Antisense Inhibition of Cyclin D1 in Human Head and Neck Squamous Cell Carcinoma

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Objective: To study the role of cyclin D1 in regulating the biological behavior of head and neck cancer.

Design: Squamous cell carcinoma of the head and neck (SCCHN) cells were stably transfected with an antisense cyclin D1 using lipofectin-mediated transfection. In vitro growth assays, cell cycle analyses, cytotoxicity assays, and in vivo tumorigenicity assays were performed.

Materials: Human SCCHN cell lines TU138, TU167, TU177, TU182, MDA183, and MDA1386 and athymic nude mice were used for this study.

Results: The antisense cyclin D1 transfected cells revealed decreased growth rates in vitro and decreased tumorigenicity in athymic nude mice. Furthermore, antisense cyclin D1 transfection enhanced the chemosensitivity against cisplatin.

Conclusions: These studies provided evidence that overexpression of cyclin D1 may play an important role in growth rates and biological behavior of human head and neck cancer. Additionally, expression of cyclin D1 may make human head and neck cancer cells resistant to platinum-based chemotherapeutic approaches. The ability to suppress the malignant phenotype by down-regulating cyclin D1 expression may provide a new gene therapy approach for patients with head and neck cancer.


The G1 phase of the cell cycle is the decision-making phase for the cells to remain in the cell cycle and proliferate or to leave the cell cycle to become quiescent, to differentiate, or to die.1 The G1 cyclins, including types C, D, and E, play an important role in this cell cycle pathway.2,4 Cyclin D1 associates with its catalytic partners cyclin-dependent kinase 4 (CDK4) or 6 (CDK6), which results in phosphorylation of the tumor suppressor retinoblastoma gene product (pRb).3 Phosphorylation of pRb results in the release of the transcription factor E2F and the subsequent activation of genes necessary for DNA synthesis, which leads the cells into the S phase.

The cyclin D1 gene, located at 11q13, is frequently amplified and/or overexpressed in several types of human cancer, including squamous cell carcinoma of the head and neck (SCCHN).5-8 Overexpression of cyclin D1 accelerates the G1 phase, reduces the dependence of cells on mitogens,9,10 and increases chemosensitivity in cultured cells.11 Also involved in the G1 progression is the tumor suppressor gene p16, which encodes a specific cyclin D1/CDK4 binding protein that inhibits CDK4 activity.12 Many human cancers harbor a deletion or a mutation of the p16 gene.13 In SCCHN, however, methylation is reportedly the major mechanism of p16 gene inactivation.14 This inactivation may act to potentiate the oncogenic drive of the dysregulated cyclin D1 in head and neck cancer. Cyclin D1 expression or amplification has been reported to correlate with the patient’s prognosis in head and neck cancer.15,16 In addition, many clinical studies suggest that cyclin D1 overexpressants exhibit greater invasion and metastasis potentials.7,17 Taken together, these facts suggest that cyclin D1 may play an important role in tumorigenesis and progression of head and neck cancer.

To assess the role of cyclin D1 in the biological regulation of head and neck cancer cells, we constitutively expressed an antisense cyclin D1 complementary DNA (cDNA) in SCCHN cell lines that exhibit a high rate of endogenous expression of cyclin D1. The effects of cyclin D1 on in vitro cell growth, in vivo tumorigenicity, and cisplatin chemosensitivity were examined.
MATERIALS AND METHODS

CELL LINES AND CELL CULTURE

Human SCCHN cell lines TU138, TU167, TU177, TU182, MDA183, and MDA1386 were established at the Department of Head and Neck Surgery, M. D. Anderson Cancer Center, Houston, Tex, and some of them have been characterized previously. Cells were cultured in Dulbecco modified Eagle medium–Ham F-12 medium supplemented with 10% heat-inactivated fetal bovine calf serum and penicillin-streptomycin. Proliferation assays of the cells were performed by direct cell count using a Coulter Counter (Coulter Corp, Miami, Fla).

VECTOR CONSTRUCTION AND TRANSFECTION

The 1.2-kilobase human cyclin D1 cDNA that contained the entire coding sequence was subcloned in its antisense orientation into BamHI-EcoRI sites of the expression vector pcDNA3 (Invitrogen, Carlsbad, Calif). Human TU138 cells were stably transfected with the pcDNA3-antisense cyclin D1 plasmid and the pcDNA3 vector control plasmid via liposome-mediated transfection using CLONfectin (CLONTECH Laboratories Inc, Palo Alto, Calif), according to the conditions described by the supplier. Forty-eight hours after transfection, transfected cells were selected in complete medium containing 600-µg/mL G418 (CLONTECH Laboratories Inc). Forty-two independent clones were randomly isolated using cloning rings, and each clone was plated separately.

WESTERN BLOT ANALYSIS

Proteins were extracted from exponentially growing cells. The cell monolayer (10⁶ cells per 100-mm dish) was rinsed twice with ice-cold phosphate-buffered saline (PBS) and collected by scraping. The cell pellets were resuspended in lysis buffer (10-mmol/L Tris buffer, pH 7.5; 130-mmol/L sodium chloride; 1% Triton X-100; 10-mmol/L sodium fluoride; 10-mmol/L sodium phosphate; and 10-mmol/L sodium diphtosphate) containing protease inhibitors (16-µg/mL benzamidine hydrochloride, 10-µg/mL phenanthroline, 10-µg/mL aprotinin, 10-µg/mL leupeptin, 10-µg/mL pepstatin A, and 1-mmol/L phenylmethylsulfonyl fluoride [PMSF]) and were incubated on ice for 30 minutes. The protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, Calif). Fifty micrograms of protein was subjected to 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The gels were electrotransferred onto a nitrocellulose membrane (Amersham, Piscataway, NJ). Filters were blocked with 5% nonfat milk in PBS for 1 hour and then incubated with monoclonal anticyclin D1 antibody (Santa Cruz Biotechnology, Santa Cruz, Calif) for 1 hour at room temperature. Immune detection was performed using a chemiluminescent detection system (Amersham).

NORTHERN BLOT ANALYSIS

Total RNA was extracted from exponentially growing cells. Twenty micrograms of total RNA was electrophoresed on 1% agarose–3% formaldehyde RNA gels and transferred onto a Hybond N+ nylon membrane (Amersham). The filter was prehybridized and hybridized in Rapidhyb buffer (Amersham). Hybridization was performed at 65°C for 2.5 hours with cyclin D1 cDNA radiolabeled with α-32 phosphate deoxyctydilidne triphosphate. The filter was initially washed in 2× SSC–0.1% SDS at room temperature and then washed twice in 1× sodium chloride/sodium citrate (SSC)–0.1% SDS at 65°C. Autoradiography was then carried out. A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was used as an internal control.

CELL CYCLE ANALYSIS

Flow cytometry was performed on exponentially growing TU138 cells stably transfected with antisense cyclin D1. The cells were washed twice with PBS, fixed in 50% methanol for 30 minutes at 0°C, treated with 1-mg/mL RNase, and then stained with 50-µg/mL propidium iodide. Flow cytometric analysis was carried out on a Coulter Epics Profile Cytometer (Coulter Corp).

TUMORIGENICITY

To assess the effect of antisense cyclin D1 on tumorigenicity, 4×10⁴ parental TU138 cells expressing vector alone or cyclin D1 antisense were injected subcutaneously in dorsal flanks of 4- to 7-week-old female athymic nude mice. Each animal received injections in 2 different flanks and served as its own internal control. The animals were monitored for tumor formation every week and killed 7 to 12 weeks after injection. Institutional guidelines regarding animal-handling protocols were followed.

CYTOTOXICITY ASSAYS

Cisplatin was obtained from Sigma, St Louis, Mo. Cells were seeded in 96-well plates and incubated without cisplatin for 24 hours. Following a 24-hour exposure to cisplatin, the wells then were washed twice with PBS and incubated for another 24 hours. Cell viability was determined by a tetrazolium-based nonradioactive assay (Cell Titer 96 AQ; Promega, Madison, Wis). The experiment was repeated twice in quadruplicate. P<.05 was considered statistically significant.

RESULTS

TRANFECTION OF ANTISENSE CYCLIN D1 IN TU138 HUMAN SCCHN CELL LINE

In our initial studies, we evaluated the expression of cyclin D1 in SCCHN cell lines. Five of the 6 cell lines examined revealed significant expression of cyclin D1 (Figure 1). To obtain evidence about whether cyclin D1 may play a critical role in maintaining the malignant phenotype of head and neck cancer cells, we overexpressed an antisense cyclin D1 cDNA construct in the TU138 human SCCHN cell line. Twenty independent clones were selected after 2 to 3 weeks of growth in medium supplemented with G418. Immu-
The results of 11 of the 20 clones showed a decrease in cyclin D1 protein level. Three representative clones, 138as1, 138as2, and 138as4, were chosen for further studies (Figure 2). Next, northern blot analysis was performed on RNA extracted from the parental TU138, vector control clones VC1 and VC2, and 138as1, 138as2, and 138as4 cell lines. As demonstrated in Figure 3, the clones expressing antisense cyclin D1 revealed a reduction in endogenous cyclin D1 mRNA. In antisense clones 138as2 and 138as4, antisense cyclin D1 mRNA was detected. In comparison, the controls (the parental TU138 cells and the 2 vector control clones) revealed comparable levels of endogenous cyclin D1 mRNA.

IN VITRO GROWTH OF THE ANTISENSE CYCLIN D1 CELL LINES

When grown in 10% fetal bovine serum medium, the parental TU138 cells and the vector control cell lines showed similar doubling times (24.8-25.9 hours). In contrast, the antisense cyclin D1 clones 138as1 and 138as4 displayed doubling times of 38.5 and 54.2 hours, respectively (Figure 4, top).

CELL CYCLE ANALYSIS OF THE ANTISENSE CYCLIN D1 CELL LINES

Since there is increasing evidence that cyclin D1 plays a critical role in the progression of the G1 phase of the cell cycle, we next examined the cell cycle parameters of the antisense cyclin D1 clones using flow cytometry. Representative flow cytometry profiles for the parental line (TU138) and for the antisense cyclin D1 transfected clone (138as4) are shown in Figure 4, bottom. The antisense cyclin D1 clone cells showed an increase in the percentage of the total cell population in the G0/G1 phase and a decrease in the percentage of total cells in the S phase. Thus, the slowed doubling time of the antisense cyclin D1 clone cells appeared to be due to the block at the G1 phase.
Effect of Antisense Cyclin D1 on Tumorigenicity in Athymic Nude Mice

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>No. of Mice Developing Tumors</th>
<th>Mean ± SD Tumor Volume at Day 30, mm³</th>
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<tbody>
<tr>
<td>TU138</td>
<td>5/5</td>
<td>499.3 ± 227</td>
</tr>
<tr>
<td>138VC1 (vector-only control)</td>
<td>5/5</td>
<td>449.6 ± 126</td>
</tr>
<tr>
<td>138as1 (antisense cyclin D1)</td>
<td>4/5</td>
<td>76.0 ± 91*</td>
</tr>
<tr>
<td>138as4 (antisense cyclin D1)</td>
<td>2/5</td>
<td>2.0 ± 1.7*</td>
</tr>
</tbody>
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*Significant compared with the vector-only control (P < .05).

**TUMORIGENICITY OF THE ANTISENSE CYCLIN D1 CELL LINES**

To examine the in vivo growth properties of the clones transfected with antisense cyclin D1, the tumorigenicity of the TU138 parental cells, the vector control cells, and cells expressing the antisense cyclin D1 were assayed in athymic nude mice by subcutaneous injection. The parental TU138 cells as well as the vector cell line 138VC1 cells produced small tumors within 1 week following injection. In contrast, the in vivo growth was significantly lower (P = .01 for 138as1 and P = .003 for 138as4) for the antisense cyclin D1 clones than for the control cells. Thirty days after injection, the mice injected with cells transfected with antisense cyclin D1 exhibited tumors 84% to 99% smaller than those found in the control mice (Figure 5 and Table). This may be related to the decreased proliferation rate observed in the antisense cyclin D1 transfectants in vitro. These nodules were stable in size for 12 weeks. Because of the continued exponential growth rates of the control cell lines TU138 and 138VC1, animals were killed because of tumor burden.

**CISPLATIN SENSITIVITY OF THE ANTISENSE CYCLIN D1 CELL LINES**

Cisplatin cytotoxic effects were measured using a tetrazolium-based nonradioactive assay. There was a marked increase in sensitivity in the 138as4 antisense cyclin D1 cell line (P = .04) and a slight increase the 138as1 cell line (P = .53). The mean ± SD IC₅₀ values (the concentration of drug inhibiting cell growth by 50%) for the clones were 5.47 ± 0.79 µmol/L for the Tu138 cell line transfected with vector alone, 4.78 ± 1.01 µmol/L for clone 138as1, and 1.94 ± 0.61 µmol/L for clone 138as4 (Figure 6). The antisense cyclin D1 clone 138as4 was significantly more sensitive than the vector control clone (P = .04).

**COMMENT**

The amplification and overexpression of the cyclin D1 gene has been implicated as having a critical role in head and neck carcinogenesis. Clinically, overexpression of cyclin D1 in cancer specimens has been shown to correlate with decreased survival of patients with multiple types of human cancer, including head and neck cancer. Cyclin D1 overexpression can occur through several mechanisms, including DNA amplification of 11q13, chromosome 11 polysomy, or posttranslational regulation of the cyclin D1 protein. Izzo et al demonstrated that dysregulated cyclin D1 expression occurs early in head and neck carcinogenesis and contributes to cellular malignant transformation by dysregulating cell proliferation and enabling gene amplification. Cyclin D1 inhibition by antisense cyclin D1 transfection or by antibody microinjection has been reported to inhibit proliferation and reverse the tumorigenicity of human colon, esophageal, pancreatic, and head and neck cancer cells and to prevent cultured fibroblasts from entering the S phase of the cell cycle.

In our present study, to reevaluate the role of cyclin D1 in the growth and chemosensitivity of SCCHN cells, we stably transfected antisense cyclin D1 cDNA in the human SCCHN cell line TU138. Although the exogenous antisense cyclin D1 transcript was not abundantly detected, we found a 60% reduction of the endogenous cyclin D1 protein, and this resulted in inhibition of cell proliferation in vitro and decreased tumorigenicity in vivo. These results suggest that cyclin D1 has a critical role in
maintaining the abnormal growth and tumorigenicity of this cell line. The expression level of the cyclin D1 protein was not completely abrogated in the antisense clones, suggesting that high-level expression of cyclin D1 is essential for maintaining the malignant phenotype of the cells.

Cisplatin is one of the most effective and commonly used drugs for the systemic treatment of head and neck cancers. However, resistance to cisplatin develops frequently in human tumors. Drug resistance can occur through a number of different mechanisms and is a major cause of chemotherapy failure. Hochhauser et al reported that cyclin D1 overexpression in a fibrosarcoma cell line was associated with increased resistance to methotrexate, indicating that cyclin D1 expression may contribute to drug resistance in cancer cells. Recently, Kornmann et al demonstrated that inhibition of cyclin D1 in pancreatic cancer cells enhances the responsiveness of those cells to multiple chemotherapeutic agents; they suggested that this may be due to the altered expression of several chemoresistance genes, including MRP and MDR1. In this study, we report that cyclin D1 suppression in an SCCHN cell line via antisense transfection enhanced the cytotoxic effects of cisplatin. The sensitivity to cisplatin was greater in the parental cell line than in the antisense transfectants above 10 mmol/L. This biphasic effect at high cisplatin concentrations may be due to the slow doubling time of the antisense transfectants. Since the dosage of cisplatin used for therapy is limited by cumulative renal, hematopoietic, and neural toxic effects, increasing the sensitivity of the cancer cells to chemotherapeutic agents is an attractive strategy.

In summary, we have demonstrated that suppression of endogenous cyclin D1 expression in a human head and neck cancer cell line is sufficient to suppress in vitro cell growth and in vivo tumorigenicity in athymic nude mice. Furthermore, we have demonstrated that antisense cyclin D1 transfection enhances cisplatin chemosensitivity. This suggests that cyclin D1 plays an important role in the growth, aggressiveness, and chemotherapy responsiveness of head and neck cancer cells, and therefore, it may provide a new target for novel therapy in patients with head and neck cancer.

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