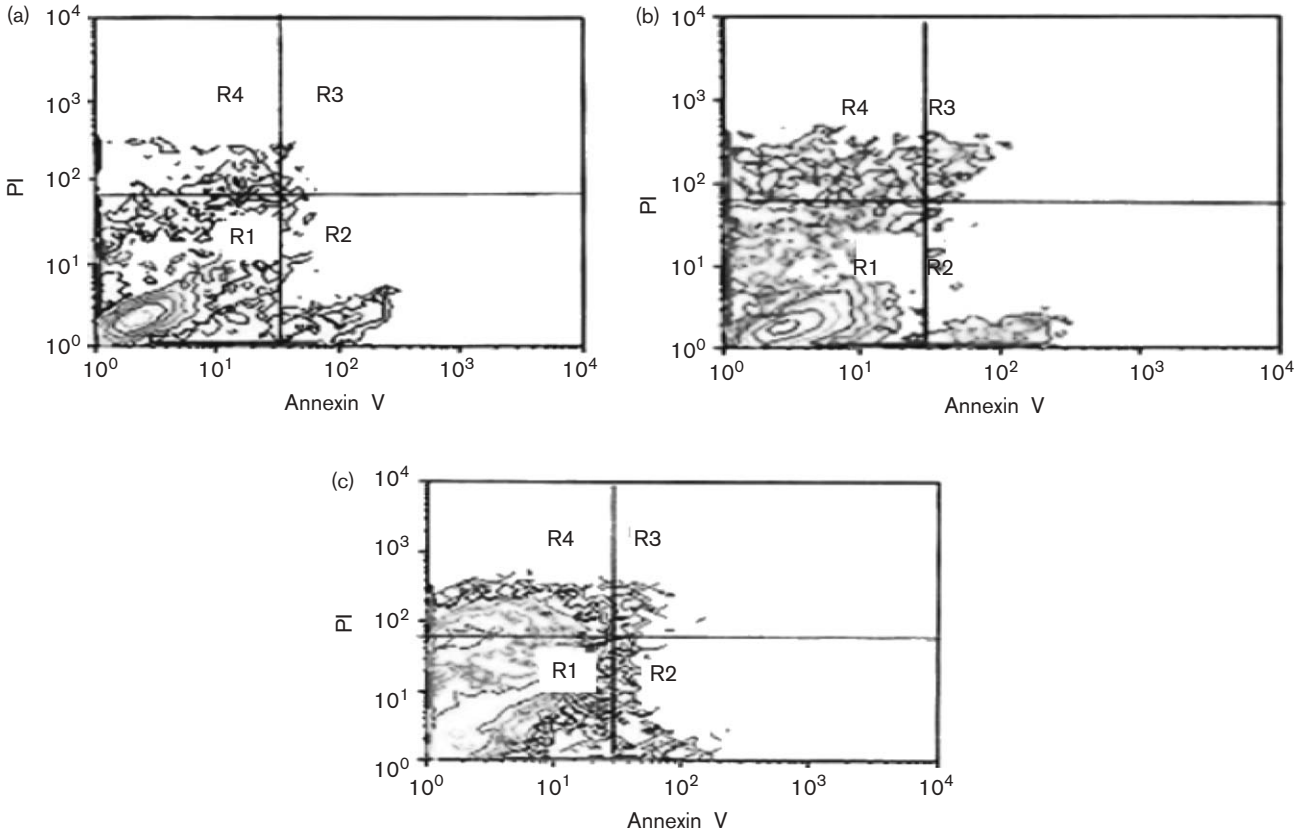
Following 1 h of *cis*-DDP treatment, the inhibition of cell growth analyzed by a clonogenic assay and expressed as percentage of ICF, was dose-dependent; we tested concentrations ranging from 1 to 5 μg/ml, that were representative of the clinical contest. The concentration that inhibited growth by 50 ± 10% over a 2-day period was 1 μg/ml (Fig. 1). At a concentration of 5 μg/ml, colony formation was inhibited by 75% over a 5-day experiment. Cells regained their ability to divide after a period dependent on the drug concentration: after 72–96 h at a concentration of 1 μg/ml and after 168 h at a concentration of 5 μg/ml.

cis-DDP at 1 μg/ml concentration induces minimal cell death, analyzed by a dye exclusion assay: cell viability decreased to 80% after 3–4 days, then increased and reached the same value as the control. Increasing the concentration of *cis*-DDP to a maximum of 5 μg/ml reduced cell viability to 60%.

The ICF after radiation exposure increased for each 2 Gy increment up to 10 Gy. At this point the ICF was 90% and cells began to divide again after 72 h. The percentage of cells killed by radiation remained constant (10%) up to 4 Gy and then increased to above 50% after administration of 10 Gy.

In the combined regimen a *cis*-DDP concentration of 1 μg/ml was used, considered to be minimally toxic, in order to avoid excessive cell damage that could mask the secondary effects of radiation. When radiation was administered after *cis*-DDP, the inhibition of cell growth increased at each dose: after 10 Gy cell death was quite

Fig. 2



	cis-DDP 48 h	4 Gy	*cis*-DDP–radiation 10 Gy
R2	16%	9.5%	7%
R4	14%	17.5%	65%

(Top). Flow cytometry analysis of Annexin V versus propidium iodide (PI) contour plot in cells treated with (a) 1 μg/ml *cis*-DDP at 48 h, (b) 4 Gy radiation and (c) combined treatment after 10 Gy. Here is reported the graphical representation of two-parameter data in which contour lines show the distribution of events and the contour lines show event frequencies as peaks and valleys. Four regions of cells are defined: R1 includes cells stained negatively for both Annexin V and PI and considered alive; R2 includes cells that are stained with Annexin V but are PI-negative and considered apoptotic; R3 includes cells that are both Annexin V and PI-positive and which are classified late apoptotic; R4 includes cells which are PI-positive and considered necrotic. (Bottom). Most significant degrees of apoptosis and necrosis, expressed as percentage of cells after: 48 h after treatment with 1 μg/ml *cis*-DDP after 4 Gy radiation and after combined treatment (1 h with 1 μg/ml *cis*-DDP and 10 Gy radiation). Apoptotic cells were identified as those contained in the R2 region of the contour plot; necrotic cells were identified as those contained in the R4 region of the contour plot.

pronounced with a viability of 20%; cells no longer divided and permanently lost their ability to form colonies (unshown data).

Effect of treatment on cell kinetics

After *cis*-DDP treatment cells became arrested in the final S phase and in the G_2 phase of the cell cycle. At a concentration of 1 μg/ml the G_2 arrest increased to up to 72 h and was transient, with reversal starting at 96–120 h. At a concentration of 5 μg/ml G_2 arrest persisted throughout the 5-day experiment. Cells treated only with radiation were arrested in both G_1 and G_2, with a decrease in the fraction of cells in S phase, with a maximal impact at 72 h; the delay in the G_1 and G_2 phases was dose related, and after 10 Gy cells were unable to replicate until 72 h after treatment. In the combined treatment, G_1 and G_2/M blocks were marked, and radiation administered 96 h after *cis*-DDP did not allow the cells to bypass G_2 arrest (Table 2).

Effect of treatment on apoptosis

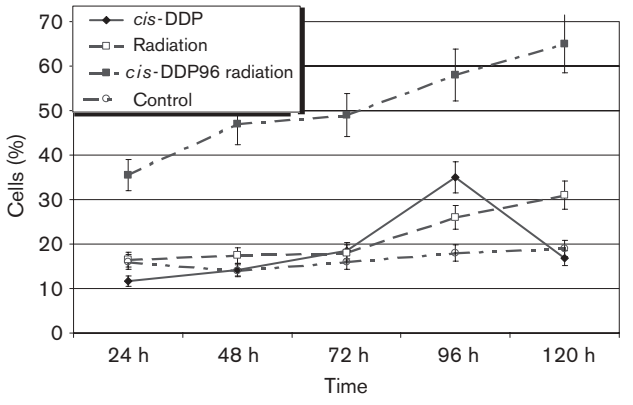
F9 untreated cells showed a small percentage of apoptotic cells that reached a value of 8% after the cells remained undivided for 120 h. In samples treated with *cis*-DDP, the percentage of apoptotic cells reached 16% at 48 h after treatment (Fig. 2a), whereas radiation induced apoptosis mostly after 4 Gy (Fig. 2b); at this time the fraction of apoptotic cells was 9.5%. In F9 cells treated with both radiation and *cis*-DDP, the apoptotic percentage was 13.5% after 4 Gy. In contrast, the percentage of necrotic cells increased substantially in the combined regimens after each radiation dose and peaked at 65% after 10 Gy (Figs 2c and 3).

It was observed that no fragmentation occurred at any drug concentration on the first day, using gel electrophoresis to resolve nucleosome; after 2 days a faint characteristic nucleosome ladder consisting of multimers of 180 base pairs was observed in F9 cells treated with *cis*-DDP after 24 and 48 h (data not shown). No nucleosome fragments were observed after radiation and radiation with *cis*-DDP.

Effect of treatment on gene expression

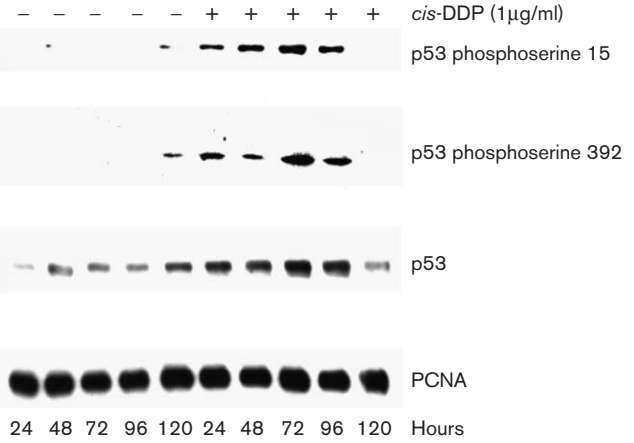
No accumulation of p53 mRNA was observed in the treated cells. Analysis of p53 levels by Western blotting was performed in cells 24 h after treatment; we observed only a modest increase in p53 protein in irradiated cells, whereas a larger and more sustained increase in p53 protein was seen in *cis*-DDP-treated cells (data not shown). p53 serine 15 and 392 were also analyzed for their phosphorylation status on cell lysates obtained from cells 24 h after treatment with ± *cis*-DDP of 1 μg/ml (Fig. 4). These results are in agreement with data of Fig. 5, where quantitative differences in gene expression revealed an upregulation of PUMA and PERP at 24–48 h following *cis*-DDP and after 6 Gy (Fig. 5). An upregulation of p21 mRNA was seen only after combined treatments. No substantial changes were seen in the expression of PIG8 and β-galactosidase (unshown data).

Fig. 3



Percentage of necrotic cells measured by flow cytometry at different harvesting times in untreated and treated F9 cells. Cells were treated as detailed in Fig. 1. Percentage plotted corresponds to the number of cells counted in the cytoflourimetric analysis with Annexin V–fluorescein isothiocyanate and propidium iodide (PI), which resulted Annexin V-negative and PI-positive (quadrant R4, as defined in Fig. 2).

Fig. 4



Studies of phosphorylation at serines 15 and 392 in p53 in the F9 cell line non treated or treated with *cis*-DDP 1 μg/ml, and analyzed 24, 48, 72, 96 and 120 h after. Proliferating cell nuclear antigen (PCNA) is used as housekeeping protein.

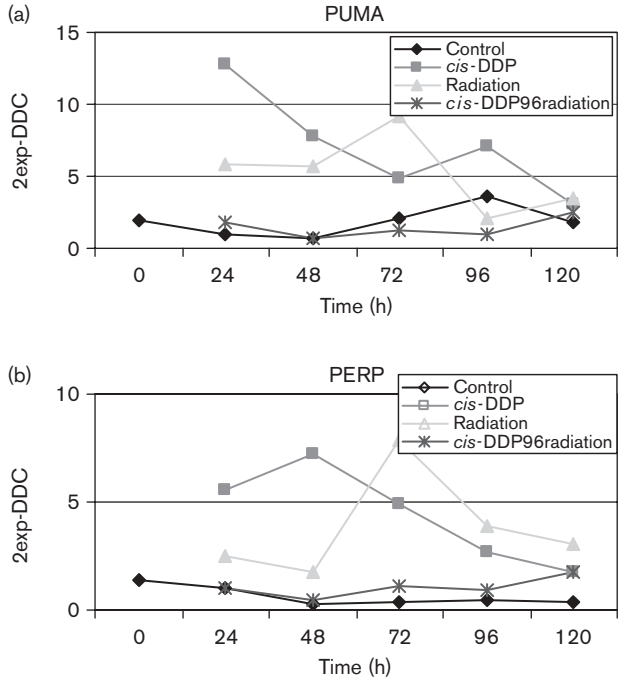
Discussion

cis-DDP has been found to sensitize tumor cells to the effects of irradiation and concurrent *cis*-DDP chemoradiotherapy is considered as the standard treatment in locally advanced HNSCC [4]. Results from *cis*-DDP chemoradiotherapy studies using exponentially growing cell cultures range from a *cis*-DDP-induced radiosensitization to only an additive effect. The effects of *cis*-DDP treatment on radiosensitivity may depend on the cell type used [15,21].

We have analyzed the effects of a combined *cis*-DDP-ionizing radiation regimen on cytotoxicity and apoptosis in the murine teratocarcinoma F9 cell line, accepting the limitation that the choice of a mouse model may lessen the translational impact of the work. *cis*-DDP alone did not exhibit marked cytotoxic effect, but, when combined with radiation, radiosensitization was put into evidence. Radiosensitization can act through the inhibition of cellular recovery of radiation damage, as recently reported by Raaphrost *et al.* [22] and Ruy *et al.* [4], who analyzed the interaction of heptaplatin and ionizing radiation in human carcinoma cell lines.

In these experiments, *cis*-DDP-induced G_2 arrest was followed by recovery at 72–96 h with passage of cells through to the next cell cycle. When radiation was

Fig. 5



Quantification in real-time reverse transcription-polymerase chain reaction of PUMA and PERP target gene transcripts performed in comparison to the reference gene transcript Eukaryotic 18S rRNA using the '$\Delta\Delta C_t$ method' for comparing relative expression results during time between treatments (*cis*-CCP, radiation and combined treatment as detailed in Table 1).

administered 96 h after *cis*-DDP no recovery was observed and cells died. Cells treated with *cis*-DDP and radiation demonstrated a continual decrease in viability; after 10 Gy cell death was quite pronounced with a viability of about 20%, indicating a clear additive interaction of radiation and *cis*-DDP. These results confirm that radiotherapy following *cis*-DDP chemotherapy has the potential to increase cell death and to reduce tumor repopulation owing to proliferating subclones.

There are two major mechanisms of cell death – necrosis and apoptosis, or programmed cell death [2–3,14]. Although understanding of the detailed signaling pathways that cause apoptosis is incomplete, this process is known to be controlled by a number of complex proteins, which are activated by various triggers and organized in sequential signaling modules [34–36]. On the contrary, necrosis is characterized morphologically by vacuolation of the cytoplasm, destruction of organelles, disruption of the plasma membrane and induction of inflammation, and it has been traditionally considered to be only a passive form of cell death, although many recent studies now describe that necrotic forms of cell degeneration may be programmed [37].

Several authors have reported a correlation between apoptosis during treatment and loss of clonogenicity of the tumor cells [15,38–40]. It appears logical that the reduction of clonogenicity and the induction of DNA damage and apoptosis may improve the efficacy of cytotoxic drug and radiation therapy. Our data on the F9 cell line, however, indicate that loss of clonogenicity after exposure in culture to radiation and *cis*-DDP is not associated with apoptosis. These results show that both *cis*-DDP and radiation induced cell death, but the level of induced apoptosis was low and there was no correlation with the results of the clonogenic assays: we noted a difference between cytotoxic effects in the clonogenic assay and the extent of apoptosis by FACS analysis, suggesting that cell killing reflected not only apoptosis but also cell cycle arrest, and that apoptosis, cell kinetics and clonogenicity suppression were independent processes. This finding is in agreement with the study by Tannock *et al.* [41], who concluded that apoptotic mechanisms had at most a minor role in leading to reproductive death of MGH-U1 and CHO cells after chemotherapy, suggesting that when apoptosis is observed following treatment with anti-cancer drugs it may be a secondary event which occurs in lethally damaged cells, leading to their lysis, rather than a primary event that leads to loss of reproductive integrity. Kumala *et al.* [42] showed the extent to which apoptosis is induced and clonogenicity reduced in three tumor cell lines of different origin and concluded that cells which readily undergo apoptosis do not necessarily show a correlated loss of clonogenicity. A similar conclusion was reached by Russell *et al.* [43], who measured the response of radioresistant human neuroblasoma cell lines to several cytotoxic agents.

The role of apoptosis in response to radiation damage is also controversial: some studies suggest its importance [44,45], whereas others demonstrate that apoptosis is not the predominant form of cell death after radiation exposure [40]. In some studies, the degree of radiation-induced apoptosis has been shown to correlate with p53 status [46]. In particular, Zeng *et al.* [28] reported that UV, but not γ-irradiation (7 or 10 Gy) caused a massive apoptosis in human tera-2 or murine F9 cell lines, both of which contain wild-type p53. The p53 tumor suppressor gene plays a central role in the regulation of the apoptotic machinery, suppressing the growth of genetically damaged cells by suspending the cell cycle and by promoting programmed cell death. Apoptosis caused by anticancer drugs or radiation has either a p53-dependent or a p53-independent pathway [47,48]. Accordingly, the cell arrest caused by radiation or anticancer drugs also has a p21-dependent or a p21-independent pathway [49].

A number of p53-regulated genes containing p53 responsive elements have been identified, and some of

these represent potential downstream mediators of p53-dependent apoptosis. These include *Bax*, *CD95* (*Fas/APO-1*), *Killer/DR5*, *Ei24/PIG8*, *Noxa*, *PERP*, *Pidd*, *p53AIP1* and *PUMA* [16]. Out of these genes, the only one found to be significantly upregulated in this study was p21 after treatment with *cis*-DDP and radiation in association. Rather than induction of apoptosis, the p53 gene seems here to be involved in regulating the stress response of F9 cells by controlling growth arrest. We did not find a p53 accumulation in the F9 cell line after treatment and these data are in agreement with the model proposed by Chen *et al.* [50] suggesting that wild-type p53 levels are important to decide whether the cells undergo cell cycle arrest or apoptosis; growth arrest has been correlated with low levels of p53. We found an upregulation of p21 that may provide an explanation for the maintenance of induced cell cycle arrest. Data reported here showed an association between the levels of p21 expression and the dominant effect of radiation and *cis*-DDP inducing growth arrest over apoptosis. Radiation and *cis*-DDP administered in association induced irreparable DNA damage; the repair processes are themselves overwhelmed and cell death occurs to eliminate damaged cells. Lee *et al.* [51] found that when the hypopharyngeal carcinoma PNUH-12 cell line is exposed to *cis*-DDP, the quantity of p53 protein increased but progressed to apoptosis without an increase in p21 protein; the S phase of the cell cycle in DNA flow cytometry increased. In this study, there were no significant changes in p53 and p21 following exposure to radiation, but the G_2-phase arrest of the cell cycle and apoptosis were put into evidence. Previously, Wang *et al.* [52] did not observe apoptosis following exposure to *cis*-DDP in the nasopharyngeal cancer cell line but only observed the senescence-like growth arrest of the G_2/M phase.

The results reported here strengthen the notion that radiation associated with *cis*-DDP can induce an improved cellular response in terms of mortality and prolonged cytostatic arrest. In addition, the importance of factors such as cell death through necrosis, senescence, DNA repair capacity and the efficiency of cell cycle controls are shown as playing here an important role in *cis*-DDP-radiation association. All these mechanisms are mediated by the molecular profile of the target tumor cells and improvements in clinical strategy may depend on the one that is the most involved in cellular response according to the molecular profile of the individual tumor to be treated.

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