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Advances in Brief

Evidence of Cisplatin-induced Senescent-like Growth Arrest in Nasopharyngeal Carcinoma Cells[1]

Xianghong Wang, Stephen C. H. Wong, Jie Pan, S. W. Tsao, Kevin H. Y. Fung, Dora L. W. Kwong, Jonathan S. T. Sham, and John M. Nicholls[2]

Departments of Pathology [X. W., K. H. Y. F., J. M. N.], Anatomy [S. C. H. W., J. P., S. W. T.], and Radiation Oncology [D. L. W. K., J. S. T. S.], University of Hong Kong, Queen Mary Hospital, Hong Kong, People's Republic of China

Abstract

Cellular senescence is a programmed cell response leading to growth arrest in human diploid fibroblasts. We have shown that a nasopharyngeal carcinoma cell line, CNE1, following treatment by the DNA-damaging agent cisplatin, can undergo cellular senescent-like growth arrest, similar to fibroblasts, judged by cellular morphological changes and the expression of senescence-associated β-galactosidase (SA-β-gal). This senescent-like change was dose related; at 0.5 μg/ml, the percentage of cisplatin-induced SA-β-gal-positive cells was high (40–96%), and the staining was intense. Higher doses (1.0 and 2.0 μg/ml) of cisplatin induced lower SA-β-gal expression (30–70%), and the process was irreversible. This cisplatin-induced cellular senescent-like response was not due to the inhibition of telomerase activity. Our results indicate that cellular senescent-like pathways exist in nasopharyngeal carcinoma cells and can be induced by cisplatin. Our evidence suggests that cellular senescent-like responses may be a cellular protection mechanism that acts differently in response to different degrees of cellular damage.

Introduction

Senescence has been suggested to be a programmed cellular response, the induction of which depends on the accumulated number of cell doublings in human diploid fibroblast cells (1, 2). Human fibroblast cells from an older adult undergo senescence sooner than the cells from a younger adult (3). Recent evidence suggests that senescence can also occur in response to stimuli other than aging, such as H-*ras* oncogene transfection (4), treatment with DNA-damaging agents such as bleomycin and mitomycin D (5), and phosphatidylinositol 3-kinase inhibitors (6). It appears that senescence might represent an alternative cellular response to apoptosis following extracellular stimuli, possibly representing a short-term response induced to block further cellular proliferation (7). Although many of the mechanisms involving cellular senescence are still not well established, a strong correlation between telomere shortening and cellular senescence has been made. Telomeric DNA shortens during the growth of human somatic cells, and senescence of these cells may occur as a result of a check point arrest in response to the shortened telomeres (8, 9). Both shortening of telomere and inhibition of telomerase activity have been reported in cells treated with DNA-damaging agents such as cisplatin (10–12).

Escape from senescence has been implicated as a mechanism of tumor development in immortalized human uroepithelial cells and bladder cancer cell lines (13). The evidence that HeLa cells became senescent after the introduction of normal human chromosome 4, which has been reported to carry gene(s) responsible for inducing senescence, also supports this hypothesis (14). Thus far there is little evidence of induction of senescence in tumor cells, either *in vitro* or *in vivo*, by extracellular stimuli. The aim of this study was to determine whether the changes induced by a DNA-damaging agent, cisplatin, in a NPC[3] cell line, CNE1, could be attributed to cellular senescence, rather than apoptosis. Our results showed that the CNE1 cells not only displayed a similar morphology to senescent fibroblasts but also expressed SA-β-gal, a biomarker for cellular senescence. In addition, the percentage of cells positive for SA-β-gal was different in cells treated with increasing doses of cisplatin.

Materials and Methods

Cell Culture and Drug Treatment. The human NPC cell line CNE1, derived from a poorly differentiated NPC (15), was maintained in RPMI 1640 (Life Technologies, Inc.) supplemented with 2 mM L-glutamine and 10% (v/v) FCS. All cultures were maintained *in vitro* for <10 passages continuously. Primary cultured tumor cells were obtained by growing biopsies directly from untreated NPC patients in the above-mentioned cell culture conditions. The primary cultured NPC cells were confirmed by positive staining with an antibody against human cytokeratin (AE1/AE3; DAKO), and the presence of EBV using the nonisotopic EBER-PNA probe (DAKO) according to the manufacturer's instructions. Cisplatin was purchased from David Bull Laboratories (Victoria, Australia).

Cell survival experiments were carried out by plating 10^4 cells using 12-well plates (Corning), 24 h before cisplatin treatment. Three concentrations of cisplatin (0.5, 1.0, and 2.0 μg/ml) were added for 24 h in duplicate wells. Cell viability was measured every 24 h up to 120 h and judged by the cells' ability to exclude trypan blue [0.5% (w/v) in PBS]. Each experiment was repeated three times, and the cell survival curves were drawn by plotting the means and SDs of three experiments.

Cell Cycle Analysis. Cell cycle analysis was performed on an EPICS profile analyzer using the ModFit LT2.0 software (Coulter). The CNE1 cells (10^6) were treated with 0.5 and 2.0 μg/ml cisplatin and harvested at 24 and 48 h. The cells were then fixed with cold 70% ethanol for at least 1 h and stored at 4°C. Before testing, the cells were treated with RNase (1 mg/ml) and stained with PI (400 μg/ml) for 30 min at 37°C.

SA-β-gal Staining. Briefly, the cisplatin-treated CNE1 cells were fixed at different postexposure time points in PBS containing 0.2% glutaraldehyde and 2% formaldehyde for 5 min. Staining for SA-β-gal activity was performed as described previously (16). The positive cells were identified by the presence of blue precipitation in the cytoplasm under a Nikon microscope. SA-β-gal intensity was assessed using a four-grade scale (from + to ++++) by scoring the intensity of the blue precipitation and comparing among all samples tested. Each experiment was repeated at least twice and the percentage of positive cells were determined by counting of 5–10 fields under ×400 magnification.

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[2] To whom requests for reprints should be addressed, at Department of Pathology, University of Hong Kong, Queen Mary Hospital, Pok Fu Lam Road, Hong Kong, People's Republic of China. Phone: (852) 2855 4883; Fax: (852) 2872 5197; E-mail: jmnichol@hkucc.hku.hk.

[3] The abbreviations used are: NPC, nasopharyngeal carcinoma; SA-β-gal, senescence-associated β-galactosidase; PI, propidium iodide.

The SA-β-gal expression curves were drawn by plotting the mean percentages and SDs of each time point.

Determination of Telomerase Activity. Telomerase activity was determined using the telomeric repeat amplification protocol assay described previously (17).

Fluorescence staining and DNA fragmentation assay were performed according to previously published methods (18).

Results and Discussion

Cisplatin is one of the most widely used DNA-damaging agents in the treatment of cancer and has been shown to induce apoptosis in many tumor types through both p53-dependent and -independent pathways (18, 19). However, the NPC cell line CNE1 did not display any evidence of apoptosis after treatment with cisplatin; we did not observe the typical apoptotic morphological changes, the formation of a DNA ladder (data not shown) or a sub-G_1 peak in flow cytometry studies. Instead, after exposure to cisplatin, the CNE1 cells underwent senescent-like cell growth arrest, first judged by morphological changes. The cisplatin-treated cells showed evidence of cellular senescence, including cessation of proliferation, enlarged cell size, flattened cell morphology, and the appearance of multinucleated and vacuolated cell forms. In addition, they expressed SA-β-gal, a biomarker for cellular senescence that has been shown to be associated with cellular senescence in numerous nonneoplastic cell types (16). There is currently little published evidence demonstrating SA-β-gal expression in tumor cells, although one recent study showed that after transfection of the *p16INK4A* gene, human glioma cells expressed SA-β-gal and underwent senescent-like growth arrest (20). The failure of the senescent-like cells to undergo apoptosis has also been observed in human fibroblasts in response to extracellular stimulation (21). Our observations were also confirmed in primary cultured NPC cells, providing evidence that it was not due to an artifact of cell culture of CNE1 cells (data not shown).

It has been suggested that the inactivation of senescence pathways might play an important role in tumorigenesis (13). However, our results indicate that cellular senescent-like response can be produced by cisplatin in a tumor cell line, CNE1. It might be possible that this pathway is reactivated by cisplatin-induced DNA damage, or cellular senescent-like pathways exist in some tumors.

Two patterns of SA-β-gal expression were observed. In CNE1 cells treated with a lower dose (0.5 μg/ml), we observed partial growth inhibition at early time points and growth recovery at later time points. Higher doses (1.0 and 2.0 μg/ml) resulted in complete growth inhibition and cell death (Fig. 1*A*). After exposure to 0.5 μg/ml cisplatin, a significant proportion of CNE1 cells expressed SA-β-gal with accompanying growth inhibition by 48 h (Fig. 2*B*). The proportion of SA-β-gal-positive cells reached almost 100% at 96 h but decreased thereafter. The intensity of staining was consistently high (Table 1). Higher doses of cisplatin (1.0 and 2.0 μg/ml) also produced an increased percentage of SA-β-gal-positive cells with increased postexposure time and decreased cell viability. However, the percentage of SA-β-gal-positive cells was lower and the intensity of SA-β-gal staining was weaker than they were in the cells treated with 0.5 μg/ml cisplatin (Fig. 2).

Explanations for the dose-related expression of SA-β-gal expression are unclear. It is possible that, at the dose of 0.5 μg/ml cisplatin, the cellular damage is such that the cells are able to respond actively, as shown when their growth was slowed down and high levels of SA-β-gal expression were induced. This is shown in cell cycle distribution: the cells were able to overcome cisplatin-induced S-phase block at 48 h postexposure time. The cell growth inhibition was persistent until a postexposure time at 96 h, allowing time to repair cellular damage. The expression of SA-β-gal and growth inhibition

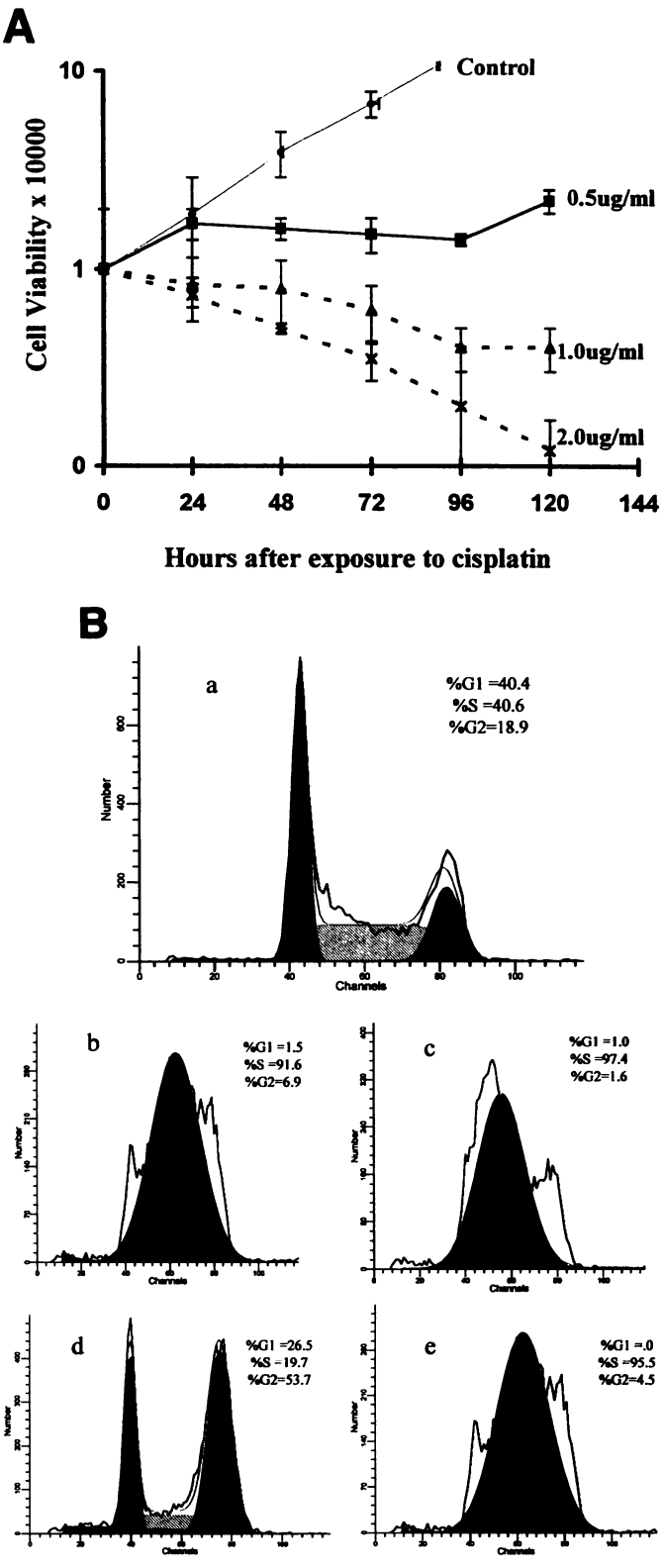

Fig. 1. Cell viability and cell cycle distribution after exposure to cisplatin. *A*, cell growth inhibition by cisplatin. CNE1 cells were plated in 12-well plates at 10^4 cells per well. The cells were exposed to three doses of cisplatin, and the cell numbers were determined at different time points. The viable cells were determined according to their ability to exclude trypan blue. *B*, cell cycle analysis after exposure to cisplatin. The CNE1 cells were fixed in 70% ethanol and stained with PI (400 μg/ml) for 30 min at 37°C. *a*, untreated CNE1 cells; *b* and *d*, cells treated with 0.5 μg/ml cisplatin; *c* and *e*, cells treated with 2.0 μg/ml cisplatin. Cells were tested at 24 (*b* and *c*) and 48 (*d* and *e*) h postexposure time.

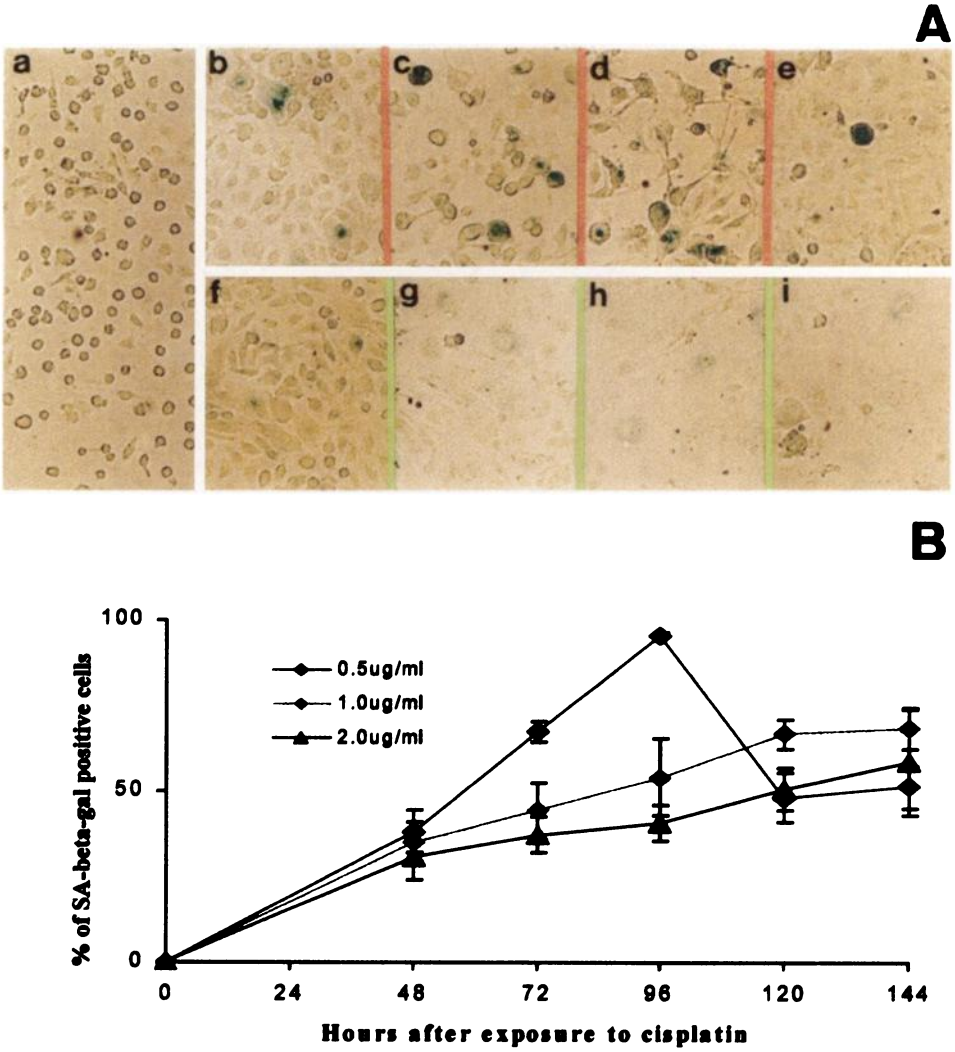

Fig. 2. The induction of SA-β-gal by cisplatin in CNE1 cells. The CNE1 cells were treated with cisplatin at 0.5, 1.0, and 2.0 μg/ml, and the cells were fixed and stained for SA-β-gal at different time points. *A*, representative photographs of the expression of SA-β-gal taken under ×100 magnification without phase contrast. *a*, untreated CNE1 cells; *b–e*, cells treated with 0.5 μg/ml cisplatin; *f–i*, cells treated with 2.0 μg/ml cisplatin. Cells were tested at 48 (*b* and *f*), 72 (*c* and *g*), 96 (*d* and *h*), and 144 (*e* and *i*) h. *B*, percentage of SA-β-gal positive cells after exposure to three doses (0.5, 1.0, and 2.0 μg/ml) of cisplatin at five time points. The untreated control showed negative staining of SA-β-gal.

appeared at the same time (48 h), suggesting that there may be a correlation between them (Figs. 1*A* and 2*B*). This indication was supported by the evidence that when CNE1 cells began to recover at 96 h after exposure to cisplatin, the percentage of SA-β-gal-positive cells decreased.

Cellular senescence is thought to be a permanent growth arrest in fibroblasts upon aging (10), but one study on human diploid fibroblasts has shown that, at nontoxic doses, a phosphatidylinositol 3-kinase inhibitor induced a cellular senescent-like response in a reversible way (6). We found that, in the CNE1 cells, the percentage of SA-β-gal-positive cells sharply decreased when the cell growth recovery began, suggesting that, at a sublethal dose, cisplatin-induced cellular senescent-like response may be reversible, although we cannot exclude the possibility that the decreased percentage of SA-β-gal-positive cells might be a result of the increased number of proliferating cells.

Table 1 *Summary of the intensity of SA-β-gal staining*

CNE1 cells were exposed to three concentrations of cisplatin (0.5, 1.0, and 2.0 μg/ml) and stained for SA-β-gal at different postexposure time points. The intensity of staining was compared among samples studied using a four-grade scale by scoring the intensity of the blue precipitation in the cytoplasm under ×400 magnification.

Postexposure time (hr)	Intensity of staining		
	0.5 μg/ml cisplatin	1.0 μg/ml cisplatin	2.0 μg/ml cisplatin
48	+++-++++	+	+
72	+++-++++	+	+
96	+++-++++	+-++	+
120	+++-++++	+-++	+
144	+++-++++	++	+

We found that, at relatively high doses (1.0 and 2.0 μg/ml) of cisplatin, the proportion of SA-β-gal-positive cells was lower and the staining was weaker compared to the cells treated with 0.5 μg/ml cisplatin. At these high doses, the cellular damage may be too great for the cells to respond in an active way, resulting in a complete growth inhibition and decreased cell viability with the increased postexposure time. This cellular response was also shown in cell cycle studies in which a permanent S-phase arrest occurred at 24 h after exposure (Fig. 1*B*). There was a correlation between the increased SA-β-gal-positive cells and decreased cell viability. Although the origin or the function of SA-β-gal is not known, it has been suggested that this expression is unlikely to be the direct cause of growth arrest and most likely reflects the cellular changes accompanying senescence (16). We feel that the SA-β-gal staining is not an artifact because the positive cells also demonstrated morphological changes of senescence. It is possible that, after exposure to toxic doses of cisplatin, cellular senescent-like responses may be an irreversible step leading to cell death.

Our results on cisplatin-induced changes in cell cycle distribution also indicate a difference between cisplatin-induced senescent-like growth arrest and aging-related senescence. It has been shown that the senescent diploid fibroblasts are arrested in either G_1 or G_2-M phase

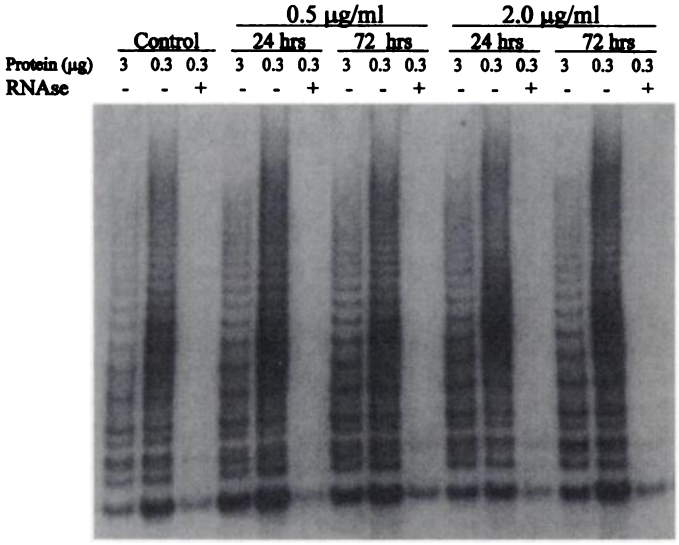

Fig. 3. Telomerase activity of CNE1 cells after exposure to cisplatin. CNE1 cells were treated with cisplatin at 0.5 and 2.0 μg/ml. The cells were harvested at 24 and 72 h after first exposure, and the telomeric repeat amplification protocol assay was performed as described by Kim *et al.* (17). Two protein concentrations (0.3 and 3 μg) were used in each experiment.

of the cell cycle and that no mitogenic stimulation is able to cause sustained cell cycle progression (10). However, the cisplatin-induced senescent-like growth arrest showed that, at 48 h postexposure time, both G_2-M and S phase accumulation occurred in CNE1 cells treated with lower and higher doses of cisplatin (Fig. 1*B*).

It has been shown that NPC cells have higher levels of telomerase activity when compared to normal tissues (22). Thus, we investigated whether cisplatin could inhibit telomerase activity in CNE1 cells. There is evidence that cisplatin causes telomere shortening (11) and cisplatin inhibits telomerase activity, resulting in cell death in testicular germ cell tumor cells (12). However, unlike normal cells, tumor cells usually have an elevated telomerase activity, which can actively extend the shortened telomeres to give advantage of survival over normal cells (10). Despite studying different time points and cisplatin doses, we did not observe any inhibition of telomerase activity in CNE1 cells (Fig. 3). Currently, we are investigating other telomerase components such as human telomerase reverse transcriptase subunit and human telomerase RNA component because the inhibition of human telomerase RNA component has been shown by cisplatin in germ cell tumor cells (12).

We provide the first report of evidence of DNA damage-induced cellular senescent-like response in a NPC cell line and primary cultured NPC cells. Our evidence suggests that this cellular senescent-like response may be a cellular protection mechanism that may act differently in response to different degrees of cellular damage. As indicated by our results, at a lower concentration of cisplatin, cellular senescent-like response may provide time for cells to repair, and this process may be transient. However, with higher doses, if the damage is beyond repair, this response may become permanent, leading to cell death. These two mechanisms may be distinguished by the levels of SA-β-gal expression.

We are currently studying additional NPC cell lines, tumor biopsies, and other tumor cell lines to investigate whether the induction of senescence by cisplatin is a phenomenon that is common both *in vitro* and *in vivo* and that is unique to NPC. Because cellular senescence is thought to be regulated by the p53 and retinoblastoma protein pathway, the expression of the related cell cycle regulators, such as p21 and p16, needs to be studied because they have been shown to play important roles in human diploid fibroblasts undergoing senescence (23).

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