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Senescence-like changes induced by expression of p21$^{Waf1/Cip1}$ in NIH3T3 cell line

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ABSTRACT

P21$^{Waf1/Cip1}$ is a potent cyclin-dependent kinase inhibitor. As a downstream mediator of p53, p21$^{Waf1/Cip1}$ involves in cell cycle arrest, differentiation and apoptosis. Previous studies in human cells provided evidence for a link between p21$^{Waf1/Cip1}$ and cellular senescence. While in murine cells, the role of p21$^{Waf1/Cip1}$ is indefinite. We explored this issue using NIH3T3 cells with inducible p21$^{Waf1/Cip1}$ expression. Induction of p21$^{Waf1/Cip}$1 triggered G1 growth arrest, and NIH3T3-p21 cells exhibited morphologic features, such as enlarged and flattened cellular shape, specific to the senescence phenotype. We also showed that p21$^{Waf1/Cip1}$-transduced NIH3T3 cells expressed β-galactosidase activity at pH 6.0, which is known to be a marker of senescence. Our results suggest that p21$^{Waf1/Cip1}$ can also induce senescence-like changes in murine cells.

Key words: *p21$^{Waf1/Cip1}$, senescence, inducible expression, cell cycle arrest.*

INTRODUCTION

Regulation of mammalian cell cycle plays an important role in cell fate determination. Many cell courses, such as proliferation, differentiation, growth arrest and senescence, have close correlation with cell cycle control. Among the regulators, several classes of cyclin-dependent kinases (CDKs) drive the cell cycle transition from the first gap (G1) to initiation of DNA synthesis (S phase), and their activities are in turn constrained by CDK inhibitors (CKIs). CKIs that govern these events can be divided into two families, the INK4 family, which includes p16^{INK4a}, p15^{INK4b}, p18^{INK4c}, p19^{INK4d}, and the Cip/Kip family, which includes p21$^{Waf1/Cip1}$, p27^{Kip1}, p57^{Kip2} [1].

P21 was first identified as a target gene of p53, mediating its roles as a tumor suppressor[2]. It was proved to interact with CDK2 associated complexes preventing the cell cycle from entering into S phase [3]. P21 can also bind to proliferating cell nuclear antigen (PCNA), a subunit of DNA polymerase d, thus is intimately involved in DNA replication and repair[4]. P21 is the first discovered gene that increases progressively during cellular senescence[5].

Consistent with above characters, p21 contributes to growth arrest, senescence, differentiation or apoptosis, depending on cell types and tissue contexts. Each outcome may provide a chance to escape from tumorigenesis, thus also demonstrating the role of p21 as a tumor suppressor mediator.

Although the importance of p21 on senescence has been well reported[6-9], the question of whether p21 is essential for the establishment of senescence still needs to be answered definitely, at least in murine cells[10-12]. In order to study the influences of p21 on murine cellular senescence, we here gen-

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erated a NIH3T3 cell line with inducible expression of p21. Our results indicate that in NIH3T3-p21 cells, inducible expression of p21 can also cause senescence- like changes, as to provide a support for previous viewpoints.

MATERIALS AND METHODS

Plasmid construction

P21cDNA cloned in pBluescript-KS (+) (kindly provided by Dr. Gordon Perters, Imperial Cancer Research Fund, UK) was isolated as 0.5kb NotI-EcoRV fragment, then was inserted into NotI-EcoRI (blunted) sites of pRetro-on-Bax (a gift from Dr. Hong-Bing Shu, National Jewish Medical and Research Center, USA). Thus the plasmid pRetro-on-p21 for transfection was prepared, in which p21 took the place of bax and its expression can be regulated by the status of tetracycline (or doxycycline).

Cell culture and transfection

NIH3T3 murine fibroblast cell line and 293-10A1 packaging line (also a gift from Dr. Hong-Bing Shu) were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Hyclone) and penicillin/ streptomycin in a 37℃ incubator with 5% CO_2. Retrovirus mediated transfection was performed as described previously[13]. In brief, by use of LipofectAMINE (Life Technologies), retroviral vectors were packaged in the 293-10A1 cell line, producing nonreplicative forms of amphotrophic virus. NIH3T3 cells were infected with viral supernatant supplemented with 4 μg/ml polybrene (Sigma) at intervals of 6 h. 24 h after the first infection, infected cells were screened using 2 μg/ml puromycin (Sigma) for a w or so. Clones of transfected cells were isolated and the expression of p21 was induced by adding 1.5 μg/ml doxycycline (Sigma) to the culture medium.

Immunoblot analysis

Cells were washed with ice-cold PBS and lysed in standard SDS sample buffer. After boiled for 5 min, lysates were cleared by centrifugation. 30 μg of total cell protein per lane were separated on 12% SDS-PAGE gel and transferred to a nitrocellulose membrane. After blocked with 3% bovine serum albumin in Tris-buffered saline-Tween buffer (0.5% Tween 20) at 37℃ for 1 h, the membrane was probed with the primary antibody p21(187) (Santa Cruz) for 2 h at 37℃, washed thoroughly with TTBS, then incubated with alkaline phosphatase-conjunct secondary antibodies, and lastly detected by the chromogenic visualization solution.

Senescence-associated b-gal (SA-b-gal) staining

The method used for SA-b-gal staining has been described previously[13].

Cell cycle analysis

Cells were harvested by trypsinization and were suspended in 0.5ml PBS (pH 7.2), then fixed in ice-cold 70% ethanol overnight at 4℃. Fixed samples were washed, resuspended in PBS containing 50 μg/ml propidium iodide (Sigma) and 50 μg/ml RNase, incubated at 37℃ for 30 min. The Epics XL-MCL flow cytometer (Beckman Coulter, Inc., CA) was used with Epics XL-MCL workstation, version 1.5, for cell cycle evaluation.

RESULTS

Inducible expression of $p21^{Waf1/Cip1}$ in NIH3T3 cells

In order to study the biological effects induced by $p21^{Waf1/Cip1}$, we utilized the retroviral vector pRetro-on-p21, in which the expression of $p21^{Waf1/Cip1}$ can be regulated by the status of tetracycline or its analog doxycycline[14]. We transferred the pRetro-on-p21 into NIH3T3 cells. Stable transfected cell lines were isolated by puromycin selection. Then we studied the inducibility of $p21^{Waf1/Cip1}$ by immunoblotting. Fig 1 shows that by 2 d, $p21^{Waf1/Cip1}$ was readily detected in NIH3T3 cells transfected with pRetro-on-p21 when 1.5 mg/ml doxycycline was added to the culture medium. In comparison, when doxycycline was not present, $p21^{Waf1/Cip1}$ was almost indiscernible, with the similar expression level as that in NIH3T3/ pRetro-on cells. Thus, NIH3T3/pRetro-on-p21 cells can be used as a sound regulative model to study the action of $p21^{Waf1/Cip1}$ in NIH3T3 cells.

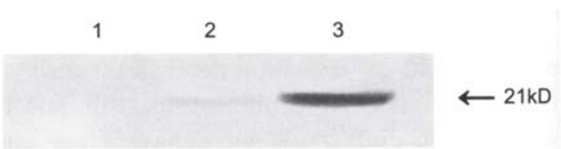

Fig 1. Protein levels of p21 in NIH3T3 cell lines. Induction duration with 1.5mg/ml doxycyclin (dox+) was 2 d.
Lane 1: NIH3T3 / pRetro-on (dox+) cells; lane 2: NIH3T3 / pRetro-on-p21 (dox-) cells; lane 3: NIH3T3/ pRetro-on-p21 (dox+) cells.

$P21^{Waf1/Cip1}$ induced growth inhibition in NIH3T3 cells

As a cyclin-dependent kinase inhibitor, $p21^{Waf1/Cip1}$ accumulates progressively in senescent cells, inactivates CDK2 associated complexes, with the results of cell cycle arrest or differentiation. To investigate the effects of $p21^{Waf1/Cip1}$ on NIH3T3 cells growth, we seeded 105 NIH3T3 cells on 60 mm plates and counted the number of the cells every 12 h. Fig 2 demonstrated the growth curves of NIH3T3 cells treated under different conditions. While NIH3T3/pRetro-on-p21 (dox-) cells proliferated just

like NIH3T3/pRetro-on (dox+) cells, the NIH3T3/pRetro-on-p21 (dox+) cells increased far more slowly. This indicates that induction of the expression of $p21^{Waf1/Cip1}$ arrested the growth in NIH3T3 cells.

Induction of senescence-like changes by $p21^{Waf1/Cip1}$

As shown in Fig 3, the expression of p21Waf1/Cip1 caused dramatic morphological alterations in NIH3T3-p21 cells. Compared with NIH3T3/pRetro-on (dox+) and NIH3T3/pRetro-on-p21 (dox-) cells, induced NIH3T3/pRetro-on-p21 (dox+) cells were much enlarged and flattened. Some cells were multinuclear, and their shape became irregular and vacuolization could also occasionally be found (Fig 3c, e, f). It has been reported before that senescent, but not presenescent, quiescent, or terminally differentiated cells express a senescence-associated β-gal (SA-β-gal) which can be detected by incubating cells at pH 6.0 with X-gal[15]. We then examined whether the cells presenting typicalsenescence-like morphology also express this senescent specific marker. Fig 3 shows that about 85% NIH3T3/pRetro-on-p21 (dox+) cells were stained positive after induction for 6 d by doxcycline, while NIH3T3/pRetro-on (dox+) and NIH3T3/pRetro-on-p21 (dox-) cells couldn't be stained. This demonstrates that senescence-like changes could be induced in NIH3T3 cells transfected with pRetro-on-p21.

G1/S cell cycle arrest caused by expression of $p21^{Waf1/Cip1}$

Ectopic expression of $p21^{Waf1/Cip1}$ has been reported to arrest cells at the G1/S or G2 phase.

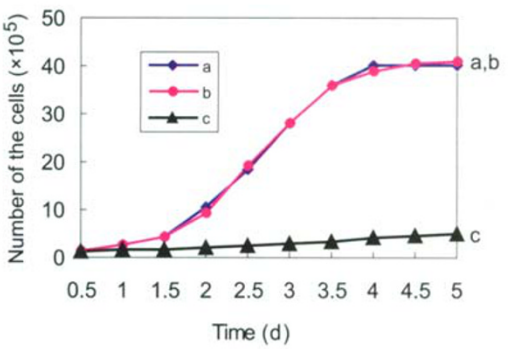

Fig 2. Growth curves of NIH3T3 cells
a, NIH3T3/pRetro-on-p21 (dox-)cells; b, NIH3T3/pRetro-on (dox+) cells; c, NIH3T3/pRetro-on-p21 (dox+) cells. Cell numbers were determined using a hemocytometer at the times indicated. All time points were performed in duplicate.

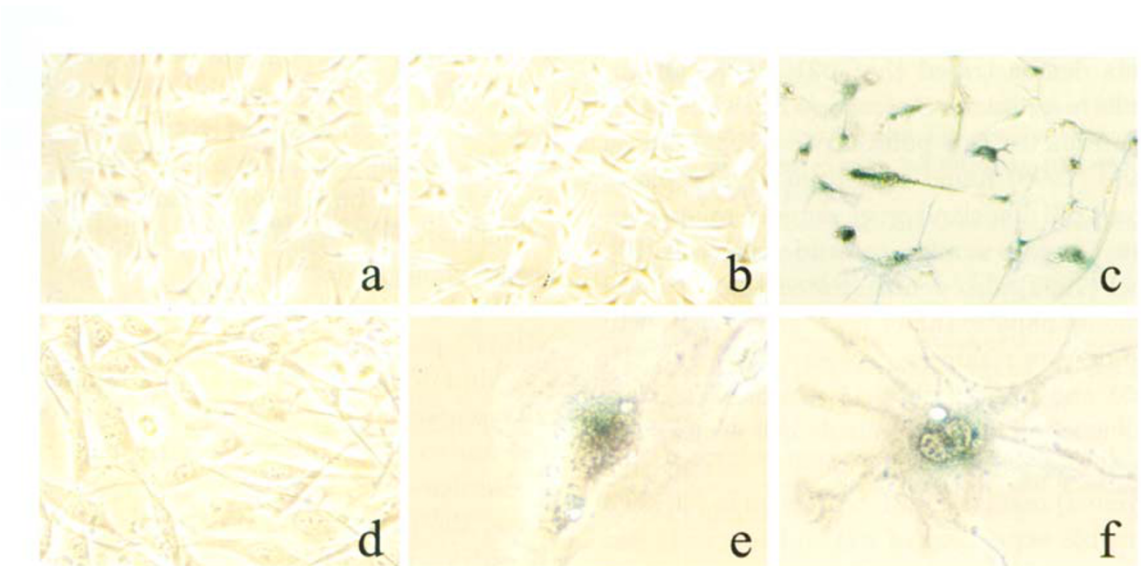

Fig 3. Morphologic changes and SA-b-gal staining following p21 induction with doxycycline for 6 d.
(a) NIH3T3/pRetro-on (dox+) cells; **(b, d)** NIH3T3/pRetro-on-p21 (dox-) cells; **(c, e, f)**NIH3T3/ pRetro-on-p21 (dox+) cells; The magnification of the upper panel is 63×, and that of the lower panel is 200×

Since G1/S cell cycle arrest is the characteristic of senescent cells, we examined the cell cycle changes of the NIH3T3 cell lines by flow cytometric analysis after 2 d induction. As shown in Fig 4, for NIH3T3/ pRe-tro-on-p21 (dox+) cells, the percentage of G1 and S phase was 70.1% and 22.5%, respectively, while for NIH3T3/pRetro-on-p21 (dox-) cells, about 48.3% cells were at G1 phase and 34.8% at S phase. This indicates that induction of $p21^{Waf1/Cip1}$ significantly increased the proportion of G1 phase cells and reduced the fraction of S phase cells. Thus ectopic expression of $p21^{Waf1/Cip1}$ arrested the cell cycle of NIH3T3 cells at G1/S phase.

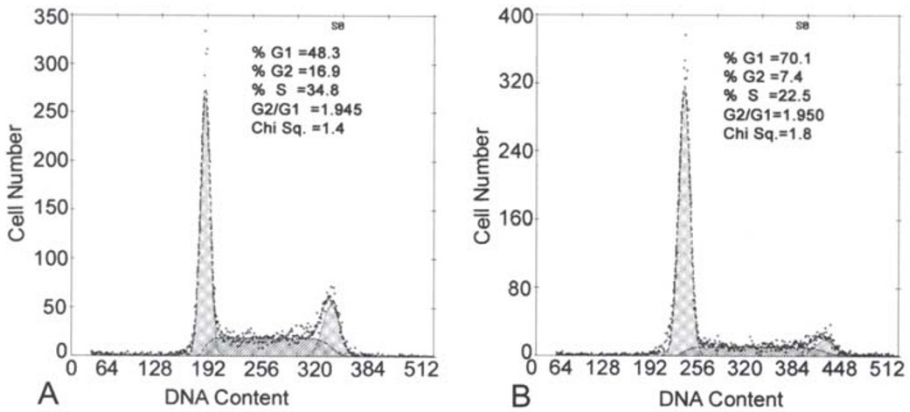

Fig 4. Cell cycle changes after induction of p21.
A, NIH3T3/ pRetro-on-p21 (dox-) cells; **B,** NIH3T3/ pRetro-on-p21 (dox+) cells. Cells were treated as described in Materials and methods.

DISCUSSION

At present, there exists discrepancy about whether p21 is indispensable in the establishment of cellular senescence, especially in murine cells. Many experiments demonstrated that p21 played an important role in cellular senescence[6-8], while some reports contradicted this point of view[12]. Pantoja and Serrano (1999) found that primary fibroblasts derived from p21 knockout mice entered senescence and had a lifespan similar to wild type cells[10]. Obata et al (1999) also demonstrated that primary cultured mouse hepatic tumor cells were resistant to senescence despite retaining expression of p16Ink4a, p19Arf, p53 and $p21^{Waf1/Cip1}$[11]. In order to clarify the influence of p21 on murine cell senescence, in this study, we generated a model of tetracycline (or doxycycline) regulated p21 expression in NIH3T3 cells. Inducible expression of p21 in these cells was shown to cause senescence-like changes characterized in terms of morphologic features, DNA content and proliferative capacity. This is evident from the altered cell shape, arrested cell cycle at G1/S phase, and the significantly slowed down rate of cell proliferation. We have also got positive result of SA-b- gal staining (pH 6.0), which is a specific marker of cellular senescence. Additionally, we have found that the induced growth arrest was to some extent reversible within 4 d of induction, but not 6 d later, when the cells was arrested irreversibly. Our data suggest that p21 may be of importance in murine cell senescence, too. We consider whether p21 can induce senescence or not depends on cell types and tissue contexts, but to elucidate the exact function of p21 more precisely needs further investigation.

We here called the state that p21 induced in NIH3T3-p21 cells senescence-like changes, because this kind of induced senescence is different from cellular senescence (or replicative senescence), though it seems to have the same appearance as replicative senescence does. Essentially, in replicative senescence, the number of cell divisions is counted so as to restrict mutations accumulated for malignancy. Thus, counting cell divisions rather than arresting growth is the distinguishing characteristic of replicative senescence[16], [17]. While in induced

senescence (now called premature senescence), instead of counting the division number, cells sense many causes, such as overexpression of CKIs, ectopic expression of some oncogenes or hyperoxygen as stress signals and make a response of achieving growth arrest with the specific senescence markers. Although premature senescence is not equal to replicative senescence, their final molecular mechanisms to arrest cell growth might be overlapped. So exploration of the mechanisms of premature senescence may contribute to elucidating the procedures of replicative senescence. By far, we still can't figure out the exact differences between irreversible cell growth arrest and cellular senescence. But no matter what phrases are used to identify the induced state, the ability of p21 to prevent the incessant proliferation of immortalized NIH3T3 cells is definite. This implies that activation of endogenous p21 gene might be a potential approach to prevent tumorigenesis, or even for cancer therapy.

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