Tumor Suppression by p21WAF1

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Abstract

The p21WAF1 gene encodes a cyclin-dependent kinase inhibitor and mediates tumor suppressor gene p53-induced cell cycle arrest. To directly test whether p21WAF1 can act as a tumor suppressor, we have expressed the p21WAF1 cDNA in several human tumor cell lines using a tetracycline-inducible system. Overexpression of p21WAF1 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 p21WAF1.

Introduction

The p21WAF1 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 SDII (senescent cell-derived inhibitor), and its expression was shown to increase more than 20-fold in senescent cells (5). Recently, it has been reported that p21WAF1 may play a role in differentiation of hematopoietic and hepatoma cells (6, 7), as well as muscle and epithelial cells (8, 9). Involvement of p21WAF1 in the p53 pathway and possibly in cell differentiation and senescence suggests that the p21WAF1 gene itself may act as a tumor suppressor. To determine the effect of p21WAF1 on tumor phenotypes, p21WAF1 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-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 p21WAF1-inducible clones were established from DLD1, DU145, and 125-IL tumor cells. Effects of p21WAF1 expression on tumor phenotypes were studied using these inducible clones.

Materials and Methods

Expression of p21WAF1. Tumor cells were transfected with a transactivator plasmid (pUHD 15-1 neo) and selected with G418. DLD1 cells were grown in DME supplemented with 5% fetal bovine serum. DU11WAF9S and IL19WAF3S were maintained in media containing 50 µg/ml of G418, 25 µg/ml of hygromycin, and 2 µg/ml of tetracycline. Inducible Expression of p21WAF1. 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 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 G1, S, and G2-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 (105) 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.

Results

Inducible Expression of p21WAF1. 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 p21WAF1 expression was detectable in DLD1 or CA4WAF14S cells cultured in the presence of tetracycline. However, p21WAF1 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 p21WAF1-inducible clones DU11WAF9S and IL19WAF3S (data not shown). A dose-dependent inhibition of p21WAF1 gene expression by tetracycline was observed. p21WAF1 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. 2A). p21WAF1 expression was induced within 6 h after the removal of tetracycline and reached a plateau at approximately 24 h (Fig. 2B). The level of p21WAF1 protein was decreased within 3 h after the addition of tetracycline and reached the basal level (endoendogenous p21WAF1 level in nontransfected cells) after 6 h (Fig. 2C). Thus, it appears that p21WAF1 has a high turnover rate.

Suppression of Tumor Cell Proliferation. The effect of p21WAF1 on tumor cells was measured by cell proliferation assay. CA4WAF14S cells were cultured under conditions that induce or do...
Fig. 1. Inducible expression of p21WAF1. 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 p21WAF1 cDNA probe. Lane 1, CA4WAF14S; Lane 2, CA4WAF14S with tetracycline; Lane 3, DLD1.

Fig. 2. Dose dependency and kinetics of p21WAF1 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 buffer, 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. Intensity of the p21 band 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. p21WAF1 expression was analyzed by Western blotting. O.D., arbitrary unit.

not induce p21WAF1. The number of cells was determined daily for a period of 10 days. Cell growth rate was significantly reduced under the p21WAF1-expressed conditions (Fig. 3A). 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 G1-S transition. Expression of p21WAF1 decreased the S-phase (Fig. 3B) and increased the G1 (Fig. 3C) cell populations. It was interesting that overexpression of p21WAF1 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 G1 (C) is shown. •, ○, and △, with tetracycline; ○, □, and △, without tetracycline. 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 p21WAFl-expressed conditions (Fig. 4). These results indicate that induction of p21WAFl suppresses anchorage-independent growth of tumor cells, whereas neither tetracycline itself nor the transfection procedure affected 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 R² 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 R² value of 0.95. These results suggest that overexpression of p21WAFl suppresses tumor formation.

Discussion

Due to the involvement of p21WAFl in the cell cycle, cell differentiation, and senescence, its biochemical functions are under intensive investigation. p21WAFl 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 p21WAFl on tumor cell phenotypes was unknown. In the present study, we have provided direct evidence for the tumor-suppressive activity of p21WAFl.

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 p21WAFl itself does not induce apoptosis of tumor cells. Therefore, it is possible that p21WAFl is not associated with the apoptotic pathway, or p21WAFl alone may not be sufficient to induce programmed cell death. In addition, overexpression of p21WAFl did not completely stop tumor cell proliferation, soft agar growth, or tumor growth. This may be due to a heterogeneity of p21WAFl expression. Although p21WAFl-inducible clones were picked up individually, cells may diverge during long-term culture. Indeed, we have observed large differences in the level of p21WAFl expression among 28 subclones isolated from CA4WAF14S cells. Nevertheless, tumorigenicity of CA4WAF14S cells in nude mice is significantly suppressed under p21WAFl-expressed conditions.

We realize that it is important to test the generality of p21WAFl effect on tumor cells. Because it is difficult to establish p21WAFl-inducible cells, only limited tumor cell lines could be tested by this method. Recently, we constructed a p21WAFl recombinant adenovirus. By using this recombinant virus, we have determined the effect of p21WAFl on the proliferation of 11 different tumor cell lines. Overexpression of p21WAFl suppresses cell growth of all 11 tumor lines in culture.4

p21WAFl is induced by wild-type p53. It can also be induced in a

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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.

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