Prolonged Response to Antisense Cyclin D1 in a Human Squamous Cancer Xenograft Model

Edward R. Sauter, Meenhard Herlyn, Shao-Chen Liu, Samuel Litwin, and John A. Ridge


ABSTRACT

Local recurrence of squamous cell cancer (SCC) causes high morbidity and is often readily accessible, making such patients potential candidates for gene therapy. Cyclin D1 (CD1), critical in the G1-S transition in the cell cycle, is amplified in 20–50% and overexpressed in up to 80% of head and neck SCC. Our earlier studies indicated that CD1 expression increased with progression from low grade to high grade dysplasia, and that treatment of established tumors with antisense cyclin D1 (AS-cyclin D1) led to tumor regression during a one week evaluation period. We hypothesized that: 1) CD1 expression increases with disease progression to advanced SCC, and 2) AS-cyclin D1 therapy would lead to prolonged tumor regression in a xenograft model of human SCC. CD1 expression, evaluated by immunostain in 30 stage III/IV head and neck SCC, increased in the basal layer from normal-dysplasia (P = 0.06) and from dysplasia-carcinoma (P = 0.004). In the germinative layer CD1 expression increased from dysplasia-carcinoma (P = 0.002) but not from normal-dysplasia. Western blotting of eight SCC and two transformed keratinocyte cell lines demonstrated CD1 overexpression in 8/10 (80%) lines. An 11th cell line (A431) had previously been shown to overexpress CD1, shrink significantly for 2–4 weeks after AS-cyclin D1 treatment, while tumors transduced with control vector grew. Cyclin D1 expression increases in frequency with disease progression, and antisense cyclin D1 was effective in a xenograft model of human cancer, independent of tumor growth rate.

INTRODUCTION

SCC is the most common malignancy in man, occurring in multiple sites in the body, including the head and neck, skin, cervix, and lung. Advanced SCC is often fatal, even when treated with multimodality therapy. SCC often presents in one area of the body, without evidence of blood-borne metastases despite extensive locoregional progression. This makes SCC an ideal substrate for gene therapy through direct treatment of the tumor, because local delivery remains possible despite advanced disease stage.

There are a variety of approaches to cancer gene therapy, including 1) local therapy, through direct treatment of the lesion; 2) regional therapy, administered through an artery or vein supplying a given organ(s) or region of the body, or through injection into a body cavity; and 3) systemic therapy, generally administered through venous access. Although limitations exist with each of these approaches, steps are being taken to overcome them. Of the approaches mentioned, direct injection has the advantage of administering a very high dose to the target while minimizing the dose to surrounding tissue.

Cytogenetic analyses of SCCHN have demonstrated a breakpoint on chromosome 11 at 11q13 (1). The oncogenes int-2, hst-1, and prad1 in the 11q13 amplicon have been reported (2). Of the three genes, only prad1 (bcl-1, CCND1, cyclin D1) is expressed. Cyclin D1 is a proto-oncogenic regulator of the G1-S checkpoint in the cell cycle that has been implicated in the pathogenesis of several types of cancer, including SCC. Amplification of the cyclin D1 gene is found in 20–50% of SCCs, and the protein is overexpressed in up to 80% of tumors (3, 4).

Amplification is only one method by which the protein product can be overexpressed. Increased expression has also been observed due to gene rearrangement (5, 6) both in parathyroid tumors (11q13 with 11p15) and B cell tumors (11q13 with 14q32). Overexpression of cyclin D1 in cultured cells leads to a more rapid transversion through the G1 phase of the cell cycle and entry into S phase (7, 8). Cyclin D1 cooperates with ras (9) and complements a defective Eta adenoviral gene (10) to function as an oncogene.

Other gene products that help regulate the G1 progression...
include pRb, CDKN2A/p16INK4A, cdk4, and cdk6 (11). pRb is functional in the vast majority of both primary (12) and immortalized SCCs (13, 14). Deletions and mutations in p16 in primary SCCs are uncommon, occurring in 0–20% of cases (15–17). Although methylation has been proposed as a major mechanism of p16 gene inactivation in SCCs (18), other investigators have not found this to be the case (15). cdk4 and cdk6 gene mutations are rare in cancer of any origin (19). We elected to study cyclin D1, which is frequently mutated and overexpressed in SCCs, suggesting that alteration of the gene is an important event in SCC development and/or progression.

Cyclin D1 overexpression occurs in preinvasive lesions, and increases from low grade to high grade dysplasia (20). An increase in intense cyclin D1 expression was observed in the basal and superficial epithelium of oral cavity mucosa from histologically normal:low:high grade (mild:moderate:severe) dysplasias (3:7:19% for basal, 0:5:4.6% for superficial epithelium), none of which had a concurrent invasive SCC.

Through the creation of a replication-deficient adenoviral vector containing cyclin D1 in antisense orientation (AS-cyclin D1), we demonstrated in a human xenograft model that AS-cyclin D1 was effective for 1 week in decreasing cyclin D1 expression both in vitro and in vivo, in increasing apoptosis, and in promoting shrinkage of established tumors (21). We hypothesized that cyclin D1 overexpression was important not only in initiation, but also with progression of SCCs. This was addressed using a two-tiered approach. First, we determined whether cyclin D1 expression increased in advanced clinical (stage III and IV) and experimental SCCs. Second, we assessed whether treatment with AS-cyclin D1 led to a persistent reduction in tumor volume. We found that the incidence of cyclin D1 overexpression increases with disease progression, that SCC cell lines with CD1 overexpression formed tumors in ≤ 80 days, whereas cells lacking increased CD1 did not, and that antisense cyclin D1 therapy leads to a persistent reduction in tumor volume over a 2- to 4-week follow-up period. These findings confirm the importance of cyclin D1 in SCC disease progression and demonstrate the potential efficacy of antisense cyclin D1 gene therapy to treat SCCs.

MATERIALS AND METHODS

Cell Culture. Nine human SCC cell lines, six from the head and neck (SCC 9, SCC 25, SCC 40, A253, Det562, FaDu), and two from facial skin (SCC 12, SCC 13), and one from the vulva (A431), and two keratinocyte cell lines, one spontaneously (HaCaT) and the other human papillomavirus (HPK1A) transformed, were kindly provided by J. Rheinwald (SCC 9, SCC 12, SCC 13, SCC 25, SCC 40; Harvard Medical School, Boston, MA), or by J. Henderson (HPK1A; Department of Medicine, McGill University, Montreal, PQ, Canada), by N. Fusenig (HaCaT; Division of Carcinogenesis and Differentiation, German Cancer Research Center, Heidelberg) or were obtained from the American Type Culture Collection (A253, Det562, A431, FaDu, ATCC, Rockville, MD). Despite initial maintenance in DMEM supplemented with 10% FCS, these were weaned off serum and grown at a 4:1 ratio in MCDB 201/L15 (Sigma Chemical Co., St. Louis, MO) supplemented with 5 μg/ml insulin (SCC medium). Cyclin D1 expression was compared when cells were grown with or without FCS, because evidence suggests that cyclin D1 has a serum response element (22). Normal keratinocytes obtained from neonatal foreskins were cultured using conditions described by Southgate et al. (23), with minor modifications.

Transcomplementing 293 cells (24), used to grow AS-cyclin D1, were obtained from the Vector Core at the Institute for Human Gene Therapy (University of Pennsylvania, Philadelphia, PA) and grown in DMEM supplemented with 10% FCS. Unless otherwise noted, all tissue culture reagents were purchased from Sigma.

Construction of Replication-Defective Adenoviral Vector. Construction of an adenoviral vector has been previously described (25). Briefly, A 1.1-kb PCR product with the entire cyclin D1 open reading frame was inserted into the modified multiple cloning site (University of Pennsylvania Vector Core) of pSL301 (Invitrogen, Carlsbad, CA). The resulting plasmid was subcloned into the adenoviral vector pAd.CMV-Link1 (Vector Core), and the cyclin D1 orientation was determined. pAd.CMV-Link1 was cotransfected into 293 cells containing the E1 gene of Ad5 with adenoviral DNA lacking the E1 and E3 regions (26), using calcium phosphate precipitation. A 10% glycerol shock was then administered, and the cells were grown with an overlay of 0.8% Bactoagar (Difco Labs, Detroit, MI). Fresh overlay was added every 3–4 days until plaques appeared.

Putative plaques were expanded and screened by restriction fragment and Southern blot analyses (27), a positive AS-cyclin D1 plaque was propagated in 293 cells (28), the virus was released by freeze-thawing and purified by CsCl gradient centrifugation, and the final plaque-forming units were determined by titration under an agar overlay (29).

In Vivo Cell Growth. Cell lines were grown in SCC media to 70–80% confluence and detached with 0.06% trypsin, the cells counted, and 2 × 10⁶ cells injected s.c. in the dorsal surface of SCID mice just medial to the right hind limb. Cell lines were considered tumorigenic if a visible tumor was identified within 80 days of injection. Established tumors from three lines (A431, SCC 13, and A253) were evaluated for their response to AS-cyclin D1. After 10–12 replicate tumors formed from each line had grown to a minimum volume of 100 mm³, a single injection of adenovirus of 5 × 10⁹ plaque-forming units and containing control vector (β-galactosidase, LacZ) or AS-cyclin D1 in 50 μl of SCC medium was administered into each tumor using a tuberculin syringe. Each tumor volume was measured (length × width × height) for a minimum of 2 weeks and a maximum of 4 weeks after treatment. Measurements were stopped at 28 days or when control tumors reached such a large size that euthanasia was required. For statistical analysis, tumor volumes at baseline and at days 4, 7, 10, 14, 17, 21, 24, and 28 after viral transduction were fitted to an exponential curve \( y = A \exp(\beta t) \), where \( \beta \) is the tumor regression rate estimated for each mouse from the eight observations. \( \beta \) values in each group were rank-ordered and analyzed by the Wilcoxon two-sample (one-sided) procedure to test the hypothesis of equal rates of tumor regression among groups versus the alternative that treated tumors regress faster.
Detection of Cyclin D1 by Western Blotting and Immunohistochemistry. After growing the cell lines to 70–80% confluence, 100 \( \mu \)g of total protein was extracted from each sample, separated electrophoretically under reducing conditions in a discontinuous 12% polyacrylamide gel, and transferred to a polyvinylidene difluoride membrane. After transfer, membranes were blocked with 5% nonfat dry milk and probed with a mouse monoclonal antibody to cyclin D1 (clone HD-11; Santa Cruz Biotechnology, Santa Cruz, CA) followed by a phosphatase-conjugated goat antimouse IgG (Jackson ImmunoResearch, West Grove, PA) and the substrates 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. Band intensity was quantified using a personal densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Thirty formalin-fixed, paraffin-embedded SCCs were cut in 5-\( \mu \)m sections and placed on poly-L-lysine-coated glass slides. After boiling in distilled water for 10 min, slides were incubated with an anticyclin D1 monoclonal Ab (Ab-3; Calbiochem). Horse antimouse IgG was then applied (Vector Laboratories) followed by an avidin-biotin-peroxidase complex (Vector Laboratories) and the chromagen 3',3'-diaminobenzidine (with a counterstain of hematoxylin).

Expression of Cyclin D1 in Clinical and Experimental SCCs. Among subjects with advanced SCCHN, cyclin D1 staining was observed in 26/30 (87%) tumor specimens, although the fraction of cells that stained varied. We evaluated cyclin D1 expression in the three layers nearest the basement membrane, otherwise known as the germinative layer. The germinative layer is divided into the basal layer, the cell layer nearest the basement membrane, and the adjacent two cell layers, the parabasal layer. We (20) and others (30, 31) have demonstrated that the proliferative activity of normal squamous epithelia is higher in the parabasal than in the basal layer. Expression of cyclin D1, as a critical regulator of G1-S in the cell cycle, helps to drive cell proliferation. Indeed, we observed a cyclin D1 labeling index of 43% in the parabasal layer and of 3% in the basal layer of normal oral mucosa (20). There was an

<table>
<thead>
<tr>
<th>Specimens</th>
<th>Basal layer</th>
<th>Germinative layer</th>
<th>Basal layer</th>
<th>Germinative layer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal(^b)</td>
<td>57</td>
<td>3 (0.4)</td>
<td>19 (6.0)</td>
<td>19 (6.0)</td>
</tr>
<tr>
<td>Adjacent normal(^d)</td>
<td>24</td>
<td>5 (3.9)</td>
<td>20 (7.3)</td>
<td>20 (7.3)</td>
</tr>
<tr>
<td>Adjacent dysplasia(^d)</td>
<td>5</td>
<td>10 (7.6)</td>
<td>0.004</td>
<td>0.002</td>
</tr>
<tr>
<td>SCC</td>
<td>24</td>
<td>20 (9.6)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Six specimens that contained only invasive cancer were not included in this table.

\(^b\) Individuals without SCCHN.

\(^c\) Not significant.

\(^d\) Histologically normal or dysplastic tissue adjacent to invasive SCCHN.

**RESULTS**

**Expression of Cyclin D1 in Clinical and Experimental SCCs.** Among subjects with advanced SCCHN, cyclin D1 staining was observed in 26/30 (87%) tumor specimens, although the fraction of cells that stained varied. We evaluated cyclin D1 expression in the three layers nearest the basement membrane, otherwise known as the germinative layer. The germinative layer is divided into the basal layer, the cell layer nearest the basement membrane, and the adjacent two cell layers, the parabasal layer. We (20) and others (30, 31) have demonstrated that the proliferative activity of normal squamous epithelia is higher in the parabasal than in the basal layer. Expression of cyclin D1, as a critical regulator of G1-S in the cell cycle, helps to drive cell proliferation. Indeed, we observed a cyclin D1 labeling index of 43% in the parabasal layer and of 3% in the basal layer of normal oral mucosa (20). There was an
increase (Table 1) in cyclin D1 expression (Fig. 1) in the basal layer from normal to dysplasia ($P = 0.06$) and from dysplasia to carcinoma ($P = 0.002$) but not from normal to dysplasia. In histologically normal epithelium adjacent to SCC, the median fraction of cells staining for cyclin D1 in both the basal and germinative layers was similar to epithelium from normal donors.

Using a relative band intensity of > 1.5 compared with normal keratinocytes, there was overexpression of cyclin D1, a 34-kDa protein, in 8/10 (80%) cell lines (Fig. 2). A nonspecific band, routinely seen using this mAb (clone HD-11; Santa Cruz Biotechnology catalogue), was visualized at 51 kDa. We did not evaluate A431 by Western blotting because of numerous prior reports documenting its being both amplified and overexpressed (8, 32, 33). In the remaining two lines (SCC 12 and SCC 40), a lesser increase in expression was noted. No significant difference was found in cyclin D1 expression when comparing cell lines grown with or without FCS. Of the lines with cyclin D1 overexpression when injected into SCID mice, 7/8 (88%) formed tumors 80 days after injection, whereas none of the three lines without cyclin D1 overexpression formed a tumor within that time period (Table 2).

**Inhibition of Growth After Transduction of AS-Cyclin D1.** In each of the three cell lines tested, tumor growth was significantly decreased (A431, $P = 0.004$; A253, $P = 0.017$; SCC 13, $P = 0.008$) after AS-cyclin D1 treatment compared with tumors treated with LacZ (Table 3). Table 3 provides information regarding tumor growth over a 14-day period from which we can determine whether tumor growth rate has any influence on the effect of antisense cyclin D1. AS-cyclin D1 was effective in shrinking A431 (a fast-growing tumor), A253 (also fast-growing once formed, although taking approximately 60 days to form a visible tumor in a SCID mouse after injecting 2 million cells versus 14 days for A431), and SCC 13 (a slow-growing tumor once formed, and taking approximately 3 months

![Fig. 2 Cyclin D1 expression in SCC cell lines and in transformed keratinocyte lines compared with normal (NL) keratinocytes. Cells were grown in serum-free media to 70–80% confluence. Each sample (100 μg) was separated electrophoretically, transferred to a polyvinylidene difluoride membrane, blocked with 5% nonfat dry milk, and probed with a mouse monoclonal antibody to cyclin D1 followed by a phosphatase-conjugated goat anti-mouse IgG and the substrates 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. A nonspecific band is seen at 51 kDa.](Image)

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cyclin D1 amplification</th>
<th>Cyclin D1 overexpression</th>
<th>Tumorigenicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>HaCaT</td>
<td>Yes</td>
<td>Increased</td>
<td>Yes</td>
</tr>
<tr>
<td>HPK1A</td>
<td>N/A</td>
<td>Increased</td>
<td>Yes</td>
</tr>
<tr>
<td>SCC 9</td>
<td>N/A</td>
<td>±</td>
<td>No</td>
</tr>
<tr>
<td>SCC 12</td>
<td>N/A</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>SCC 13</td>
<td>No</td>
<td>Increased</td>
<td>Yes</td>
</tr>
<tr>
<td>SCC 25</td>
<td>No</td>
<td>Increased</td>
<td>No</td>
</tr>
<tr>
<td>SCC 40</td>
<td>No</td>
<td>±</td>
<td>No</td>
</tr>
<tr>
<td>A253</td>
<td>Yes</td>
<td>Increased</td>
<td>Yes</td>
</tr>
<tr>
<td>Det562</td>
<td>Yes</td>
<td>Increased</td>
<td>Yes</td>
</tr>
<tr>
<td>FaDu</td>
<td>Yes</td>
<td>Increased</td>
<td>Yes</td>
</tr>
<tr>
<td>A433</td>
<td>Yes</td>
<td>Increased</td>
<td>Yes</td>
</tr>
</tbody>
</table>

*a* All results except HaCaT (38) were provided by one of the authors (SCL, unpublished data).

*b* Defined as a relative band intensity of >1.5 compared to normal keratinocytes.

*c* Each cell line formed a malignant tumor except HaCaT, which formed a benign tumor.

*d* Not available.

*e* SCC 12 formed tumors only if $1 \times 10^7$ cells (5 times the normal number) were injected.
for a visible tumor to form). Although the degree of significance for the different tumor types varied, this is primarily related to sample size.

Fig. 3 visually demonstrates the effect of AS-cyclin D1 on each of the xenograft tumor types. The Y axis is different for each tumor type, due to the different growth rates. Each of the tumors responded to AS-cyclin D1, and the tumor size remained below baseline for the entire evaluation period (17–28 days). After treatment with the control (LacZ) vector the tumors continued to grow, although at different rates.

DISCUSSION

Our hypothesis was that cyclin D1 was critical to squamous cell carcinogenesis, and that suppression of cyclin D1 protein expression would lead to either growth arrest or cell death. To test the hypothesis, we created an adenoviral vector containing cyclin D1 in antisense orientation, believing that the antisense product could suppress cyclin D1 protein production in the SCC cells. After treatment with AS-cyclin D1, both cyclin D1 protein production and cell growth were substantially suppressed, and apoptosis dramatically increased, both in vitro and in vivo (21).

The response after in vivo treatment of established tumors is promising. Prior reports of in vivo treatment of SCCs using wild-type p53 (34, 35) document a delay in tumor growth after single injection or stable disease after repeat therapy, rather than demonstrating tumor shrinkage. Our earlier work only addressed tumor response for 1 week after treatment. The current report evaluates the longer term response (up to 4 week) of SCCs to antisense cyclin D1.

Prior reports have demonstrated in vitro (36–39) and/or ex vivo (38, 40–42) inhibition of cell growth with antisense cyclin D1. Our plan was to evaluate the ability of antisense cyclin D1 to inhibit cell growth in established tumors derived from human cancer cells. We chose squamous cell carcinoma, a human tumor system in which the importance of cyclin D1 has been clearly demonstrated. The gene is amplified in up to half of the tumors and the RNA and protein are overexpressed in most cases. Our findings indicate that in vivo transduction with AS-cyclin D1 leads to prolonged tumor shrinkage in a xenograft model of human SCC. Our findings document that: 1) cyclin D1 expression continues to increase with disease progression both in clinical and experimental SCCs and 2) antisense cyclin D1 can shrink established human SCCs, including those from the

Table 3  Tumor response after treatment with antisense cyclin D1 or β-galactosidase (LacZ) control

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment</th>
<th># Mice</th>
<th>Mean (±SE) change in tumor volume (mm³)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A431</td>
<td>LacZ</td>
<td>5</td>
<td>342.2 (107.4) 1055.4 (358.7) 2422.2 (863.2)</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>AS-cyclinD1</td>
<td>5</td>
<td>432.2 (195.4) 195.2 (63.6) 305.5 (222.2)</td>
<td></td>
</tr>
<tr>
<td>SCC 13</td>
<td>LacZ</td>
<td>5</td>
<td>137.6 (48.3) 167.6 (50.0) 151.2 (70.0)</td>
<td>0.017</td>
</tr>
<tr>
<td></td>
<td>AS-cyclinD1</td>
<td>3</td>
<td>994.3 (521.7) 174.0 (105.6) 141.5 (101.6)</td>
<td></td>
</tr>
<tr>
<td>A253</td>
<td>LacZ</td>
<td>4</td>
<td>420.0 (150.5) 1160.4 (143.4) 2091.4 (86.1)</td>
<td>0.008</td>
</tr>
<tr>
<td></td>
<td>AS-cyclinD1</td>
<td>5</td>
<td>881.7 (273.2) 236.0 (99.5) 148.0 (68.0)</td>
<td></td>
</tr>
</tbody>
</table>

* Some mice were evaluated for up to 28 days (see Fig. 3).

Fig. 3  Inhibition of SCC (A431, A253, and SCC 13) growth after transduction in vivo of AS-cyclin D1. Two million cells were injected s.c. into the dorsum of SCID mice until a visible tumor formed. Tumors were allowed to grow to a minimum volume of 100 mm³ and then injected with adenoviral vectors (5 × 10⁸ plaque-forming units) in 50 μl of SCC media.
face, head and neck, and vulva. This suppression persists for at least 2–4 weeks.

We observed regression both in tumors with (A431 and A253) and without (SCC 13) cyclin D1 overexpression. There was not a significant difference in response related to the level of cyclin D1 expression within the tumor. Nonetheless, a greater difference in size (treated versus controls) was observed 2–4 weeks after treatment in tumors overexpressing cyclin D1 (A431: 620% growth versus 62% shrinkage; A253: 324% growth versus 79% shrinkage) than in tumors lacking cyclin D1 overexpression (SCC 13: 36% growth versus 87% shrinkage). Further studies are required to determine whether the level of cyclin D1 expression is an important predictor of response to AS-cyclin D1.

Our model of injecting formed tumors recapitulates more accurately the clinical situation than experiments documenting tumor shrinkage after ex vivo therapy. SCCs of the skin (both facial and vulvar) and the head and neck can be seen in the vast majority of cases. As such, these tumor types are readily accessible to direct injection with antisense cyclin D1. Antisense cyclin D1 treatment could prove to be an appropriate adjunct to currently available therapy, especially when unresectable tumors persist or recur locally after surgery and radiotherapy. Indeed, significant shrinkage could potentially complement standard treatments such as surgery and radiation.

In a prior report (21) we demonstrated that cyclin D1 induces apoptosis, leading to tumor shrinkage. It is likely that residual viable tumor cells divide and the tumors will eventually regrow. Whether these viable tumor cells transiently undergo G1 arrest or continue to divide in the period immediately following treatment is unclear. Our in vitro experiments in SCC cells did not demonstrate G1 arrest, suggesting that the viable cells continue to divide but that tumor shrinkage occurs as long as cell death exceeds cell replication. Hence, multiple treatments may lead to a further reduction in tumor volume. Prior reports with an adenoviral vector containing wild-type p53 show that multiple doses are more effective than a single treatment (35). In addition, combination gene therapy may provide a greater reduction in tumor volume.

Although SCC 25 demonstrated increased cyclin D1 expression, it failed to form tumors in immunodeficient mice, contrary to our findings in the other cell lines that had increased cyclin D1 expression. Although it is not entirely clear why findings for SCC 25 are seemingly in conflict with the remaining cell lines, some factors are worth mentioning. First, we found that, although SCC 25 cells grown in serum-free medium did not form tumors in SCID mice, others have found that SCC 25, after in vitro growth in 10% fetal bovine serum, was tumorigenic in nude mice (source: American Type Culture Collection catalogue no. CRL-1628). Another interesting observation is that SCC 25 was one of only two cell lines with cyclin D1 overexpression that, when analyzed, lacked cyclin D1 amplification. The other, SCC 13, formed tumors in SCID mice only after 3 months or more, a longer period of time than for any cell line with cyclin D1 amplification that we studied. Nonetheless, we are not aware of published data demonstrating that the method leading to overexpression (gene amplification versus translocation) affects the tumorigenicity of the cells.

An important question arises as to whether or not the tumor suppressive effect of antisense cyclin D1 is temporary or permanent. As mentioned in “Materials and Methods,” measurements were stopped at 28 days or when control tumors reached such a large size that euthanasia was required. Tumors formed from A253 and A431 cells treated with control vector became very large after 2–3 weeks. Although we could have extended the length of time for the cyclin D1-treated tumors, we would not have had a control. We stopped observation of SCC 13 cells after 28 days to be in concordance with the observation time of the other two cell lines. Although we cannot determine from the data available whether the growth suppressor effects of cyclin D1 are transient or permanent, we are concerned that the tumors did not vanish by 21–28 days. In a different tumor system, we are evaluating the possible synergism of two adenoviral vectors with different mechanisms of action. In some of the lesions treated with both vectors, the tumor has vanished within the first 28 days.

In summary, cyclin D1 expression increases with disease progression at all stages of SCC carcinogenesis. Established tumors undergo dramatic shrinkage after treatment with antisense cyclin D1. The response persists for at least 2–4 weeks.

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