[CANCER RESEARCH 55, 4536–4539, October 15, 1995]

Advances in Brief

Tumor Suppression by $p21^{WAF1}$ [1]

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Abstract

The $p21^{WAF1}$ gene encodes a cyclin-dependent kinase inhibitor and mediates tumor suppressor gene *p53*-induced cell cycle arrest. To directly test whether $p21^{WAF1}$ can act as a tumor suppressor, we have expressed the $p21^{WAF1}$ cDNA in several human tumor cell lines using a tetracycline-inducible system. Overexpression of $p21^{WAF1}$ suppresses proliferation and soft agar growth of tumor cells *in vitro*, as well as tumorigenicity *in vivo*. Our data provide direct evidence for the tumor-suppressive activity of $p21^{WAF1}$.

Introduction

The $p21^{WAF1}$ gene has been shown to mediate *p53* functions (1, 2) and has been proven to be an inhibitor of cyclin-dependent kinases (3, 4). The gene was also named *SDI1* (senescent cell-derived inhibitor 1), and its expression was shown to increase more than 20-fold in senescent cells (5). Recently, it has been reported that $p21^{WAF1}$ may play a role in differentiation of hematopoietic and hepatoma cells (6, 7), as well as muscle and epithelial cells (8, 9). Involvement of $p21^{WAF1}$ in the *p53* pathway and possibly in cell differentiation and senescence suggests that the $p21^{WAF1}$ gene itself may act as a tumor suppressor. To determine the effect of $p21^{WAF1}$ on tumor phenotypes, $p21^{WAF1}$ cDNA was expressed in colon carcinoma DLD1 and prostate carcinoma DU145 and 125-IL cells using a tetracycline-inducible system (10). The system consists of a transactivator plasmid and a cloning vector. The transactivator is a fusion protein comprising the TRE[3] binding domain and the activation domain of VP16 from herpes simplex virus. The cloning vector has seven repeats of TRE sequence upstream of the basal cytomegalovirus promoter. The transactivator binds to the TRE and activates gene expression; however, its DNA-binding capacity is abolished by tetracycline. Therefore, a transfected gene is expressed in the absence and inhibited in the presence of tetracycline. Several $p21^{WAF1}$-inducible clones were established from DLD1, DU145, and 125-IL tumor cells. Effects of $p21^{WAF1}$ expression on tumor phenotypes were studied using these inducible clones.

Materials and Methods

Expression of $p21^{WAF1}$. Tumor cells were transfected with a transactivator plasmid (pUHD 15-1 neo) and selected with G418. Clones with functional transactivator proteins were identified by transient transfection of a inducible vector containing the luciferase gene (*pUHC13-3*). Clones with transactivator were then transfected with $p21^{WAF1}$-inducible vector (pUHG10–3 WAF-S) and pTK-hyg and selected with hygromycin. Inducible expression of $p21^{WAF1}$ was confirmed by Northern and Western blotting using the human $p21^{WAF1}$ cDNA probe (a gift from Dr. Bert Vogelstein, The Johns Hopkins Oncology Center) and the monoclonal anti-$p21^{WAF1}$ antibody clone CP74 (a gift from Dr. Wade Harper, Baylor College of Medicine).

Tumor Cell Lines. DU145 and 125-IL cells were cultured in RPMI 1640 supplemented with 5% fetal bovine serum. DLD1 cells were grown in DMEM with 5% fetal bovine serum. $p21^{WAF1}$-inducible clones CA4WAF14S, DU11WAF9S and IL19WAF3S were maintained in media containing 50 μg/ml of G418, 25 μg/ml of hygromycin, and 2 μg/ml of tetracycline.

Cell Proliferation Assay. $p21^{WAF1}$-inducible cells were grown in the presence or absence of 2 μg/ml of tetracycline for 10 days. Media were changed every other day, and the number of cells was counted every day with a hematocytometer under microscope. Three dishes were used for each point, and three independent experiments were performed.

Cell Cycle Distribution. CA4WAF14S cells were cultured with or without 2 μg/ml of tetracycline for 0–72 h. Cells were collected and fixed in 3 ml of cold 70% ethanol. Cells were washed, treated with RNase, stained with 60 μg/ml of propidium iodide, and analyzed in a fluorescence-activated cell sorter (Becton Dickinson). Percentage of cells in G_1, S and G_2-M was calculated using the CellFit program.

Anchorage-independent Growth Assay. Six-well plates were coated with 1 ml of medium-0.5% agarose, with or without 5 μg/ml of tetracycline. CA4WAF14S and DLD1 cells (10^3) were suspended in medium-0.15% agarose, with or without 5 μg/ml of tetracycline, and seeded onto coated wells. Tumor cells were grown in soft agar culture for 2 weeks, and 100 μl of fresh medium or medium with 5 μg/ml of tetracycline was carefully added into each well every 3 days. Colonies with ≥30 cells (5 cell doublings) were considered positive and scored by three individuals.

Tumorigenicity. DLD1 and CA4WAF14S cells were mixed with an equal volume of matrigel (Collaborative Biomedical Products) injected s.c. into BALB/C nude mice (10^7 cells/site, 2 sites/mouse, and 5 mice/group) on day 0. Tumors were periodically measured with Vernier calipers, and two perpendicular diameters were used to estimate tumor volume with the formula $ab^2/2$, where a is the larger and b the smaller diameter.

Results

Inducible Expression of $p21^{WAF1}$. CA4WAF14S from DLD1 cells, DU11WAF9S from DU145 cells, and IL19WAF3S from 125-IL cells were used in this study. Fig. 1 illustrates that little or no $p21^{WAF1}$ expression was detectable in DLD1 or CA4WAF14S cells cultured in the presence of tetracycline. However, $p21^{WAF1}$ expression was strongly induced in CA4WAF14S cells in the absence of tetracycline. Identical results were obtained with parental cell lines DU145 and 125-IL and their respective $p21^{WAF1}$-inducible clones DU11WAF9S and IL19WAF3S (data not shown). A dose-dependent inhibition of $p21^{WAF1}$ expression by tetracycline was observed. $p21^{WAF1}$ expression was maximal at 0 μg/ml of tetracycline, decreased at 0.001–0.01 μg/ml of tetracycline, and inhibited at ≥0.1 μg/ml of tetracycline (Fig. 2*A*). $p21^{WAF1}$ expression was induced within 6 h after the removal of tetracycline and reached a plateau at approximately 24 h (Fig. 2*B*). The level of $p21^{WAF1}$ protein was decreased within 3 h after the addition of tetracycline and reached the basal level (endogenous $p21^{WAF1}$ level in nontransfected cells) after 6 h (Fig. 2*C*). Thus, it appears that $p21^{WAF1}$ has a high turnover rate.

Suppression of Tumor Cell Proliferation. The effect of $p21^{WAF1}$ on tumor cells was measured by cell proliferation assay. CA4WAF14S cells were cultured under conditions that induce or do

Received 6/27/95; accepted 8/31/95.

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[1] Supported in part by American Cancer Society Grant IN-162 and a fund from the Pathology Department, Wayne State University.

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[3] The abbreviation used is: TRE, tetracycline-responsive element.

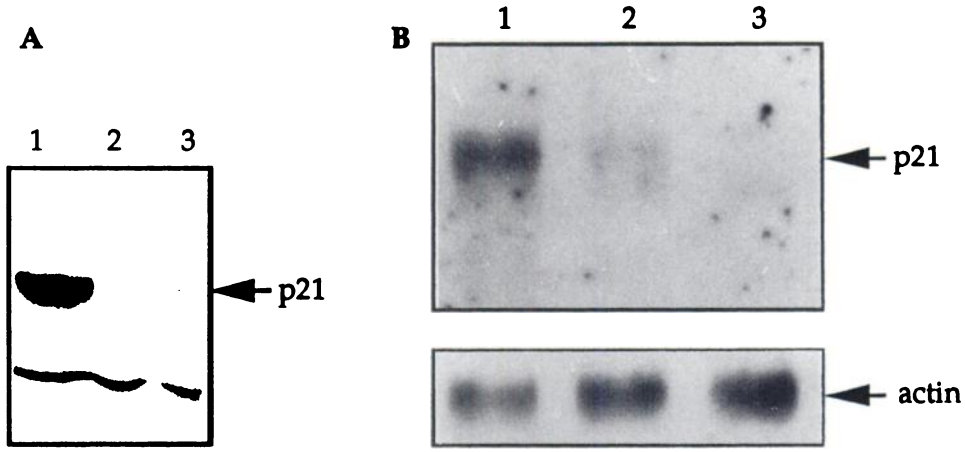

Fig. 1. Inducible expression of $p21^{WAF1}$. DLD1 and CA4WAF14S cells were cultured for 2 days with or without 2 μg/ml of tetracycline. Total protein and RNA were isolated and used for Western blotting (*A*) with anti-p21 monoclonal antibody and Northern blotting (*B*) with $p21^{WAF1}$ cDNA probe. *Lane 1*, CA4WAF14S; *Lane 2*, CA4WAF14S with tetracycline; *Lane 3*, DLD1.

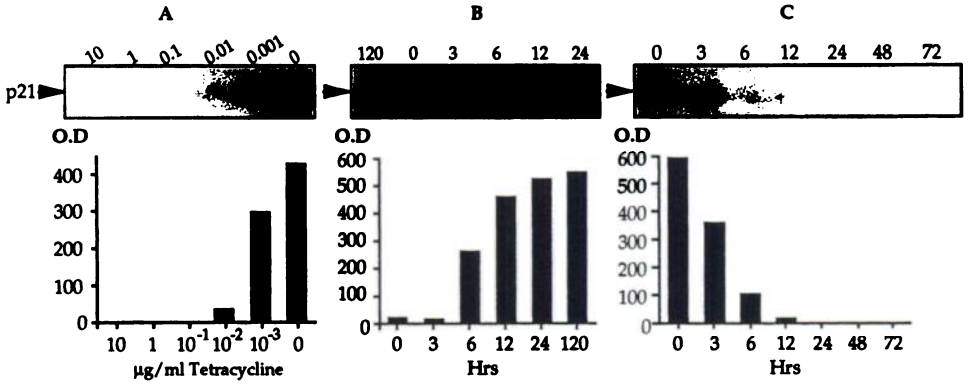

Fig. 2. Dose dependency and kinetics of $p21^{WAF1}$ expression. *A*, CA4WAF14S cells were cultured in the presence of 0–10 μg/ml of tetracycline for 2 days. Cells were lysed in 1X protein sample bufter, and protein concentration was measured using the BCA protein assay reagent (Pierce). Ten μg of protein from each condition were used for Western detection of $p21^{WAF1}$. Intensity of the $p21^{WAF1}$ signal was measured by densitometric scanning of X-ray films using AMBIS image analysis system (AMBIS, San Diego, CA). *B*, CA4WAF14S cells were released from 2 μg/ml of tetracycline and harvested 0–120 h after tetracycline withdrawal. Equal amounts of proteins were used for Western blotting. *C*, CA4WAF14S cells were cultured for 2 days in the absence of tetracycline. Cells were harvested 0–72 h after addition of 2 μg/ml of tetracycline. $p21^{WAF1}$ expression was analyzed by Western blotting. *O.D.*, arbitrary unit.

not induce $p21^{WAF1}$. The number of cells was determined daily for a period of 10 days. Cell growth rate was significantly reduced under the $p21^{WAF1}$-expressed conditions (Fig. 3*A*). Tetracycline had no effect on the growth rate of the parental lines. This reduced cell growth was, at least partially, due to a blockage of G_1-S transition. Expression of $p21^{WAF1}$ decreased the S-phase (Fig. 3*B*) and increased the G_1 (Fig. 3*C*) cell populations. It was interesting that overexpression of $p21^{WAF1}$ did not completely stop tumor cell growth. Similar results were obtained in DU11WAF13S and IL19WAF3S cells (data not shown).

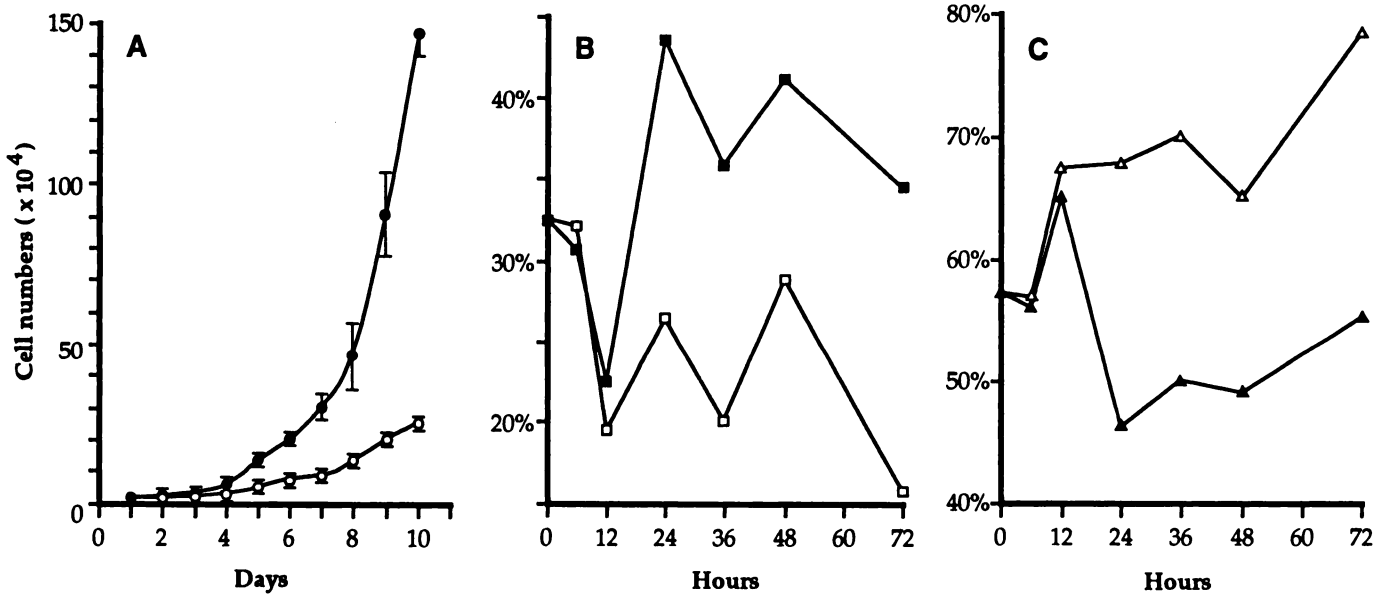

Fig. 3. Suppression of cell proliferation. *A*, CA4WAF14S cells were grown in the presence or absence of 2 μg/ml tetracycline for 10 days. Media were changed every other day, and the number of cells was counted every day. Three dishes were used for each point, and three independent experiments were performed. *B* and *C*, CA4WAF14S cells were cultured with or without 2 μg/ml of tetracycline for 0–72 h. Cells were collected, fixed, and analyzed in a fluorescence-activated cell sorter (Becton Dickinson). Percentage of cells at S (*B*) and G_1 (*C*) is shown. ●, ■, and ▲, with tetracycline; ○, □, and △, without tetracycline. *Bars*, SD.

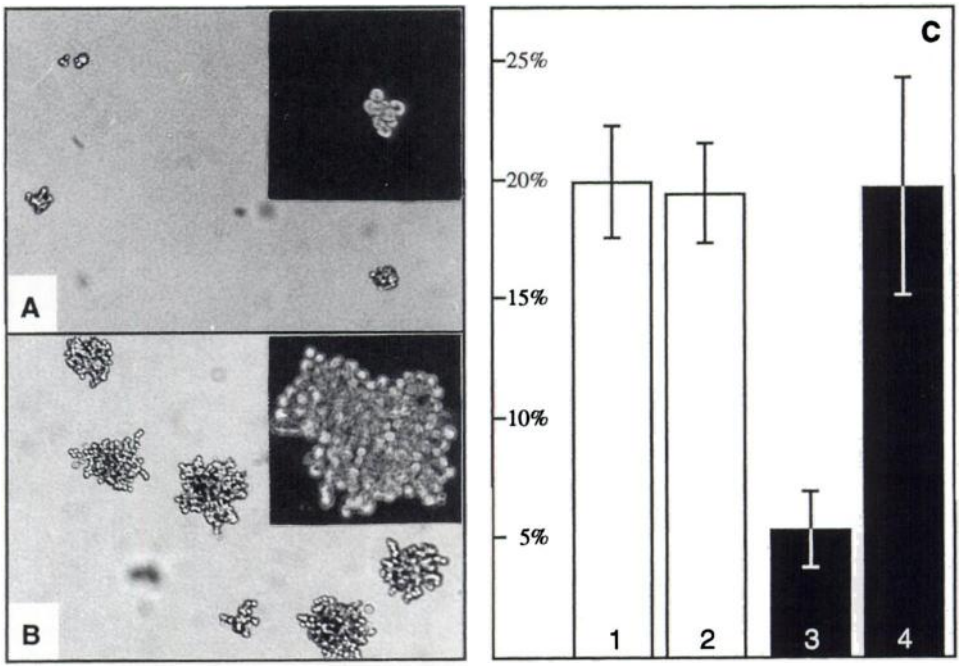

Fig. 4. Suppression of anchorage-independent growth. CA4WAF14S and DLD1 cells were grown in soft agar culture for 2 weeks. *A*, CA4WAF14S cells; *B*, CA4WAF14S with 5 μg/ml of tetracycline; *C*, colony formation of DLD1 cells in the absence (*Column 1*) or presence (*Column 2*) of tetracycline and CA4WAF14S in the absence (*Column 3*) or presence (*Column 4*) of tetracycline. Average of three independent experiments. *Columns*, mean; *bars*, SD.

Suppression of Anchorage-independent Growth. CA4WAF14S and parental cell line DLD1 were tested for their anchorage-independent growth. DLD1 cells with or without tetracycline had approximately 20% colony-forming efficiency (Fig. 4). In the presence of tetracycline, CA4WAF14S cells had the same colony-forming efficiency (Fig. 4). However, CA4WAF14S had colony-forming efficiency reduced to 5% under $p21^{WAF1}$-expressed conditions (Fig. 4). These results indicate that induction of $p21^{WAF1}$ suppresses anchorage-independent growth of tumor cells, whereas neither tetracycline itself nor the transfection procedure effected tumor cell growth in soft agar.

Suppression of Tumorigenicity. CA4WAF14S and DLD1 cells were tested in tumorigenicity assay. DLD1 and CA4WAF14S cells mixed with matrigel were injected s.c. into nude mice, and tumor volume was measured 5 days postinoculation. Fig. 5 depicts the growth of tumors in nude mice. DLD1 cells formed tumors that grew exponentially with a volume-doubling time of 6.6 days (95% confidence interval, 5.8–7.6 days) and a R^2 value of 0.99. CA4WAF14S cells formed tumors that grew exponentially with a volume-doubling time of 15.5 days (95% confidence interval, 13.5–18.0 days) and a R^2 value of 0.95. These results suggest that overexpression of $p21^{WAF1}$ suppresses tumor formation.

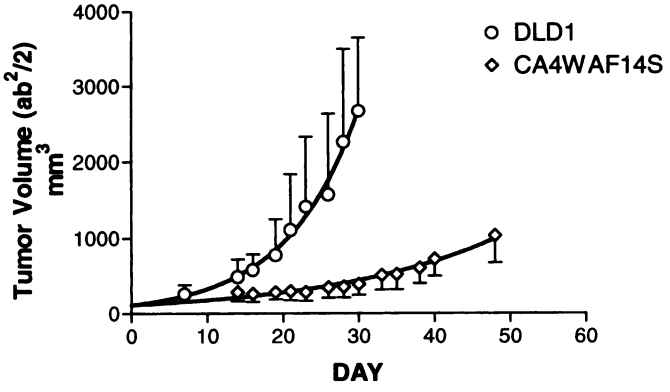

Fig. 5. Suppression of tumorigenicity. Tetracycline was washed off of DLD1 and CA4WAF14S cells, and cells were mixed with equal volume of matrigel and injected s.c. into nude mice. Tumor volume was measured with Vernier calipers. *Points*, mean; *bars*, SD.

Discussion

Due to the involvement of $p21^{WAF1}$ in the cell cycle, cell differentiation, and senescence, its biochemical functions are under intensive investigation. $p21^{WAF1}$ has been shown to mediate p53-induced cell cycle arrest (1, 2), and to inhibit cyclin-dependent kinase activity (3, 4) and proliferating cell nuclear antigen-dependent DNA replication (11) through two separated domains (12–14). However, the effect of $p21^{WAF1}$ on tumor cell phenotypes was unknown. In the present study, we have provided direct evidence for the tumor-suppressive activity of $p21^{WAF1}$.

During the course of this study, we have noticed several interesting phenomena. The *p53* tumor suppressor gene is known to be involved in programmed cell death; however, overexpression of $p21^{WAF1}$ itself does not induce apoptosis of tumor cells. Therefore, it is possible that $p21^{WAF1}$ is not associated with the apoptotic pathway, or $p21^{WAF1}$ alone may not be sufficient to induce programmed cell death. In addition, overexpression of $p21^{WAF1}$ did not completely stop tumor cell proliferation, soft agar growth, or tumor growth. This may be due to a heterogeneity of $p21^{WAF1}$ expression. Although $p21^{WAF1}$-inducible clones were picked up individually, cells may diverge during long-term culture. Indeed, we have observed large differences in the level of $p21^{WAF1}$ expression among 28 subclones isolated from CA4WAF14S cells. Nevertheless, tumorigenicity of CA4WAF14S cells in nude mice is significantly suppressed under $p21^{WAF1}$-expressed conditions.

We realize that it is important to test the generality of $p21^{WAF1}$ effect on tumor cells. Because it is difficulty to establish $p21^{WAF1}$-inducible cells, only limited tumor cell lines could be tested by this method. Recently, we constructed a $p21^{WAF1}$ recombinant adenovirus. By using this recombinant virus, we have determined the effect of $p21^{WAF1}$ on the proliferation of 11 different tumor cell lines. Overexpression of $p21^{WAF1}$ suppresses cell growth of all 11 tumor lines in culture.[4]

$p21^{WAF1}$ is induced by wild-type *p53*. It can also be induced in a

[4] Y. Q. Chen, J. T. Hsieh, F. Yao, and S. C. Cipriano, unpublished data.

p53-independent manner (15–18). Recently, we have found that *p53*-independent induction of $p21^{WAF1}$ pathway is preserved in tumor cells, regardless of the status of *p53* (*i.e.*, wild-type, wild-type inactivated by SV40T, or mutant) or the state of tumor cells (*i.e.*, immortal, tumorigenic, or metastatic; Ref. 19). Because inactivation of *p53* has been found in approximately 50% of human cancers (20, 21), the *p53*-dependent induction of $p21^{WAF1}$ pathway is disrupted in these tumor cells. In principle, activation of $p21^{WAF1}$ through the *p53*-independent pathway could bypass the requirement for functional *p53* in cell cycle arrest and inhibition of DNA replication. Therefore the *p53*-independent induction of the $p21^{WAF1}$ pathway could be a potential target in suppressing proliferation and stimulating differentiation of tumor cells.

Acknowledgments

The authors thank Dr. Bert Vogelstein for providing $p21^{WAF1}$ cDNA, Dr. Hermann Bujard for providing the tetracycline inducible system, Dr. Wade Harper for anti-$p21^{WAF1}$ antibody, and Dr. Steven Reed for the Tk-hygromycin plasmid. We also thank Matthew Fox for his assistance in Northern and Western blotting. The mice used in this study were housed in American Association for Accreditation of Laboratory Animal Care approved rooms. All protocols involving animal care and handling were approved by the Wayne State University Human and Animal Investigation Committee.

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