Rapid senescence induced by overexpression of p53 in NIH3T3 cells

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Abstract **An NIH3T3 cell line which overexpresses temperature-sensitive p53Val135 was constructed by introduction of p53Val135 gene. It exhibited rapidly characteristic morphological and biochemical alterations related to replicative senescence when being cultured in 32 °C. We suggested that the overexpression of p53 activated probably the onset of senescence in NIH3T3 cells, which induced a rapid cellular senescence.**

Keywords: p53, p53Val135, NIH3T3, senescence, overexpression.

Since it was found that p53 prevents some types of cells from carcinogenesis[1], its function has become the center of intensive study in biology. p53 plays an important role in maintaining genomic stability and suppressing cellular aberration [2]. It has been proved that more than 50% tumors are related to abrogation of p53 function [3]. Moreover, p53 can not only induce apoptosis or growth arrest of some cells, but also is related to replicative senescence[2–7]. We constructed an NIH3T3 cell line which overexpresses the temperature-sensitive p53Val135 through introducing p53Val135 gene into NIH3T3 cell line by employing a retrovirus vector system. We showed that the NIH3T3 cell line, when being cultured in 32℃, exhibited characteristic morphological alterations corresponding to replicative senescence: an increase in cell size and nuclear size; flattenation of cytoplasm and formation of dendritic protuberance; multinucleation; accumulation of lipofuscin granules; vacuolation of cytoplasm; and ventation of nuclei. Such NIH3T3 cells showed positive staining for SA-β-gal. These results demonstrated that p53 overexpression activated the rapid onset of senescence in NIH3T3 cells.

1 Materials and methods

(ⅰ) Materials. All reagents except those specially marked were obtained from Sigma Company.

(ⅱ) Amplification and identification of plasmids. DH5α strains were transformed respectively with plasmids PLXSNp53Val135[5] and PLXSN (presented by Prof. Moshe Oren in Weizman Institute, Israel), and single colonies were isolated by spreading a plate containing ampicillin. The colonies were amplified and plasmids were extracted, digested by *Eco*RⅠ, and identified by agarose gel electrophoresis.

(ⅲ) Cell culturing, transfection and infection. Cells, including the packaging cell line GP+E-86 (presented by Dr. Wu Xiaobing with the Chinese Academy of Medical Sciences) and the target cell line NIH3T3, were grown in Dulbecco modified Eagle medium (DMEM) with high glucose (supplied by Gibico Company) supplemented with 10% fetal bovine serum (FBS, supplied by Hyclone Company), at 37℃ in a humidified incubator with 5% CO_2.

A confluent dish of packaging cells GP+E-86 was split 1∶10, and the next day liposomes were used to deliver respectively plasmids PLXSNp53Val135 and PLXSN into the cells. After incubating for 6 h, medium was replaced with normal growth medium containing serum and cells were incubated for additional 48 h. Then medium was removed and filtered through a 0.45 μm filter to obtain supernatant containing the transiently produced retrovirus. Or the transfected cells were split 1∶5, plated in selective medium containing 100 μg/mL G418, and incubated until colonies were visible. The same way was employed to get the supernatant containing retrovirus. The retroviruses were stored at −70℃.

NIH3T3 cells were split 1∶5, and the next day the medium containing virus stock was added, which is diluted by complete DMEM with 10% FBS (DMEM-10) at 1∶4 ratio. On the 3 d, the medium was removed and replaced with the DMEM-10 to grow cells until confluent. Infected cells were split 1∶4 into the complete medium containing 150 μg/mL G418 to obtain the NIH3T3 cell lines carrying a drug resistance gene.

(ⅳ) Immunofluorescence. Cells were grown in coverslips. After confluent, coverslips were removed, and treated by the methods as follows: Coverslips with attached cells were washed in PBS and fixed with 3% paraformaldehyde in PBS for 20 min at room temperature, rinsed in PBS, and subsequently permeated with 0.2% Triton X-100 in PBS for 5 min on ice, and then were washed extensively with 1% BSA in PBS. The cells were incubated with the primary antibody PAb1801 diluted in PBS for 1 h washed in PBS, and incubated with appropriate secondary antibodies conjugated with FITC for 50 min. The cells were washed extensively with PBS, mounted on the slides with 50% glycerol, detected by fluorescent microscopy.

(ⅴ) Western blotting. For Western blotting, 1×10^6 cells were lysed directly in 100 μL of 1× reducing SDS sample buffer (NEB) and incubated at 100℃ for 5 min. Ten microliters of the boiled extract were run on a 12% polyacrylamide gel, transferred to a nylon membrane. The membrane was washed in TTBS for 10 min, incubated with the primary antibody PAb1801 for 120 min at room temperature, washed in TTBS for four times, then incu-

bated with the secondary antibodies labeled by alkaline phosphatase for 110 min, washed sufficiently in PBS, and at last developed by the chromogenic visualization solution.

(vi) Induction of senescence, cell counts and SA-β-gal staining. Cells were split 1∶5 and incubated for 8 h. The medium was replaced with the DMEM-10 at 32℃. Grown in a humidified incubator with 5% CO_2, cells were counted at 0, 12, 24, 36, 48 h to draw the growth curve. Medium was changed every two days and cells were split only if the dishes were confluent. After being cultured for 10—14 d, cells were treated according to the following methods: washed in PBS (pH 7.2), fixed with 0.5% glutaraldehyde for 5 min, and washed in PBS (pH 7.2) containing 1 mmol/L $MgCl_2$. Then X-gal solution containing 1 mg/mL X-gal was added to cover cells, incubating overnight at 37℃, and cells were detected by a microscope.

2 Results and discussion

(i) Identification of plasmid PLXSNp53Val135. The plasmids extracted from DH5α strains transformed by PLXSNp53Val135 and PLXSN were digested by *Eco*R Ⅰ and electrophoresed. According to the construction map[8] (shown in fig. 1), p53Val135 gene about 1.5 kb and the linear vector fragment about 5.7 kb were obtained after plasmid PLXSNp53Val135 was cleaved by *Eco*R Ⅰ, which was consistent with our result (fig. 2).

(ii) Expression and identification of p53Val135 in NIH3T3 cells. The expression of p53 was detected in NIH3T3 cells, PLXSN NIH3T3 cells and PLXSNp53Val135 NIH3T3 cells by immunofluorescent microscopy and Western blotting (shown in figs. 3 and 4).

p53 plays an important role in regulation of cell cycle checkpoints, apoptosis induced by DNA damage, terminal differentiation and other physiological processes, so it is essential to regulate rigidly its activity in cells[3,4]. p53 is expressed at a low level under normal conditions (see fig. 4-1) so that it is hardly detected by immunofluorescent microscopy (fig. 3(a)), so are the PLXSN NIH3T3 cells into which only *neo* gene was introduced (shown in fig. 3(b) and fig. 4-2). However, the expression of p53 increased obviously in p53Val135 NIH3T3 cells (shown in fig. 3(c) and fig. 4-3).

(iii) Growth of NIH3T3 cells, PLXSN NIH3T3 cells and PLXSNp53Val135 NIH3T3 cells at 32℃. p53Val135 is a mutant protein with a substitution from alanine to valine at position 135 of wild type p53, whose conformation is a mutant form at 37.5℃ but a wild type one at 32℃, and whose activity is similar to authentic wt p53 at 32℃[9]. As shown in fig. 5, at 32℃ PLXSN- p53Val135 NIH3T3 cells showed growth arrest obviously, but NIH3T3 cells and PLXSN NIH3T3 cells were still normal. The growth arrest of PLXSNp53Val135 NIH3T3 cells is related to the overexpression of p53. This result suggests that the overexpression of p53 induce the growth arrest, which is consistent with those reported in refs. [10—12].

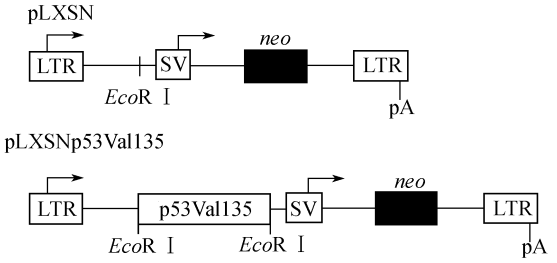

Fig. 1. Construction map of plasmid PLXSNp53Val135. p53 gene was inserted in the *Eco*R Ⅰ site of PLXSN vector. SV, SV40 early promoter; pA, polyadenylation signal.

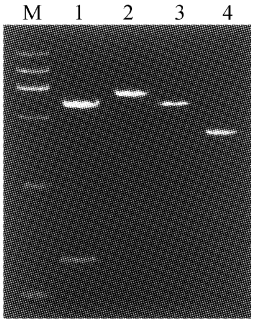

Fig. 2. Electrophoretogram of plasmids and those digested by *Eco*R Ⅰ. M, DNA marker; 1, the fragment of plasmid PLXSNp53Val135 digested by *Eco*R Ⅰ; 2, plasmid PLXSNp53Val135; 3, fragment of plasmid PLXSN digested by *Eco*R Ⅰ; 4, plasmid PLXSN.

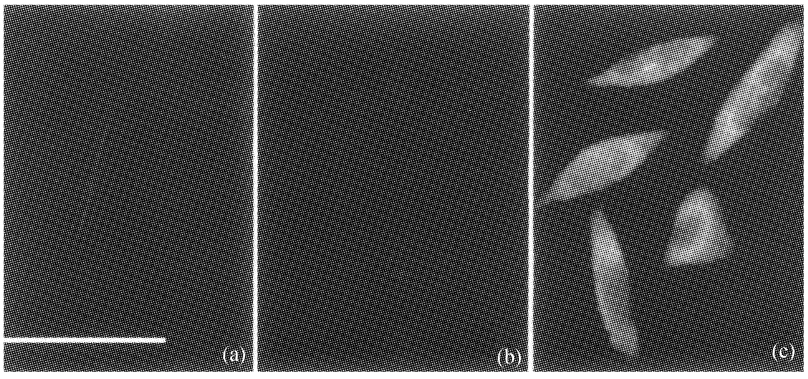

Fig. 3. Expression of p53 was detected in NIH3T3 cells, PLXSN NIH3T3 cells and PLXSNp53Val135 NIH3T3 cells by immunofluorescent microscopy. (a) NIH3T3 cells; (b) PLXSN NIH3T3 cells; (c) PLXSNp53Val135 NIH3T3 cells. Bar = 50 μm.

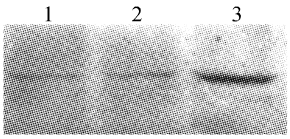

Fig. 4. Expression of p53 was detected in NIH3T3 cells, PLXSN NIH3T3 cells and PLXSNp53Val135 NIH3T3 cells by Western blotting. 1, NIH3T3 cells; 2, PLXSN NIH3T3 cells; 3, PLXSNp53Val135 NIH3T3 cells.

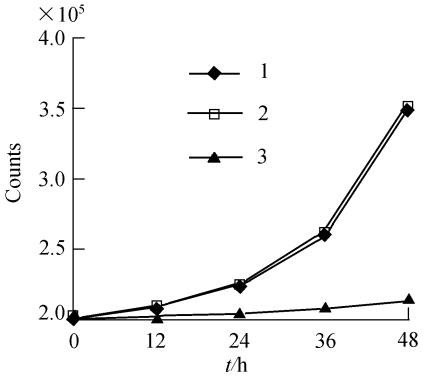

Fig. 5. Growth curves of NIH3T3, PLXSN NIH3T3 and PLXSNp53Val135 NIH3T3 cells in DMEM-10 at 32℃. 1, NIH3T3 cells; 2, PLXSN NIH3T3 cells; 3, PLXSNp53Val135 NIH3T3 cells.

(ⅳ) Overexpression of p53Val135 in NIH3T3 cells triggers a rapid senescence. Normal cells deviate from cell cycle, irreversibly lose the ability of proliferation and enter a comparatively stable state, which is termed replicative senescence. Compared with cells in other state, senescent cells still remain active metabolically, but there are many fundamental changes in morphology or function, such as the insensitivity to mitogen stimulation and resistance to apoptosis [6,13,14]. In senescent cells, the activity of β-galactosidase increases obviously, which can be detected by SA-β-Gal staining. But SA-β-Gal activity is undetectable in young cells which have the ability to proliferate or which is in the nondividing state by withdrawal of growth factors. So SA-β-Gal is a good marker of senescence in some cells[15]. Shown in Plate Ⅰ-1, PLXSN p53 NIH3T3 cells were stained blue (positive) by SA-β-Gal after incubating at 32℃ for 10—14 d, showing that PLXSNp53 NIH3T3 cells entered senescent phase. However, under the same condition, negative results of SA-β-Gal staining were obtained in NIH3T3 cells and PLXSN NIH3T3 cells, showing that these kinds of cells did not enter senescent phase. In summary, we made a conclusion: when PLXSNp53 NIH3T3 cells were transferred from 37.5℃ to 32℃, plenty of p53Val135 mutant converted into wild type conformation, activated the senescent program, and finally resulted in cellular senescence.

We further investigated the structural and morphological changes in senescent PLXSNp53 NIH3T3 cells (shown in Plate Ⅰ-2). After the senescence was triggered in PLXSNp53 NIH3T3 cells, nuclear and cellular size increased; then cytoplasm spread and formed some protuberances; subsequently cells multinucleated; cytoplasm vacuolated; nuclei approached cytoplasmic membrane and were excluded; eventually cells were lysed. In this process, lipofuscin vesicles increased gradually in cytoplasm. These morphological alterations accorded with those in the replicative senescent cells[14], which demonstrated further that overexpression of p53 activated the senescence program in NIH3T3 cells.

It is possible that p21 up-regulated by overexpression of p53[5,16—18] functions in the NIH3T3 cells senescence, which requires more investigations. Since NIH3T3 cells are immortal mouse embryonic fibroblasts, which are different from tumor cells and from primary cells, can the overexpression of p53 induce a rapid senescence of these cells? It is necessary for cells to break through the restriction of cellular senescence mechanism to proliferate during their carcinogenesis[6]. So the mechanism of senescence has been destroyed in tumor cells. Only these tumor cells in which the downstream pathway of p53 maintained integral in senescence mechanism can be induced rapidly into senescence by overexpression of p53. On the contrary, since there is an integral senescence mechanism in primary cells[6,14], it is suggested that overexpression of p53 can induce a rapid onset of senescence, which also requires further investigations.

Acknowledgements We thank Prof. Moshe Oren in Weizman Institute of Israel for providing the plasmids PLXSNp53 NIH3T3 and PLXSN, and Dr. Wu Xiaobing with the Chinese Academy of Medical Sciences for providing packaging cell line GP+E-86. Also we thank Dr. Tao Wei, Zhao Yun, Liu Xiaoling and others in our lab for their help. This work was supported by the National Natural Science Foundation of China (Grant No. 39300075) and the National Basic Sciences Research Program (Grant No. G19990539).

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(Received July 14, 2000)