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SHORT COMMUNICATION

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Sensitivity to cisplatin treatment of human K1 thyroid carcinoma cell lines with altered p53 function

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Abstract *Aim*: p53 is the most frequently altered gene in human cancer. It was expected to be the principle marker for chemo/radiosensitivity, resistance, tumor recurrence and ultimate survival, but is no longer considered a universal prognostic factor. Different alterations in the p53 gene have led to conflicting results depending on cell/tissue specificities and on radiation and drug specificities. *Methods*: We evaluated the properties of isogenic and isophenotypic cell lines from the K1 papillary thyroid carcinoma in which p53 function was disrupted either by mutation (expression of dominant-negative p53, 143^{ala}) or by inactivation (expression of human papilloma virus protein HPV16 E6). Their proliferation, their propensity to trigger apoptosis and their survival were analyzed after treatment with cisplatin (CDDP). *Results*: Only K1 lines with wild-type p53 had significantly accumulated apoptotic bodies 72 h after treatment. Despite their incapacity to trigger apoptosis in response to CDDP treatment, the survival of K1 cell lines in which p53 expression was altered was not significantly different from the survival of K1 cell lines expressing wild-type p53. In addition, the order of magnitude of resistance of K1 cells in which p53 was mutated was similar to that in which p53 was totally inactivated, although the pathways involved may be different. *Conclusions*: These results show that the means by which p53 expression is disrupted and the consequence on downstream pathways regulated by p53 deserve to be considered in order to elucidate some apparently conflicting responses of this gene.

Keywords K1 thyroid carcinoma cells · p53 status · Cisplatinum · Apoptosis · Survival

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Introduction

Despite numerous strategies to develop new treatments, cancer is still one of the main causes of mortality in developed countries. A significant effort has been made to identify specific molecular markers which would help predict either resistance to treatment or post-treatment recurrence. About 20 years ago, a new family of genes involved in the control of cancer emerged: the tumor suppressor genes. Among them the p53 gene appeared to be the most prominent marker in human cancer pathology being mutated in over 50% of tumors [6, 9, 23]. It was expected to be a valuable marker for predicting chemo/radiosensitivity or resistance of tumors especially on the basis of its capacity to activate apoptosis following DNA damage [1, 3]. Being a transcription factor, p53 activates multiple downstream genes. Several approaches attempting to identify p53-regulated genes have revealed the existence of over 200 p53 target genes [5, 21].

Numerous in vitro and in vivo studies have been conducted with the aim of determining the general mechanism correlating p53 status and the response(s) of tumors to chemo/radiotherapy. Among the several experiments designed to address this question are in vitro experiments using normal or transformed cell lines in which p53 is mutated, inactivated, deleted or transfected and in vivo experiments using genetically modified animals [23]. Experiments trying to reactivate mutant p53-specific DNA binding have been attempted [14, 15] as well as pharmacological suppression of p53 in order to reduce the side effects induced by cancer treatment [8]. p53 tumor suppressor gene therapy has also recently emerged and is currently in clinical trials [7, 13, 26].

To date these experiments have yielded conflicting results and do not allow the delineation of a clear-cut mechanism. It remains difficult to reconcile in vitro and in vivo results and even more importantly clinical data [11, 23]. Existing data suggest that the correlation between response of tumors to anticancer therapy and p53 status is not only largely cell/tissue specific but also depends on the type of treatment. We evaluated the behavior of isogenic and isophenotypic cell lines from human K1 papillary thyroid carcinoma displaying either a mutated or an inactivated p53 status [4, 24, 25]. The various cell lines were examined for proliferation, propensity to trigger apoptosis and survival after treatment with cisplatin (CDDP).

Materials and methods

Materials

Cell culture media (Dulbecco's modified Eagle's medium with sodium pyruvate and pyridoxine, Ham's F12 and MCDB 104), phosphate-buffered saline (PBS) Dulbecco's formulation, Hank's balanced salt solution (HBSS), trypsin-EDTA, antibiotics (penicillin and streptomycin) and fetal calf serum (FCS) (myoclone plus, virus and mycoplasma screened) were from Gibco (Paisley, UK). Culture dishes were from Falcon Corning (Corning, N.Y). Cis-dichlorodiammine platinum (cisplatin, CDDP) was from Laboratoires Qualimed (Puteaux, France). All other chemicals were of analytical grade from Sigma (L'Isle d'Abeau Chesnes, France) or Merck (Darmstadt, Germany).

Cell culture

The wild-type human thyroid carcinoma cell line K1 (K1 wt) was purchased from ECACC, Cerdic (Sophia Antipolis, France) and its derivatives K1 neo11 (K1 neo), K1 SCX3 expressing a dominant-negative human mutant p53 143 ^{ala} (K1 μt) and K1 E6.4 expressing the E6 gene from human papilloma virus type 16 (K1 E6) were kindly provided by Dr. Wynford-Thomas [24, 25]. The parental cell line and its derivatives were grown in medium consisting of a mixture of Dulbecco's modified Eagle's medium, Ham's F12 and MCDB 104, (2:1:1, v/v/v) supplemented with 2 m*M* L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS.

Colorimetric proliferation and cytotoxicity assay

Confluent cultures were trypsinized, cells were seeded in 96-multiwell plates at a density of 15,000 cells/cm^2 and allowed to adhere. The cells were exposed 24 h later to 10 or 20 μg/ml CDDP (33 and 67 μ*M*) for 1 or 4 h. At the end of the incubation period, the plates were rinsed with HBSS and then further incubated in complete culture medium. Drug-induced cytotoxicity was measured 24 and 72 h after treatment using the Sulforhodamine B (SRB) colorimetric end point assay. Quantification of cell proliferation by this method results in a linear response at cell densities ranging from sparse to multilayered confluence [16]. Optical density was measured in a microplate reader (MRX, Dynex Technologies, Issy-les Moulineaux, France) using Biolinx software. The results are expressed as percentages of the values from untreated control cells set at 100%.

Detection of apoptotic cells

Apoptosis induced by CDDP treatment of K1 cells was assessed by visualization of the morphological changes, especially chromatin condensation, after staining the cells with the fluorescent dye Hoechst 33258. Experimental conditions were identical to those used in the proliferation and survival assays. At 24 and 72 h after CDDP treatment the cells from supernatants and adherent cells were collected, centrifuged and washed twice with cold PBS. After a final centrifugation the cells were processed according to the recommendations of the manufacturer. An aliquot (20 μl) representing one-fifth of the suspension of each cell suspension was placed on a microscope slide, covered with a coverslip and examined by fluorescent microscopy (Axiophot Zeiss, Germany). Bright condensed nuclei of apoptotic cells were enumerated and expressed as percentages of the total number of nuclei.

Clonogenic survival

The effects of CDDP on K1 cell survival were investigated in a clonogenic assay according to established procedures [20]. Clonogenic survival experiments were performed in conditions comparable to those of the proliferation experiments. For each condition an appropriate number of cells which would yield approximately 50 to 100 clones per dish was seeded in 100/20 mm Petri dishes. The cells were treated 24 h later with CDDP at 10 or 20 μg/ml for 1 or 4 h. After CDDP removal the dishes were incubated. After 15 days incubation without a medium change, the cultures were carefully rinsed with PBS and stained with May-Grünwald Giemsa dye and colonies containing more than 50 cells were counted [2]. The results are expressed as percentages of the values obtained from untreated control cultures and plotted on a semilogarithmic scale.

Results

The cytostatic/cytotoxic effects of CDDP on K1 cell lines exposed to 10 or 20 μg/ml of CDDP for 1 or 4 h were estimated 24 and 72 h after treatment using the SRB colorimetric assay. Between 24 and 72 h, the average cell density of control cells increased threefold (data not shown). Exposure of K1 cells to 10 μg/ml CDDP for 1 h led to a mean cell density decrease at 72 h of about 35% (Fig. 1). This decrease reached 50% when the duration of exposure was 4 h. Increasing the concentration of CDDP to 20 μg/ml led to a diminution in cell density of 46% in cultures exposed to CDDP for 1 h and this decrease reached almost 70% when the treatment lasted 4 h. Apoptosis was estimated under these conditions and appeared also to be time and dose dependent. A 1-h exposure to 10 or 20 μg/ml CDDP had failed to trigger significant nuclear chromatin condensation by 24 h after treatment. Even after 72 h, only less than 5% of apoptotic nuclei could be detected.

Treating the cells with 20 μg/ml CDDP for 4 h induced the development of apoptotic bodies in K1 wt and K1 neo cells. The proportion of apoptotic bodies had reached 20% of total nuclei 24 h later and increased with time leaving only 40% nonapoptotic residual cells 72 h after exposure to the drug. Apoptotic bodies were also detected in the K1 E6 cell line but at a much lower rate: they had reached less than 20% at the 72-h time point. No apoptotic bodies could be detected in the K1 μt cell line under these conditions. We next investigated whether the capacity of cells to activate apoptosis would influence their subsequent survival. Exposure of the cells to 10 μg/ml CDDP for 1 h inhibited the survival

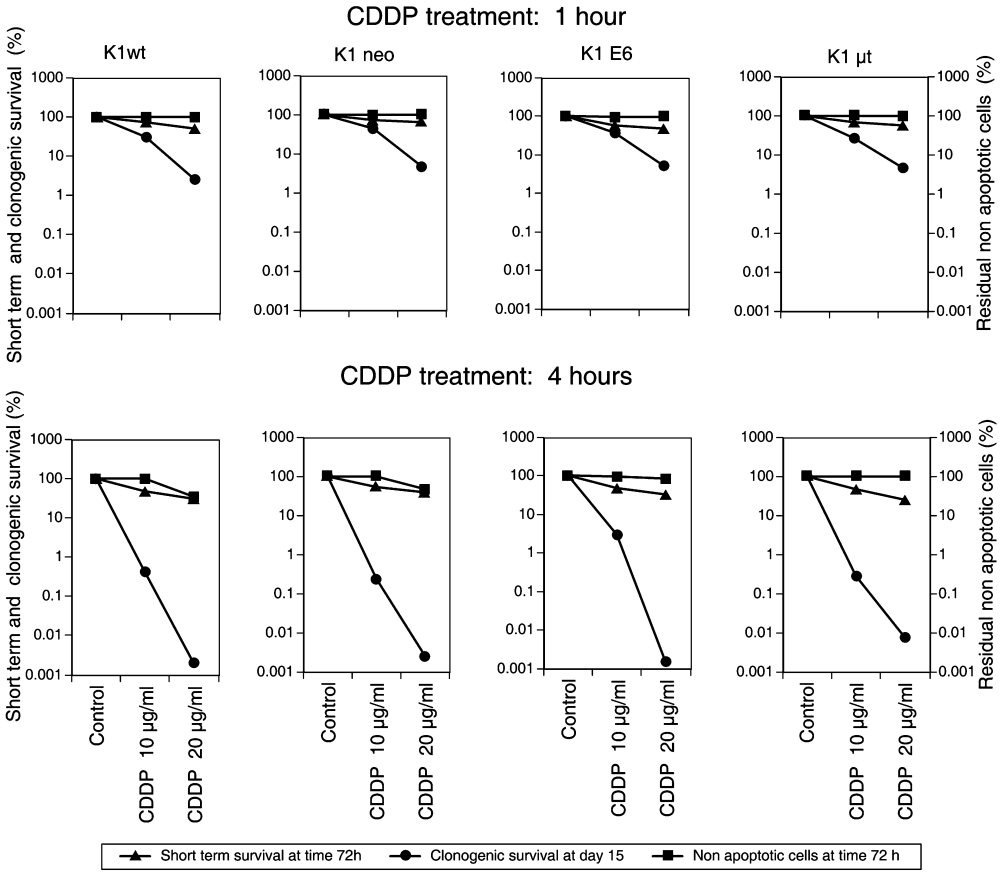

Fig. 1 Effects of exposure to CDDP on K1 cell lines. K1 cell lines were treated with 10 and 20 µg/ml CDDP for 1 or 4 h. Short-term survival was estimated using the SRB colorimetric assay 72 h after CDDP treatment. The data presented for each cell line correspond to the means ± SEM from two separate experiments performed in sextuplicate. Apoptosis was visualized by staining of condensed nuclear chromatin with Hoechst 33258. Residual nonapoptotic cells were estimated at 72 h. Clonogenic survival was estimated 15 days after exposure to CDDP. The data for each cell line are percentages of the value of the control set a 100% presented on a semilogarithmic scale and are the means ± SEM from three or four separate experiments performed in triplicate. The error bars were too small to appear on the graphs on the logarithmic scale

of each K1 cell line by about 70%. Increasing the concentration of CDDP to 20 µg/ml enhanced the inhibitory effects of the drug to about 95%. When the cells were exposed to 10 µg/ml CDDP for 4 h, the residual survival ranged between 3% for K1 E6 and around 0.3% for K1 wt, K1 neo and K1 µt cells, while with 20 µg/ml CDDP the clonogenic survival was decreased by about 4 logs for K1 µt cells and almost 5 logs for K1 wt, K1 neo and K1 E6 cells.

Discussion

Normal cells possess wild-type p53 allowing damaged cells to arrest in the G_1 cell cycle phase [9] and eventually in G_2 [19] for the time necessary to restore intact DNA. Following DNA damage certain cell types undergo p53-dependent programmed cell death or apoptosis. This property of p53 was initially demonstrated by restoring wild-type activity of p53 to cells in which p53 had been altered (reviewed in references 3 and 9). Many cancer cells although transformed retain wild-type p53 activity and do not undergo apoptosis despite extensive DNA damage. Furthermore, cells in which the functions of p53 have been disrupted lose their capacity to activate p53-driven apoptosis [1]. Whether p53 disruption sensitizes or renders cells more resistant to treatments still remains a matter of debate.

In the study reported here, using isogenic and isophenotypic human thyroid cancer K1 cell lines displaying different p53 statuses, we demonstrated that the ability of these cells to activate p53-dependent apoptosis is not predictive of their long-term survival after CDDP treatment. The response of tumor cells to chemo- or radiotherapy not only depends on their p53 status but also appears to be tissue-specific (reviewed in references 11 and 23). To address this, we analyzed the sensitivity of K1 cell lines displaying various p53 statuses to treatment with one of the most widely used chemotherapeutic agents, CDDP. In particular, we investigated the extent to which apoptosis is involved in the death of K1 cell lines after exposure to CDDP. The four K1 cell lines displaying different p53 statuses have been described previously [4, 24, 25].

On the whole, these cells appeared to be rather resistant to apoptosis since the rate of apoptotic nuclei in K1 cell lines displaying wild-type p53 (K1 wt and K1 neo) was only of 20% 24 h after treatment with 20 µg/ml CDDP for 4 h and had reached 50–60% by 72 h. The conditions used here to induce apoptosis are extremely drastic and far from conditions achievable in vivo [12]. As expected, in cell lines in which p53 expression was altered the induction of apoptosis, determined by chromatin condensation, was either significantly diminished or totally abolished. Consequently, we aimed to correlate the capacity of K1 cell lines to trigger apoptosis

after CDDP treatment with their subsequent clonogenic survival. All four K1 cell lines, independent of their p53 status, displayed similar resistance to CDDP treatment. Extended (4 h) exposure to high concentrations of CDDP (20 μg/ml) inhibited the survival of K1 wt, K1 neo and K1 E6 by nearly 5 logs. The K1 μt cell line, which expressed a dominant-negative mutant p53 and for which the survival was diminished by about 4 logs, seemed slightly more resistant.

In this study, although the K1 cell lines expressing wild-type p53 were able to activate apoptosis in response to CDDP treatment, this did not allow prediction of their final survival. Despite the capacity of K1 wt and K1 neo cells to induce apoptosis in response to CDDP treatment leading to the formation of over 50% apoptotic nuclei 72 h after treatment, the clonogenic survival of these cells was not significantly modified as compared to the survival of K1 E6 and K1 μt in which apoptosis was only moderately triggered or not at all. Brown and Wouters have demonstrated that cell proliferation inhibition detected by short-term toxicity assays do not allow the prediction of their long-term survival [2]. In particular, cells which do not die immediately after treatment accumulate damage which prevents their final survival after several generations. We confirm the need to perform clonogenic survival experiments in order to evaluate the long-term toxicity of a drug.

Short-term cytotoxicity assays allow the analysis concurrently of several phenomena including cytostasis, cell death and proliferation of surviving cells, but the resulting data do not allow the evaluation of the respective impact of these separate events. In accordance with these observations, Steel [18] recently emphasized the lack of correlation between the extent of apoptotic death and the loss of reproductive integrity as assayed by clonogenic survival assays. The results of the three approaches (short-term colorimetric assay, measure of apoptosis and clonogenic survival) used to assess the putative role of p53 alterations in cell apoptosis and survival are summarized in Fig. 1 and suggest the following conclusions. First, assessing cell survival by short-term cytotoxicity assays lacks sensitivity and does not reflect the reproductive integrity of surviving cells probably giving, at best, evidence of the therapeutic efficacy of exposure of cancer cells to a given cytotoxic DNA-damaging agent. Second, regarding the lack of correlation between apoptotic death and cell survival, our results show that the ability to develop surviving clones may be decreased by over 4 logs even in the absence of any observable apoptosis up to 72 h after exposure to CDDP. Conversely, the occurrence of visible apoptosis does not ultimately increase cell death as measured by clonogenic assays. Finally, with regard to the role of p53 in apoptosis and cell survival in K1 cells after exposure to CDDP, p53 integrity is required to induce apoptosis in K1 wt cells but the latter was only observed after a long exposure to high concentrations of CDDP. In the present model, p53-induced apoptosis does not increase the overall cytotoxicity induced by CDDP.

Altering p53 function in cells does not allow prediction of its subsequent sensitivity or resistance. The means used to disrupt p53 expression (degradation, mutation, deletion) and the specificity of the cells in which these experiments are performed are important. The consequences induced downstream by p53 disruption are difficult to anticipate as this tumor suppressor gene stands at the junction of highly connected signaling pathways [22] and thus regulates numerous downstream genes [5, 17, 21]. Examining which path of the network is affected as a function of the means by which p53 has been altered may help to elucidate some apparently opposite reactions/responses of this gene [10]. In any case, interpretation of data in this field should take into account the type of experimental methods used to assess cell toxicity and cell survival.

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