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Cisplatin-induced premature senescence with concomitant reduction of gap junctions in human fibroblasts

Wei ZHAO, Zhong Xiang LIN*, Zhi Qian ZHANG

Peking University School of Oncology, and Beijing Institute for Cancer Research, Beijing 100034, China.

ABSTRACT

To examine the role of gap junctions in cell senescence, the changes of gap junctions in cisplatin-induced premature senescence of primary cultured fibroblasts were studied and compared with the replicative senescent human fibroblasts. Dye transfer assay for gap junction function and immunofluorescent staining for connexin 43 protein distribution were done respectively. Furthermore, cytofluorimetry and DAPI fluorescence staining were performed for cell cycle and apoptosis analysis. p53 gene expression level was detected with indirect immunofluorescence. We found that cisplatin (10 m*M*) treatment could block cell growth cycle at G1 and induced premature senescence. The premature senescence changes included high frequency of apoptosis, elevation of p53 expression, loss of membranous gap junctions and reduction of dye-transfer capacity. These changes were comparable to the changes of replicative senescence of human fibroblasts. It was also concluded that cisplatin could induce premature senescence concomitant with inhibition of gap junctions in the fibroblasts. Loss of functional gap junctions from the cell membrane may account for the reduced intercellular communication in the premature senescent fibroblasts. The cell system we used may provide a model useful for the study of the gap junction thus promoting agents against premature senescence.

***Keywords*:** cisplatin, premature senescence, gap junction, intercellular communication, connexin 43, fibroblasts.

INTRODUCTION

Most somatic cells lose the ability to proliferate after a finite number of cell divisions and end in a growth-arrest state known as replicative senescence. This is considered to be a genetically controlled process[1, 2]. But young proliferating cells may arrest growth and develop some aspects of the senescence phenotype in response to sub-lethal doses of various compounds[3-5]. This has been considered to be the induction of premature senescence. Cisplatin has been known as an anti-neoplastic drug against tumor cells[6, 7]. Cisplatin-induced DNA damage was also reported in human fibroblast cells[7, 8]. However, it is not known if cisplatin will trigger a cascade of senescence responses in normal cells. Intercellular communication through gap junctions has long been known to play an important role in cell proliferation, as well as in cell differentiation[9, 10]. Several lines of evidence suggested that gap junctions are defective in replicative senescent human cells[11, 12]. In this work, changes of gap junctions were investigated in the study of the drug-induced premature senescence. It was found that cisplatin could induce premature senescence in young human fibroblasts, which exhibited phenotypes comparable to those seen in replicative senescent fibroblasts. The dye-transfer capacity that is representative of gap junctional cell-cell communication reduced in both drug-induced premature senescent cells and in untreated replicative- senescent cells. Immunofluorescent staining showed that connexin 43 (Cx43), the major gap junction protein of lung fibroblastic cells[13, 14], reduced dramatically in the membrane of both kinds of cells.

*Corresponding author: Dr. Zhong Xiang LIN,
Tel: 86-10-6617-9250 (Lab), or 86-10-6617-6447 (Inst. Office)
Fax: 86-10-6617-5832,
E-mail: zhongxianglin@Yahoo.com

MATERIALS AND METHODS

Cell culture

Normal human lung fibroblasts were obtained from early subcultures of the primary human lung cells cultured in this laboratory [14]. Cells were cultured in RPMI 1640 medium (GIBCO) supplemented with 15% (v/v) fetal bovine serum, 100 units of penicillin/ml, and 100 μg of streptomycin/ml at 37°C in a humidified CO_2 (5%) incubator. Cells were grown on the bottom surface of glass flask to subconfluency (about 85-90%) and then passaged every 3 days. The *in vitro* life span of these cells is expressed in passage number. Each

passage represents approximately 2.5-3 population doublings (PD, see following description). Cultures younger than passage 10 (<G10) which showed high proliferative ability were designated young. Cultures around G20 showed a growth-delay and were designated intermediate. Cultures of G30-G37 had virtually ceased growth and thus could represent the replicative senescent cells. G37 showed no detectable growth for 3 weeks after plating. Ultimately all cells that reached the finite lifespan of 37 passages were in a state of growth arrest. Cells from every other passage were frozen and stored in liquid nitrogen for further investigation. Normal fibroblasts were also obtained from primary culture of late chicken embryo (11-day old) tissue. After exposure of chick fibroblasts to cisplatin, cell growth were arrested. Concomitantly the fibroblasts expressed the premature senescent phenotype which resembled that of human fibroblasts. Therefore these chick fibroblasts were used for comparative purposes in some experiments. Cisplatin (Sigma Chemical Co., 10 μ*M*) was added to the complete culture medium and incubated further as indicated in the figure legends.

Cell population doubling time (Gt)

Trypsinized cells were plated onto a 24-well culture plate (Falcon Co.). Five different cell concentrations (Ni) ($2x10^4$/well, $1x10^4$/well, $5x10^3$/well, 2×10^3/well, $1x10^3$/well) were inoculated into triplicate wells. Cell counts were made 1 day later. Following the sequence from high Ni to low Ni, a triplicate of wells were trypsinized and counted each day. Cell number (Nf) was obtained by counting on a hemocytometer and from the average of the triplicate wells.

The population doubling (PD) was calculated following the formula:

$$PD = In (Nf/Ni) / In 2$$

In: natural logarithm[15]. Population doubling time (Gt) was calculated from the formula: Gt = t / PD, t: the time length (hr) of the day after inoculation. Average Gt was obtained from the Gt of the counted days.

Cell growth curve

Fifteen wells were plated with 2×10^4 cells/well. Cells from each well of the triplicate were trypsinized and counted daily as described above , and the mean number of cells /well was obtained every day from the triplicate average.

Cell mitotic activity

The mitotic index (MI) was examined. Cells were fixed in 2% formaldehyde in PBS, extracted with 0.5% Triton X-100 (Sigma Chemical Co.) in PBS for 30 min , and then were stained with a DNA specific binding fluorescent dye DAPI (4, 6-diamidino-2-phenylindole dihydrochloride; Polysciences, Warrington, PA). Mitotic figures were counted under an Olympus fluorescence reflective microscope, 40× lens. MI= mitotic figures /1000 nuclei. N = 1000.

DNA fragmentation examination

In DAPI stained cells, nuclei with fragmented DNA were conspicuously different from the normal nuclei[16] . Number of fragmented DNA /1000 nuclei was taken as the apoptotic index (AI). N = 1000.

In vitro cell cycle analysis-cytofluorimetry (CFM)

Cells (~ 2×10^6 cells) were harvested and centrifuged (800 rpm) for 5 min. 0.5 ml PBS was added to resuspend the cells. Then 95% ethanol was added to a final volume of 2 ml, and was stored at 4ºC overnight. The cell sample was centrifuged and washed twice with PBS, then was suspended and stained in 0.5 ml PBS containing Propidium iodide (PI , 1 mg/ml) for 30 min in the dark. Cell cycle analysis was performed on a Model Coolper XL cytofluorimeter and analyzed by a multicycle software. DNA content and the hypoploidy were also measured [17].

Scrape loading and dye transfer (SLDT) technique

To detect cell-cell communication via gap junctions, a fluorescent dye, Lucifer Yellow (Sigma) was used. This technique introduces macromolecules into cells by a transient perturbation of the cell membrane that does not affect cell viability. Lucifer Yellow does not diffuse through intact cell membranes, but its low molecular mass permits diffusion through patent gap junctions. Cells along the scraped line are loaded with dye and thus transfer dye to their neighboring cells in a few minutes through gap junctions. If gap junction function is inhibited, cells do not transfer dye. Only the loaded cells along the scraped line would show fluorescence. Rhodamine dextran (Mr≈10, 000) is used as control, because it cannot diffuse through intact cell membranes or gap junctions. When added simultaneously, they can be used to verify that dye transfer occurs through intercellular gap junctions only. Several studies have demonstrated that such dye transfer is related to the morphological presence of gap junctions, radioactive metabolite transfer, and electrical coupling between cells [18, 19].

Antibodies

Connexin 43 (Cx43) antibody (kindly provided by Dr. Bruce Nicholson, SUNY at Buffalo) is a poly-clonal against the synthesized Cx43 peptide containing the amino sequence 252-271[20]. Anti-Cx43 antibody has no cross reaction with other connexin proteins. Monoclonal antibody against p53 (Clone BP53-12, Sigma) stains nuclei of apoptotic cells but it does not stain the normal nuclei.

Immunofluorescence staining and microscopy

For immunofluorescence microscopy, cells were rinsed with PBS and fixed with 2% formaldehyde in PBS for 3 min at room temperature. They were then permeablized with 0.5% Triton X-100 (Sigma Chemical Co.) in PBS for 30 min. This PBS-Triton solution was also used for all subsequent antibody-washing steps. All primary antibodies were used at an appropriate dilution for 1 h at 37ºC in humid chamber. Afterwards, stained samples were washed with washing solution for three times 10 min each. All secondary antibodies (Jackson ImmunoResearch Labo-ratories, West Grove, PA) were affinity-purified and tagged with rhodamine, or fluorescein (FITC) and used at a 1:100 dilution. The nuclear dye, DAPI was used at 2 μg/ml in 0.9% NaCl for 5 min. Specimens were mounted in 60% glycerol in PBS containing 2.5% DABCO (1,4-diazabicyclo (2,2,2) octane; Sigma Chemical Co.). Cell samples were examined with epifluorescence microscope (Olympus), using filter sets that are selective for rhodamine, fluorescien, or DAPI. Color photographs were taken by an equipped digital camera and printed through Photoshop soft disc to a Fujifilm printer 3500. Black and white micrographs were taken

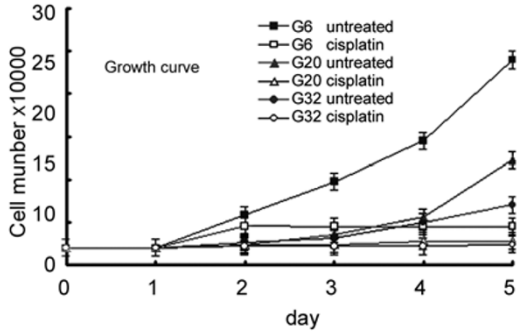

Fig 1. Growth curves of human lung cells. $2x10^4$ cells per well were plated in 24-well culture plate. Cells were trypsinized every day from the next day and counted by using a Hemocytometer. Average of cell countings from the triplicate was obtained and SD value was calculated statistically. In the cisplatin treatment, cisplatin (10 μ*M*) was added to the culture media by the 6h after plating when cells were attached to the substrate.

Tab 1. Comparison between young and senescent human fibroblast cells.

	G6	G20	G32	Cisplatin@
Gt (h)*	23±2.345	47±4.10	59±4.266▲	175±10.47■
MI	19	12	0**	0**
AI*	0**	40±2.01	112±56	330±16.5
P53+*	0**	60±3.15	129±45	780±38.5
Cx43	membrane	membrane	reduced	disappear
Dye Transfer***	4+	3+	2+	low

@: Cisplatin(10 μ*M*, 24 h) treated G 20 cultures; *: Results are presented as the mean ± SEM. Statistical significance was determined using independent Student's t test for paired samples by SPSS. ▲G32 vs. G6, P <0.01. ■G20-cisplatin vs. G20-control, P<0.01; **: total counting 1000 nuclei, repeatable in 3 separate cultures; ***: Dye transfer through over 4 gradients of the adjacent cells scored 4+; 3 or more gradients scored 3+; 2 or more gradients scored 2+. Less than 2 gradients scored low. Abbreviations: MI: mitotic index(‰). AI: apoptotic index(‰). Gt: population doubling time.

with either 40x or 100x 1.3 oil immersion objectives using 400 ASA film (T-max, black and white; Eastman Kodak Co., Rochester, NY).

RESULTS

Cisplatin arrested cell growth

As shown in the cell growth curve, cells stopped growing as early as 24 h exposure to 10 μ*M* cisplatin. Different passages of cisplatin-treated cells showed an inhibition of the growth curve comparable to the untreated growth arrested replicative senescent cells. In contrast to both the cisplatin treated- and the senescent cells, the untreated young cultures showed high proliferative ability while the middle-aged cultures showed a growth-delay (Fig 1).

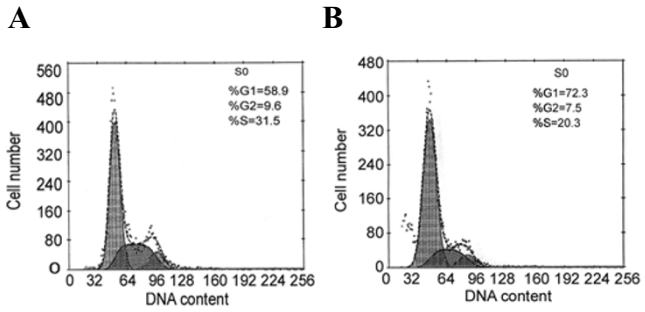

Fig 2. Cell cycle analysis of propidium iodide stained human fibroblasts. Propidium iodide staining was performed as described in Materials and Methods. The samples were analyzed by flow cytometry. (**A**) Cell cycle analysis of untreated normal young G8 cells. (**B**) Cell cycle analysis of G8 cells after exposure to 10 μ*M* cisplatin for 48 h.

The cisplatin effect on cell growth was also reflected by the delayed population doubling time (Gt, Tab 1). For the cisplatin treated cells, their Gt was 175±10.4 h. While there was a gradual increase of Gt during the process of aging in normal untreated human fibroblasts; eg. Gt of 23 ±2.3 h in young cells; Gt of 47±4.1 h in middle-aged; Gt of 59±4.2 h in senescent cells, and Gt of the cisplatin treated cells reached the longest (Tab 1).

Cisplatin inhibited mitotic activity

The frequency of mitotic figures in 1000 randomly counted DAPI stained nuclei was called mitotic index (MI). MI was relatively high in young cells (G6, MI =19) and it decreased in intermediate-aged cells (G20, MI =12). The MI was reduced to 0 in both replicative senescent fibroblasts and in cells exposed to cisplatin (Tab 1). The cisplatin effect on MI was observed not only in human fibroblasts but also in chicken fibroblasts (data not shown).

Cell-cycle blockade of cisplatin-treated fibroblasts -- CFM analysis

DNA content analysis showed typical diploid cycle phases in untreated control cells of young G8. These cells were in a constant replication condition, about 1/3 of the population were in DNA replication phase S (%S= 31.5). After 48 h exposure to cisplatin, G1 (%G1= 72.3) increased ~ 13.4% and S (%S= 20.3) decreased 11.2%. Cisplatin treatment blocked the cell cycle at the G1 and inhibited cells from entry into S phase. (Fig 2).

High frequency of nuclear DNA fragmentation in cisplatin treated cells

Nuclear DNA fragmentation of apoptotic cells was identified morphologically by nuclear dye DAPI fluorescent

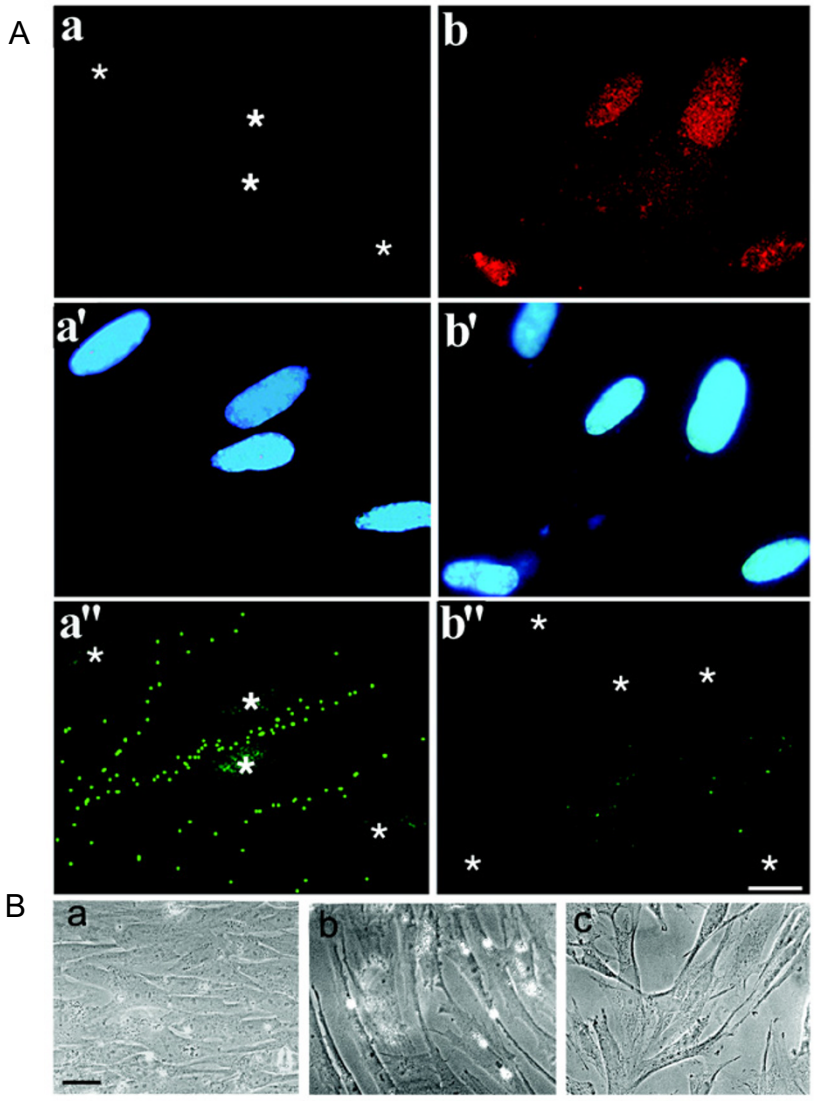

Fig 3 (**A**). Immunofluorescent triple stained micrographs. Column **a-a''**, normal young G8 human fibroblasts. Stars mark the nuclei area. Column **b-b''**, cisplatin 10 μM, 24 h treated G8 cells. Stars mark the nuclei area. **a** & **b**, p53 immunofluorescence stained nuclei in rhodamine channel. **a'& b'**, DAPI stained nuclei. **a'' & b''**, Cx43 immunofluorescence stained gap junction protein in FITC channel. Bar = 10 μm. (**B**), phase contrast micrographs. **a**, normal young fibroblasts. **b**, cisplatin treated 24 h fibroblasts. **c**, cisplatin treated 48 h fibroblasts.
Bar = 50 μm.

staining pattern of chromatin condensation . The number of the DAPI stained DNA fragmentation in 1000 counted nuclei is called Apoptotic Index (AI) in the following description. According to our studies, AI was not found in normal untreated young cells (G6-G8, AI =0). Middle-agged cells showed a modest number of AI (G20, AI=40±2.01), but after exposure to cisplatin their DNA fragmentation frequency reached the higest (AI=330±16.5), which was consistent with the increase of AI in untreated replicative senescent cells (G32, AI=112±56) (Tab 1).

Increased p53 immunofluorescence in cisplatin-treated cells

The monoclonal anti-p53 antibody stained positive only in part of the cell samples. Labelled with rhodamine, the p53 immunofluorescence displayed an amorphous red diffusion feature that distributed exclusively in nuclear area. Cytoplasmic staining of p53 was never observed in the samples detected. The p53 positive frequency varied in different samples. As shown in Tab 3 and Fig 3, the p53 staining was negative in the nuclei of untreated normal young cells but became positive in the nuclei of aged cells. The ratio of p53- positive nuclei in 1000 counted nuclei was obtained. The ratio rose along with aging of the untreated fibroblasts, and reached to the highest of 129±45 in the untreated replicative senescent fibroblasts. The ratio of p53-positive nuclei can reflect early changes of p53 expression level when the emerged smaller number of p53 positive nuclei is hard to detect in analysis from an average sample of a mixed cell population. In the cisplatin treated fibroblasts we observed a dramatic increase of the p53 expression: the p53-positive nuclei ratio was as high as

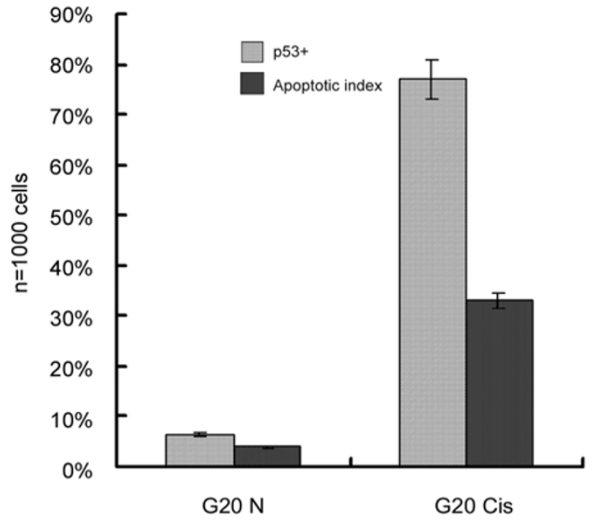

Fig 4. Relative levels of p53 positive nuclei to DAPI/fragmented nuclei in passage 20 normal fibroblast cells (G20 N) and cisplatin(10 uM, 24 h) treated passage 20 cells (G20 Cis) in double staining of p53 and DAPI fluorescence.

780±38.5 (Tab 1) (Fig 3). Among the p53-positive stained nuclei, the immunofluorescence intensity differed from weak to intense. In triple-fluorescent stained samples it was noted that the frequency of p53-positive nuclei was higher than that of DNA fragmented nuclei. Although some of the p53-positive nuclei did not exhibit DNA/DAPI fragmentation (Fig 3), the overall frequency of p53- positive nuclei was in parallel with the increasing tendency of the DNA fragmentation in the cell samples of cisplatin-induced premature senescence as well as in the progress of cell replicative senescence (Tab 1) (Fig 4).

Inhibition of introcellular communication by cisplatin

Normal untreated young fibroblast cells (G8) were well coupled by gap junctions. Luciferous Yellow dye was transferred from the loaded cells at the scraped line to the adjacent cells over 4-5 gradients (Fig 5a, a'). Middle-aged (G20) cells decreased in their dye transfer capacity. Dye was transferred to the adjacent cells only through 2 or 3 gradients (Fig 5b, b'). G8 or G20 cells after exposure to cisplatin (10 μ*M,* 24 h) were uncoupled to a certain degree, as shown in their reduced capacity for dye transfer, through 2 or less layers of the adjacent cells only (Fig 5c, c') (Tab 1). These results indicated that gap junction intercellular communication function declined during the development of the replicative senescence of human fibroblasts. Cisplatin treatment induces premature senescence concomitant with the inhibition of gap junction communication.

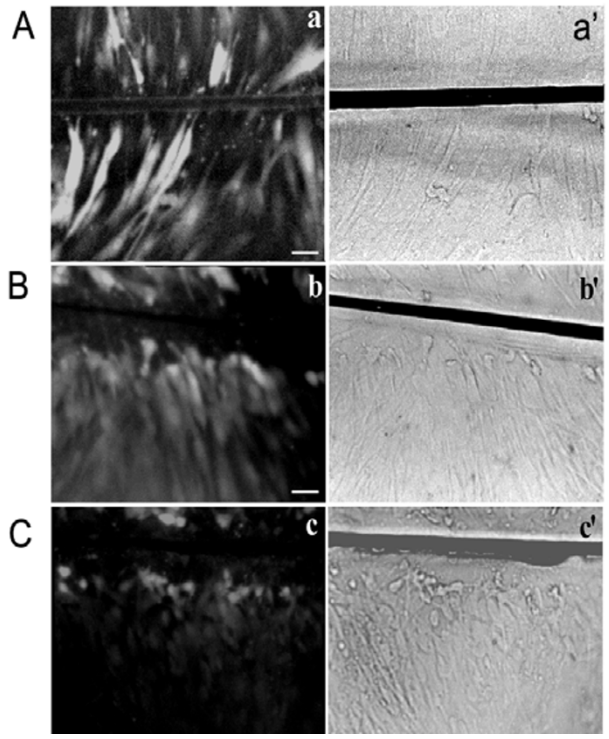

Fig 5 Lucifer Yellow fluorescence dye transfer micrographs. Monolayer cell cultures were loaded with Lucifer Yellow as described in Matirials and Methods. Dye transfer form scraped line to adjacent cells were recorded. (A), Young untreated G8 cells. (B), untreated G20 cells. (C), cisplatin (10 μ*M*, 24 h) treated G8 cells. a, b & c are fluorecent micrographs of dye transfer result. a', b' & c' are the respective light field micrographs. bar= 50 μm.

Cx43 immnuofluorescence in cisplatin-induced premature senescent cells

Cells were triple-stained with (1) Cx43 poly-clonal antibody; (2) Monoclonal antibody (Mab) against p53; and (3) DAPI nuclear dye. FITC-conjugated goat-anti-rabbit IgG, and rhodamine-conjugated goat-anti-mouse IgG were used as the secondary antibodies. The combination of the three colors of FITC-, Rhodamine- immunofluorescence with DAPI blue fluorescence is a useful method in studying the correlation between gap junction expression and the different ages of the cells in a mixed population. Cx43-FITC immunofluorescence stained dotted lines outlined the cell-cell contact membrane border of both human and chicken normal fibroblasts. The Cx43 immunofluorescent dots at the cell membrane very likely labelled the membrane gap junctions (Fig 3a"). After treatment with cisplatin, Cx43 immunofluorescent staining reduced obviously or entirely disappeared from the cell membrane. Although dispersed Cx43 immunofluorescent dots in the cytoplasm were observed in some cells, the overall Cx43 staining was much lower than in untreated normal fibroblasts (Fig. 3, compare b" with a"). The loss of membrane Cx43 im-

munofluorescence in the cisplatin-treated cells resembled the Cx43 staining observed in the untreated late passaged human replicative senescent fibroblasts (photo not shown).

Morphological changes of the cisplatin-induced premature senescent cells

Under the phase contrast microscope, normal fibroblasts showed full cell body with thick and opaque cytoplasm and cell processes, but in rare cases, filopodia. The cisplatin-treated human and chick fibroblasts showed morphological changes that were very similar to those in the untreated replicative senescent fibroblasts: they developed a large, flattened cell-shape and extended filopodia. Granules and vacuoles in the cytoplasm increased overtime (Fig 3 c, d, and e).

DISCUSSION

This work was designed to confirm the importance of gap junction cell-cell communication in cisplatin induced premature senescence. Cisplatin is a member of DNA-damaging agents used in the treatment of malignant tumors. Some cancer cells can be killed by cisplatin via a p53-independent route with some similarities to replicative senescence[6]. The cisplatin-treated WI38 fibroblasts and fibroblasts of other species have been used as controls for cytotoxicity in the studies of DNA repair and the cisplatin resistance[7, 8]. The present work demonstrated for the first time that exposure of human fibroblasts to cisplatin induced premature senescent changes similar to those seen in the replicative senescent fibroblastic cells. In normal fibroblasts the cisplatin treatment arrested cell growth at G1 phase of the cell cycle as well as up-regulated p53 expression. The p53 gene has been known as a negative regulator of growth in mammalian cells. Under normal conditions p53 levels are maintained at a low state by the virtue of the extremely short-half-life of the polypeptide . This may explain why p53 protein immunofluorescence was undetectable in the normal young cells used in this work (Fig 3, a)[21]. The increased p53 expression detected by immunofluorescent staining in the nuclei in senescent cells was consistent with the analytical results reported in the drug-induced premature senescence and in the replicative senescent diploid fibroblasts[4, 22, 23]. After exposure to cisplatin, unlike in some cancer cells[6], the changes of normal human fibroblast cells included elevation of p53 expression, G1 arrest and apoptosis. These are very likely to be the correlated events that developed in the cisplatin-induction of premature senescence. In fact, there was evidence that activation of p53 may cause cell cycle arrest at the G1 phase or lead to apoptosis of normal fibroblasts[23]. It is noteworthy that the response of the cultures to cisplatin was independent of their passage levels (Fig 1, Tab 1). Therefore, these cells establish a useful system for studying the mechanisms involved in the regulation of cell premature senescence.

Gap junctions of intercellular communication function in the exchange of molecules of less than 1200 Da, including secondary messengers, nutrients, and metabolites between adjacent cells. These molecules may carry signals of proliferation, differentiation and death. Relatively less is known about how gap junctions are involved in the control of cell death. Reduced gap junction cell-cell communication and down-regulated expression of Cx43 were reported in the senescent endothelial cells [12] and in the senescent human fibroblasts[11]. The current results support the previous findings. In addition, this paper demonstrates that: (1) There is a gradual reduction of gap junctions as the cells are getting age; comparing with young cells, the middle-aged show less gap junction activity while in senescent cells the gap junction activity is the lowest (Fig 5); (2) A similar reduction in gap junctions is also seen in human fibroblasts undergoing a cisplatin-induced premature senescent changes; (3) Cisplatin-treatment of chicken fibroblasts also induces some premature senescent phenotypes as well as the loss of the membranous Cx43. Promising roles of gap junctions in the controling of cell death have been suggested by reports from others, though different kinds of experiments have led to different conclusions[24-27]. It is necessary to design further experiments to resolve this mechenism.

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