Cyclin D1 Proteolysis: a Retinoid Chemoprevention Signal in Normal, Immortalized, and Transformed Human Bronchial Epithelial Cells

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Background: **Retinoids (derivatives of vitamin A) are reported to reduce the occurrence of some second primary cancers, including aerodigestive tract tumors. In contrast, β-carotene does not reduce the occurrence of primary aerodigestive tract cancers. Mechanisms explaining these effective retinoid and ineffective carotenoid chemoprevention results are poorly defined. Recently, the all-*trans*-retinoic acid (RA)-induced proteolysis of cyclin D1 that leads to the arrest of cells in G_1 phase of the cell cycle was described in human bronchial epithelial cells and is a promising candidate for such a mechanism. In this study, we have investigated this proteolysis as a common signal used by carotenoids or receptor-selective and receptor-nonselective retinoids.** ***Methods:*** **We treated cultured normal human bronchial epithelial cells, immortalized human bronchial epithelial cells (BEAS-2B), and transformed human bronchial epithelial cells (BEAS-2B$_{NNK}$) with receptor-selective or receptor-nonselective retinoids or with carotenoids and studied the effects on cell proliferation by means of tritiated thymidine incorporation and on cyclin D1 expression by means of immunoblot analysis. We also examined whether calpain inhibitor I, an inhibitor of the 26S proteasome degradation pathway, affected the decline (i.e., proteolysis) of cyclin D1.** ***Results:*** **Receptor-nonselective retinoids were superior to the carotenoids studied in mediating the decline in cyclin D1 expression and in suppressing the growth of bronchial epithelial cells. Retinoids that activated retinoic acid receptor β or retinoid X receptor pathways preferentially led to a decrease in the amount of cyclin D1 protein and a corresponding decline in growth. The retinoid-mediated degradation of cyclin D1 was blocked by cotreatment with calpain inhibitor I.** ***Conclusions:*** **Retinoid-dependent cyclin D1 proteolysis is a common chemoprevention signal in normal and neoplastic human bronchial epithelial cells. In contrast, carotenoids did not affect cyclin D1 expression. Thus, the degradation of cyclin D1 is a candidate intermediate marker for effective retinoid-mediated cancer chemoprevention in the aerodigestive tract. [J Natl Cancer Inst 1999;91: 373–9]**

Lung cancer is the leading cause of cancer mortality for men and women in the United States. Because curative therapy for disseminated non-small-cell lung cancer does not yet exist, effective chemoprevention of lung cancer in patients with prior tobacco exposure would have a major impact on reducing lung cancer mortality. Retinoids, natural and synthetic derivatives of vitamin A, have *in vitro* and *in vivo* chemoprevention activities. Clinical trials have shown that retinoids are active in treatment of some second cancers. Treatment with 13-*cis*-retinoic acid (13-*cis*-RA) has reduced second aerodigestive tract cancers in patients with previously resected head and neck cancers *(1)*. Treatment with retinyl palmitate has reduced second primary lung cancers in patients who have had completely resected lung cancers *(2)*. An acyclic retinoid, polyprenoic acid, inhibited development of second hepatocellular carcinomas in patients with resected or ablated

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primary hepatocellular carcinomas *(3)*. The mechanisms used by retinoids to produce these beneficial cancer chemoprevention results are poorly defined, and vitamin A-associated toxic effects have limited chronic retinoid treatments of individuals at high risk of developing cancer. The desire to reduce retinoid toxicity led to large cancer chemoprevention trials of β-carotene, a related compound that is clinically well tolerated; unfortunately, these trials did not result in the prevention of lung cancer in high-risk individuals *(4–6)*. These findings underscore the potential value of *in vitro* carcinogenesis studies in model systems that can be used to determine cancer prevention mechanisms, to identify intermediate markers, and to select appropriate agents for testing in clinical cancer chemoprevention trials.

We have reported that all-*trans*-retinoic acid (RA) can prevent the transformation of human bronchial epithelial cells *in vitro (7);* i.e., RA inhibited the transformation of immortalized human bronchial epithelial cells (BEAS-2B) that had been treated with tobacco-derived carcinogens. This *in vitro* chemopreventive activity was associated with a decline in the expression of cyclin E, the arrest of cells in G_1 phase of the cell cycle, and the concomitant suppression of growth *(7)*. A decline in the amount of cyclin D1 protein also followed treatment of these human bronchial epithelial cells with RA and was blocked by inhibition of the 26S proteasome degradation pathway *(8)*, a finding that suggests that proteasome-dependent degradation of cyclin D1 is a mechanism used by retinoids to prevent the carcinogen-induced transformation of human bronchial epithelial cells.

In this study, we have investigated whether this is a common mechanism by studying additional types of human bronchial epithelial cells and various carotenoids and retinoids to determine whether these compounds use the degradation of cyclin D1 as a common chemoprevention mechanism.

MATERIALS AND METHODS

Human Bronchial Epithelial Cells

Primary normal human bronchial epithelial cells (Clonetics, San Diego, CA) were cultured as previously described *(8)* in modified LHC-9 medium in the presence or absence of the indicated retinoids, carotenoids, or appropriate vehicle controls. Cells were passaged every 5–7 days and were used for experiments after two to four passages. BEAS-2B cells were derived from human bronchial epithelial cells immortalized with an adenovirus 12–simian virus 40 hybrid virus *(9)*. BEAS-2B$_{NNK}$ cells are carcinogen-transformed human bronchial epithelial cells derived from BEAS-2B immortalized bronchial epithelial cells after treatment with *N*-nitrosamine-4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, as described *(7)*. These immortalized or transformed human bronchial epithelial cells were cultured in serum-free medium by established techniques *(10)*. These cells were treated with the indicated retinoids or carotenoids in subdued light and then cultured in the dark in an incubator with humidified air at 37 °C with 5% CO_2, as previously described *(7–10)*.

Retinoid Treatments

The receptor-nonselective retinoids used were RA, 9-*cis*-retinoic acid (9-*cis*-RA), and 13-*cis*-RA (Sigma Chemical Co., St. Louis, MO). The receptor-selective retinoid agonists were Am80 (selective for retinoic acid receptor α [RARα]) *(11)*, SR11254 (RARγ/β) *(12)*, SR11246 and SR11345 (retinoid X receptor [RXR]) *(13)* (SRI International, Menlo Park, CA), and BMS-189453 (RARβ) *(14)* (Bristol-Myers Squibb, Buffalo, NY). Stock solutions of retinoids (10^{-2} *M*) dissolved in dimethyl sulfoxide were stored in the dark at −70 °C until used.

Carotenoids

Stock solutions of the carotenoids α-carotene (10^{-2} *M*) and β-carotene (10^{-2} *M*) (Sigma Chemical Co.) were individually dissolved in tetrahydrofuran. These stock solutions were added to LHC-9 medium at room temperature with vigorous stirring to achieve a final concentration of 10^{-5} *M*. The concentrations of these stock carotene solutions were verified spectrophotometrically, and the solutions were distributed in aliquots and stored in the dark at −70 °C *(15,16)*. Before use, the carotene stock solutions were diluted to the indicated concentrations with LHC-9 medium.

Immunoblot Analysis

The human bronchial epithelial cells examined were treated with the indicated retinoids, carotenoids, or appropriate vehicle solutions for 0–24 hours before lysis on 10-cm tissue culture plates (Falcon, Franklin Lakes, NJ) with buffer containing protease inhibitors, as described *(7)*. Total cellular protein was measured by the Bradford assay, and 100–200 μg of total cellular protein was denatured, subjected to electrophoresis through 10% polyacrylamide gels containing sodium dodecyl sulfate, and electroblotted to nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH). The following primary antibodies were used: for human cyclin D1, M-20; for RARα, C-20; for RARβ, C-19; for RARγ, C-19; and for RXRα, D-20 (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA). A polyclonal anti-rabbit immunoglobulin antibody was used as the secondary antibody (Amersham Life Science Inc., Arlington Heights, IL). Antibody was detected with the Amersham chemiluminescence assay.

To verify the specificity of each anti-retinoid receptor antibody, preincubation of the antibody with a 10-fold excess of a specific blocking peptide at room temperature for 2 hours was found to abolish the expected immunoblot signal. The blocking peptides used for each antibody (Santa Cruz Biotechnology, Inc.) were the immunogenic peptides used to produce the corresponding antibodies.

Thymidine Proliferation Assay

Growth of human bronchial epithelial cells was assessed by tritiated thymidine incorporation. Approximately 5×10^4 cells (plated per well in a six-well tissue culture plate [Falcon]) were incubated for 1 hour in tritiated thymidine (4 μCi/mL; Du Pont NEN, Boston, MA) at 37 °C, washed with phosphate-buffered saline, treated with 5% trichloroacetic acid, washed with 70% ethanol, air dried, and lysed in a solution of 10 m*M* NaOH and 1% sodium dodecyl sulfate. Lysates were collected, and the amount of tritiated thymidine incorporated was measured by scintillation counting. The amount of radioactivity detected (decays per minute) in each treatment sample was expressed as a percentage of the amount of the radioactivity in control samples. Data are reported as the average of six data points from three independent experiments. Error bars represent the standard deviation for each average value.

Inhibition of the Proteasome Degradation Pathway

To inhibit the 26S proteasome degradation pathway, we treated BEAS-2B immortalized human bronchial epithelial cells with calpain inhibitor I (100 μ*M*) with or without a retinoid for 4–6 hours at 37 °C before cell lysis and immunoblot analysis of the isolated total cellular protein. No cytotoxicity was detected as a result of the calpain inhibitor I treatment under these culture conditions.

RESULTS

Compared with a treatment with the vehicle control, a 24-hour treatment of BEAS-2B immortalized human bronchial epithelial cells with 4 μ*M* RA, 4 μ*M* 13-*cis*-RA, or 4 μ*M* 9-*cis*-RA resulted in appreciable growth suppression, as measured by tritiated thymidine incorporation (Fig. 1, A). Treatment of BEAS-2B cells with α-carotene or β-carotene at concentrations up to 8 μ*M* did not suppress bronchial epithelial cell growth (Fig. 1, B). These concentrations of carotenoids and retinoids in plasma are clinically achievable levels for each agent *(17,18)*. If the carotenoid treatment was examined for up to 72 hours, no effect on BEAS-2B cell growth was observed (data not shown).

Treatment with retinoids but not with carotenoids suppressed the growth of BEAS-2B cells, normal human bronchial epithelial cells in short-term cultures, and carcinogen-transformed BEAS-2B$_{NNK}$ cells (Fig. 1, C). These normal, immortalized, and transformed human bronchial epithelial cells represent *in vitro* models for lung tissue of carcinogen-exposed individuals at risk for developing primary or secondary aerodigestive tract cancers.

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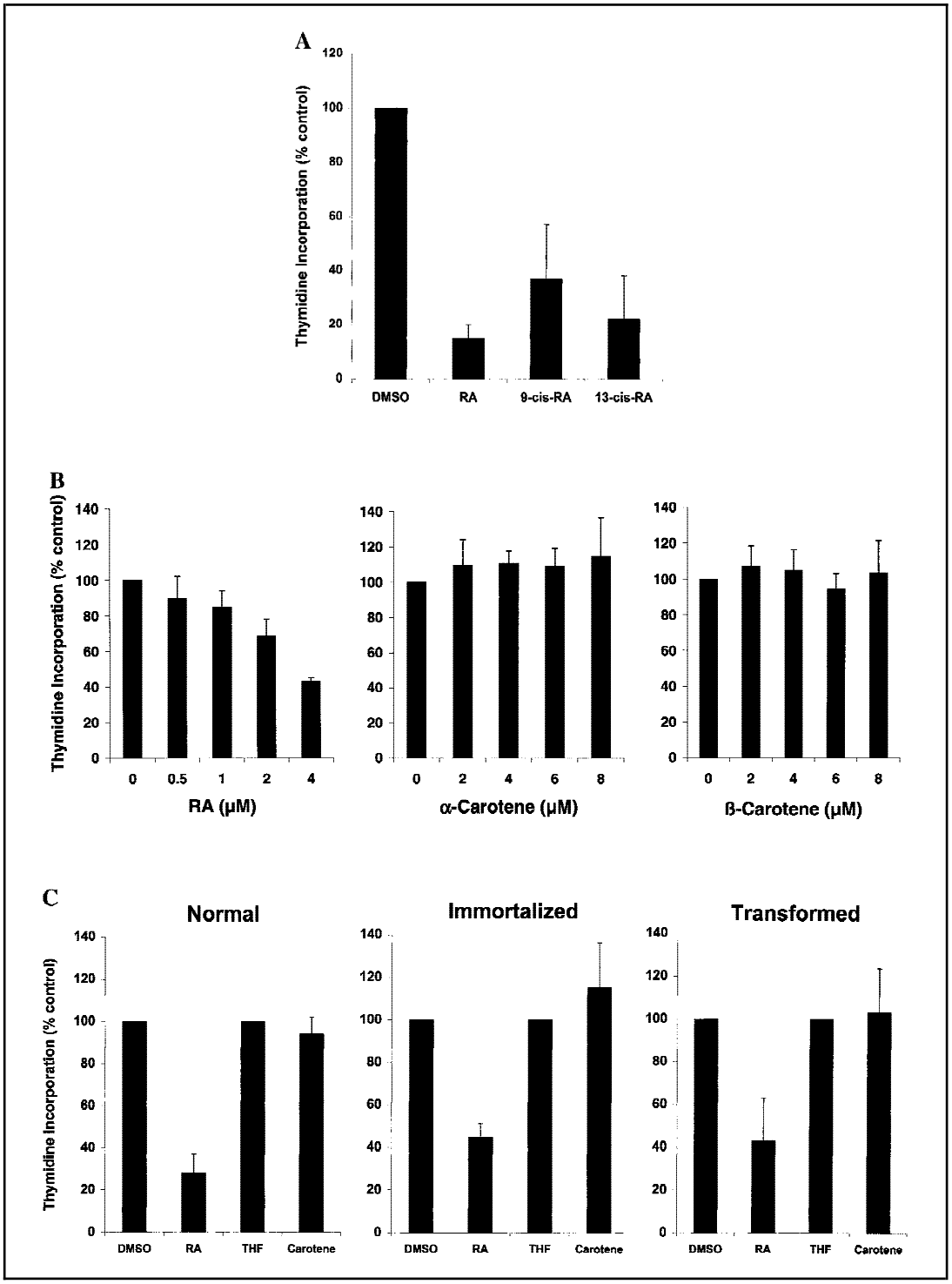

Fig. 1. Growth response of immortalized human bronchial epithelial (BEAS-2B) cells treated with retinoids or carotenoids. **A)** Cells were treated for 24 hours with 4 μ*M* all-*trans*-retinoic acid (RA), 4 μ*M* 9-*cis*-retinoic acid (9-*cis*-RA), 4 μ*M* 13-*cis*-retinoic acid (13-*cis*-RA), or dimethyl sulfoxide (DMSO; 1 : 1000 dilution; the vehicle control). Treatment with the retinoids resulted in considerable growth suppression, as measured by tritiated thymidine incorporation. **B)** Cells were treated with various concentrations of RA, α-carotene, β-carotene, or DMSO (1 : 1000 dilution; shown as 0 μ*M* drug). Twelve hours after addition, RA, but not the carotenoids, caused a dose-dependent growth suppression. Similar results were observed 24 hours after addition (data not shown). **C)** Normal, immortalized, and transformed human bronchial epithelial cells were treated for 12 hours with 2 μ*M* RA or 2 μ*M* β-carotene or their corresponding vehicles. The vehicle for RA was DMSO, and the vehicle for β-carotene was tetrahydrofuran (THF). RA, but not β-carotene (carotene) or the vehicles, caused considerable growth suppression in all three types of cells. In **panels A–C,** the error bars are the standard deviation for each mean value.

These models could be used for the preliminary testing of clinical cancer chemoprevention agents.

Earlier work indicates that a link exists between cyclin D1 proteolysis and RA-mediated prevention of carcinogen-induced transformation of immortalized human bronchial epithelial cells *(7,8).* To extend this work, we measured cyclin D1 protein by immunoblot analysis in normal, immortalized, and transformed human bronchial epithelial cells after treatment with RA or vehicle alone to determine whether the observed RA-induced growth suppression parallels a decline in the abundance of cyclin D1. In these human bronchial epithelial cells, the amount of cyclin D1 protein declined after treatment with 2 μ*M* RA, as shown in Fig. 2. In marked contrast, when these human bronchial epithelial cells were treated with 2 μ*M* β-carotene, the amount of cyclin D1 protein detected did not change appreciably. Similar findings were observed after treatment with α-carotene (data not shown). Thus, the suppression of the growth of human bronchial epithelial cells after treatment with retinoids but not with carotenoids is tightly linked to the expression of cyclin D1 protein.

Biologic effects of retinoids are mediated through the nuclear retinoid receptors RARs and RXRs. Expression of spe-

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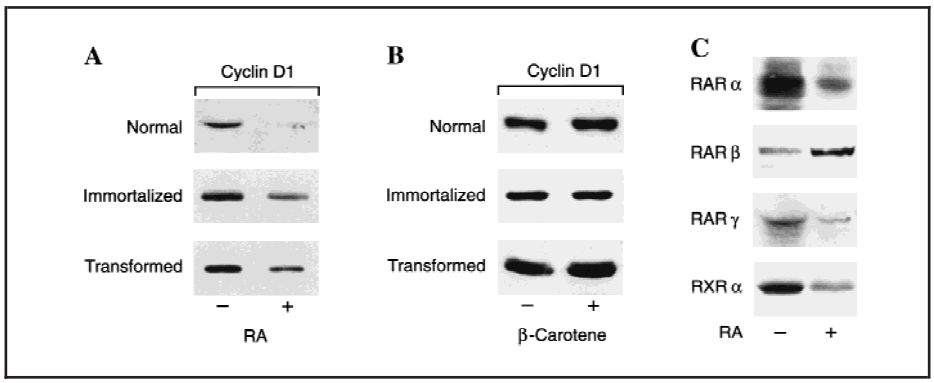

Fig. 2. A and **B)** Changes in expressed cyclin D1 protein in normal, immortalized, and transformed human bronchial epithelial cells after all-*trans*-retinoic acid (RA) or β-carotene treatments. **A)** Immunoblot analysis of cyclin D1 in the indicated cells treated for 12 hours with 2 μ*M* RA (+) or dimethyl sulfoxide vehicle (−) (1 : 1000 dilution) revealed a decrease in the amount of cyclin D1 in cells treated with RA. **B)** Immunoblot analysis of cyclin D1 in the indicated cells treated for 12 hours with 2 μ*M* β-carotene (+) or tetrahydrofuran vehicle (−) showed no change in the amount of cyclin D1. **C)** Induction of retinoic acid receptor β (RARβ) protein occurs in immortalized human bronchial epithelial cells after treatment with 2 μ*M* RA for 12 hours. At this time, the expression of RARα and retinoid X receptor α (RXRα) was repressed and the expression of RARβ was increased by treatment with RA. Expression of RARγ was repressed at 12 hours (data not shown) and at 24 hours (shown) by treatment with RA.

cific retinoid receptors is regulated by RA *(19)* at the level of messenger RNA in transformed human bronchial epithelial cells, and specific retinoid receptors are involved in regulation of basal cell growth. To determine which of these receptor proteins are involved in the retinoid-mediated decrease in the amount of cyclin D1 and resulting growth suppression in human bronchial epithelial cells, we assessed the effect of RA on the basal expression of retinoid receptor proteins by immunoblot analysis. Treatment with 2 μ*M* RA led to a decrease in the expression of RARα, RARγ, and RXRα protein, as shown in Fig. 2, C. In contrast, the expression of RARβ protein was induced after treatment with RA. Relative to basal expression, both RARα1 and RARα2 isoforms were repressed by RA treatment as shown in Fig. 2, C. Basal expression of RARγ protein was quite low in BEAS-2B cells and decreased further 12 and 24 hours after treatment with RA (Fig. 2, C; data not shown). Expression of RXRα protein was also repressed by treatment with RA (Fig. 2, C; data not shown). The induction of RARβ protein by RA was undetected at 3 hours, was maximal from 6 hours to 12 hours, and then decreased from 24 hours to 48 hours. The specificity of the anti-retinoid receptor antibodies was confirmed through blocking experiments in which receptor-specific peptides were incubated with their corresponding antibodies before immunoblot analysis. In resulting immunoblots, the specific retinoid receptor was not detected (data not shown), thus demonstrating the specificity of the various retinoid receptor antibodies.

When BEAS-2B cells were treated with ligands specific for each type of RARs and for RXRs, only ligands (agonists) that activated the RARβ or RXR pathway suppressed the growth of these cells (Fig. 3). When BEAS-2B cells were treated with the RARβ-specific agonist BMS-189453 (0–1.0 μ*M*) or with the RXR-selective agonist SR11246 (0–2.0 μ*M*), a dose-dependent suppression of growth was observed. When BEAS-2B cells were treated with another RXR agonist, SR11345 (0–2 μ*M;* data not shown), a dose-dependent decline in growth was also observed. When BEAS-2B cells were treated with the RARα-specific agonist Am80 (0–1.0 μ*M*) or with the RARγ/β-selective agonist SR11254 (0–0.1 μ*M*), cell growth was not suppressed. When BEAS-2B immortalized human bronchial epithelial cells were treated with the RARβ (BMS-189453) and the RXR (SR11246) agonists,

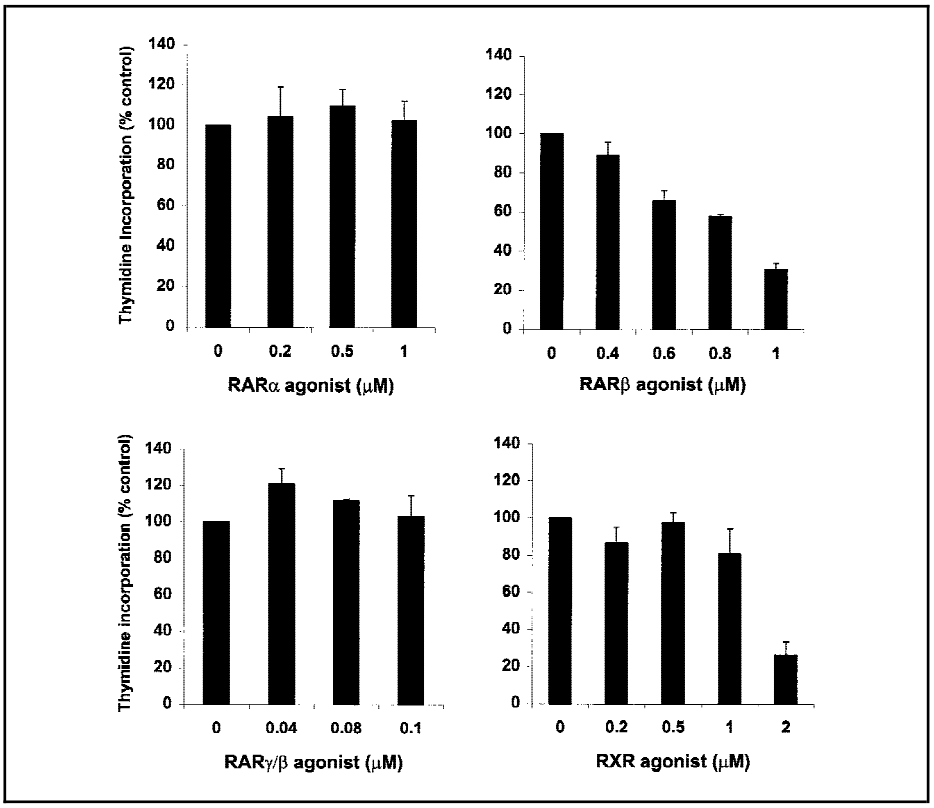

Fig. 3. Growth suppression of immortalized human bronchial epithelial BEAS-2B cells treated with retinoid receptor-selective agonists for retinoic acid receptor β (RARβ; BMS-189453) or retinoid X receptor (RXR; SR11246) but not with ligands selective for RARα (Am80) or RARγ/β (SR11254). Dose-dependent growth suppression by the indicated receptor-selective ligands, compared with dimethyl sulfoxide vehicle controls (indicated by 0 μ*M* drug), is shown for the RARβ-selective and RXR-selective ligands after 12 hours of treatment. Appreciable growth suppression was not observed at noncytotoxic doses for the RARα- or RARγ-selective ligands, compared with 12 hours of vehicle control treatments (indicated by 0 μ*M* drug). The standard deviation is indicated by error bars.

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additive effects on growth suppression were observed (data not shown).

After immortalized human bronchial epithelial (BEAS-2B) cells were treated with agonists selective for RARβ or for RXRs, the abundance of cyclin D1 protein was assessed by immunoblot analysis to determine whether the growth suppression induced by each of these ligands was linked to a decline in the level of cyclin D1. Each agonist tested (RA, the RXR-selective agonist SR11246, and the RARβ-selective agonist BMS-189453) at growth-suppressing doses caused a dose-dependent decline in the abundance of cyclin D1 protein (Fig. 4). This confirmed that a tight link exists between the growth suppression signaled by these selective retinoid receptor agonists and the level of cyclin D1 protein. To determine whether the observed growth suppression triggered by RA or a receptor-selective or receptor-nonselective agonist was due to the induction of apoptosis, we performed terminal deoxynucleotidyltransferase assays either after RA treatment or independently after treatment with the RXR agonist SR11246. Appreciable apoptosis was not detected at growth-suppressive concentrations of either retinoid examined (data not shown).

To explore whether the mechanism of the RARβ- or RXR-mediated decrease in cyclin D1 involved cyclin D1 proteolysis, which is known to be activated by RA treatment of immortalized human bronchial epithelial cells *(8)*, we treated BEAS-2B cells with 100 μ*M* calpain inhibitor I, the inhibitor of the 26S proteasome pathway, with or without the addition of 1 μ*M* RARβ agonist (BMS-189453) or 2 μ*M* RXR agonist (SR11246). A dose-dependent decline in cyclin D1 was observed after treatment with these agonists (Fig. 4) and after treatment with the RXR agonist SR11345 (data not shown). However, when cells were treated with calpain inhibitor I and with the RARβ- or RXR-selective agonist, calpain inhibitor I inhibited the decline in the expression of cyclin D1 protein induced by the receptor-selective agonists (Fig. 4). Thus, these retinoids mediate the decline of cyclin D1 through a proteolytic pathway similar to that reported for RA *(8)*.

Discussion

RA inhibits bronchial epithelial cell transformation *in vitro (7)*, and clinical trials report that retinoids reduce some second primary cancers, including aerodigestive tract cancers *(1,2)*. However, it is not clear why retinoids have cancer chemoprevention activity *(3)*, whereas the examined carotenoids do not *(4–6)*. RA signaling involves the RARs and RXRs. The RARs and RXRs are ligand-dependent transcription factors that have the potential to homodimerize and heterodimerize and are known to alter the expression of "downstream" species that directly mediate the biologic effects of retinoids. The regulation of cell cycle progression is also under active study and involves cyclins, cyclin-dependent kinases, and cyclin-dependent kinase inhibitors [reviewed in *(20–25)*]. This report confirms and extends prior work that demonstrates that these two growth-regulatory programs, the cell cycle machinery and retinoid signaling, are coupled through post-translational regulation of cyclin D1, the G_1-phase cyclin *(7,8)*. Notably, treatment with retinoid receptor-nonselective agonists, but not α-carotene and β-carotene, led to growth suppression and a decrease in the expression of cyclin D1 protein. The findings obtained with these carotenoids may also relate to their cellular permeability properties or to the ability of cells to generate active metabolites in culture. These *in vitro* findings, however, parallel observations of the clinical inactivity of these carotenoids in clinical cancer chemoprevention trials *(4–6)*.

Deregulated expression of cyclin D1 protein occurs in epithelial carcinogenesis [*(26,27);* Lonardo F, Langenfeld J, Rusch V, Dmitrovsky E, Klimstra DS: unpublished data]. Immunohistochemical findings indicate that cyclin D1 overexpression is frequent in neoplastic and carcinogen-exposed human lung epithelial cells [*(27);* Lonardo F, Langenfeld J, Rusch V, Dmitrovsky E, Klimstra DS: unpublished data]. Amplification of the cyclin D1 gene locus is often detected in squamous cell carcinomas of the lung or head and neck by comparative genomic hybridization or Southern blot analysis *(26,28,29)*. It has been hypothesized *(8)* that cyclin D1 overexpression in human bronchial epithelial cells triggers aberrant cellular proliferation. The retinoid-mediated decrease in cyclin D1 likely restores a more normal proliferation state to these neoplastic bronchial epithelial cells.

The RA-mediated prevention of carcinogen-induced transformation of immortalized human bronchial epithelial cells has been shown to be linked to a post-translational regulation of G_1 cyclins *(7,8)*. The current study extends this work by revealing that retinoids but not carotenoids act through a common cancer chemoprevention signal: cyclin D1 proteolysis by activation of a proteasome-dependent degradation pathway. Growth suppression signaled by retinoid receptor-selective agonists was also found to be associated with cyclin D1 proteolysis by a post-translational mechanism that is similar to the mechanism used by RA, as shown in Fig. 4. This pathway is activated by retinoid treatment of normal, immortalized, and transformed human bronchial epithelial cells. Similar findings were obtained by treatment of immortalized human bronchial epithelial cells with another proteasome inhibitor, lactacystin *(8)*. Thus, these findings indicate that a decline in the expression of cyclin D1 protein with concomitant growth suppression can be viewed as a retinoid chemo-

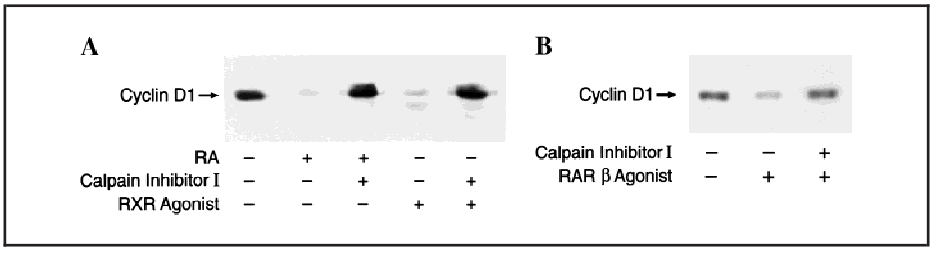

Fig. 4. Treatment with calpain inhibitor I, which inhibits the 26S proteasome-dependent degradation pathway, prevented the decline in cyclin D1 protein after treatment with all-*trans*-retinoic acid (RA) or the retinoid X receptor (RXR)- or retinoic acid receptor β (RARβ)-selective agonists (SR11246 and BMS-189453, respectively). **A)** Treatment of immortalized human bronchial epithelial (BEAS-2B) cells for 6 hours with 100 μ*M* calpain inhibitor I prevented the decrease in cyclin D1 protein that follows treatment with either 2 μ*M* RA or 2 μ*M* RXR-selective agonist for 6 hours. The immunoblot detection of cyclin D1 is shown. **B)** An independent cyclin D1 immunoblot analysis was performed after treatment with 1 μ*M* RARβ agonist. This immunoblot shows that degradation of cyclin D1 mediated by treatment with an RARβ agonist was also prevented by calpain inhibitor I.

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prevention signal active in human bronchial epithelial cells. Whether other candidate chemoprevention agents active in the prevention of lung cancer use a mechanism involving the post-translational degradation of cyclin D1 is the subject of future work.

The induction of RARβ protein by RA and the observed effects of an RARβ agonist in human bronchial epithelial cells are consistent with the hypothesis that RARβ plays an important role in signaling a retinoid chemoprevention response in this cell context. These findings support and extend the work of others *(30)*. Repression of RARβ expression is frequently observed during epithelial cell transformation *(30,31)*, and induction of RARβ by treatment with 13-*cis*-RA is associated with a beneficial clinical response in oral leukoplakia, a premalignant lesion *(30)*. Our results are consistent with the hypothesis that RARβ plays a role in retinoid-induced growth suppression of human bronchial epithelial cells. These findings suggest a role for RARβ agonists in the treatment of these neoplastic cells.

RXRs are known to heterodimerize with RARs and other members of the steroid receptor superfamily *(32)*. The growth-suppressive effect of an RXR agonist (Fig. 3) is consistent with the hypothesis that an RXR-dependent signal mediates the growth suppression observed in these examined human bronchial epithelial cells. Notably, findings depicted in Fig. 4 indicate that cyclin D1 proteolysis by a proteasome-dependent degradation pathway follows treatment with an RARβ agonist or an RXR agonist. The signaling of this degradation pathway by retinoid receptor-selective ligands is consistent with the view that cyclin D1 proteolysis plays an important role in the chemoprevention of human bronchial epithelial cells. Perhaps RXR agonists will exhibit lung cancer prevention activity when tested in appropriate clinical trials.

In this study, the growth-suppressive effects of RARβ-selective and RXR-selective agonists on immortalized human bronchial epithelial cells are consistent with results obtained when RARβ or RXRα is individually overexpressed in transformed human bronchial epithelial cells *(19)*. Growth-suppressive effects were not observed after treatment of BEAS-2B cells with the RARγ/β-selective agonist (Fig. 3) or after transfection of RARγ into transformed human bronchial epithelial cells *(19)*. These findings argue against RARγ activation playing a major role in the retinoid-induced growth suppression of these human bronchial epithelial cells.

Studies of retinoid-dependent lung cancer prevention mechanisms in *in vitro* models are useful to identify intermediate markers of effective retinoid chemoprevention, to highlight potential pharmacologic targets, and to select agents appropriate for testing in clinical trials. Changes in intermediate markers represent candidate surrogate end points for clinical cancer chemoprevention trials, and these may prove useful to predict beneficial clinical responses. Validated surrogate end points will enhance conduct of short-term clinical cancer chemoprevention trials by revealing early chemoprevention responses before the long-term clinical end point of reduced cancer incidence is available. This will expedite testing of candidate clinical cancer chemoprevention agents. The finding that cyclin D1 proteolysis is a common retinoid-dependent signal in normal, immortalized, and transformed human bronchial epithelial cells suggests that cyclin D1 proteolysis is a candidate intermediate end point for future retinoid-based clinical cancer chemoprevention trials.

REFERENCES

(1) Hong WK, Lippman SM, Itri LM, Karp DD, Lee JS, Byers RM, et al. Prevention of second primary tumors with isotretinoin in squamous-cell carcinoma of the head and neck. N Engl J Med 1990;323:795–801.

(2) Pastorino U, Infante M, Maioli M, Chiesa G, Buyse M, Firket P, et al. Adjuvant treatment of stage I lung cancer with high-dose vitamin A. J Clin Oncol 1993;11:1216–22.

(3) Muto Y, Moriwaki H, Ninomiya M, Adachi S, Saito A, Takasaki KT, et al. Prevention of second primary tumors by an acyclic retinoid, polyprenoic acid, in patients with hepatocellular carcinoma. Hepatoma Prevention Study Group. N Engl J Med 1996;334:1561–7.

(4) Hennekens CH, Buring JE, Manson JE, Stampfer M, Rosner B, Cook NR, et al. Lack of effect of long-term supplementation with beta carotene on the incidence of malignant neoplasms and cardiovascular disease. N Engl J Med 1996; 334:1145–9.

(5) Omenn GS, Goodman GE, Thornquist MD, Balmes J, Cullen MR, Glass A, et al. Effects of a combination of beta carotene and vitamin A on lung cancer and cardiovascular disease. N Engl J Med 1996;334:1150–5.

(6) The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group. The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. N Engl J Med 1994;330:1029–35.

(7) Langenfeld J, Lonardo F, Kiyokawa H, Passalaris T, Ahn MJ, Rusch V, et al. Inhibited transformation of immortalized human bronchial epithelial cells by retinoic acid is linked to cyclin E down-regulation [published erratum appears in Oncogene 1996;13:2743]. Oncogene 1996;13:1983–90.

(8) Langenfeld J, Kiyokawa H, Sekula D, Boyle J, Dmitrovsky E. Posttranslational regulation of cyclin D1 by retinoic acid: a chemoprevention mechanism. Proc Natl Acad Sci U S A 1997; 94:12070–4.

(9) Reddel RR, Ke Y, Gerwin BI, McMenamin MG, Lechner JF, Su RT, et al. Transformation of human bronchial epithelial cells by infection with SV40 or adenovirus-12 SV40 hybrid virus, or transfection via strontium phosphate coprecipitation with a plasmid containing SV40 early region genes. Cancer Res 1988;48: 1904–9.

(10) Lechner JF, Haugen A, McClendon IA, Pettis EW. Clonal growth of normal adult human bronchial epithelial cells in a serum-free medium. In Vitro 1982;18:633–42.

(11) Kagechika H, Kawachi E, Hashimoto Y, Himi T, Shudo K. Retinobenzoic acids. 1. Structure–activity relationships of aromatic amides with retinoidal activity [published erratum appears in J Med Chem 1989;32:2583]. J Med Chem 1988;31:2182–92.

(12) Yu KL, Spinazze P, Ostrowski J, Currier SJ, Pack EJ, Hammer L, et al. Retinoic acid receptor β,γ-selective ligands: synthesis and biological activity of 6-substituted 2-naphthoic acid retinoids. J Med Chem 1996;39:2411–21.

(13) Dawson MI, Jong L, Hobbs PD, Cameron JF, Chao WR, Pfahl M, et al. Conformational effects on retinoid receptor selectivity. 2. Effects of retinoid bridging group on retinoid X receptor activity and selectivity. J Med Chem 1995; 38:3368–83.

(14) Chen JY, Clifford J, Zusi C, Starrett J, Tortolani D, Ostrowski J, et al. Two distinct actions of retinoid-receptor ligands. Nature 1996; 382:819–22.

(15) Cooney RV, Kappock TJ 4th, Pung A, Bertram JS. Solubilization, cellular uptake, and activity of β-carotene and other carotenoids as inhibitors of neoplastic transformation in cultured cells. Methods Enzymol 1993;214:55–68.

(16) Levy J, Bosin E, Feldman B, Giat Y, Miinster A, Danilenko M, et al. Lycopene is a more potent inhibitor of human cancer cell proliferation than either α-carotene or β-carotene. Nutr Cancer 1995;24:257–66.

(17) Micozzi MS, Brown ED, Edwards BK, Bieri JG, Taylor PR, Khachik F, et al. Plasma carotenoid response to chronic intake of selected foods and β-carotene supplements in men. Am J Clin Nutr 1992;55:1120–5.

(18) Chen GQ, Shen ZX, Wu F, Han JY, Miao JM, Zhong HJ, et al. Pharmacokinetics and efficacy of low-dose all-*trans* retinoic acid in the treatment of acute promyelocytic leukemia. Leukemia 1996;10:825–8.

(19) Ahn MJ, Langenfeld J, Moasser MM, Rusch V, Dmitrovsky E. Growth suppression of transformed human bronchial epithelial cells by all-*trans*-retinoic acid occurs through specific retinoid receptors. Oncogene 1995;11:2357–64.

(20) Roberts JM, Koff A, Polyak K, Firpo E, Col-

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lins S, Ohtsubo M, et al. Cyclins, Cdks, and cyclin kinase inhibitors. Cold Spring Harb Symp Quant Biol 1994;59:31–8.
(21) Sherr CJ. G_1 phase progression: cycling on cue. Cell 1994;79:551–5.
(22) Nurse P. Ordering S phase and M phase in the cell cycle. Cell 1994;79:547–50.
(23) Sherr CJ, Roberts JM. Inhibitors of mammalian G_1 cyclin-dependent kinases. Genes Dev 1995;9:1149–63.
(24) Nasmyth K. Viewpoint: putting the cell cycle in order. Science 1996;274:1643–5.
(25) Resnitzky D, Hengst L, Reed SI. Cyclin A-associated kinase activity is rate limiting for entrance into S phase and is negatively regulated in G_1 by $p27^{Kip1}$. Mol Cell Biol 1995;15:4347–52.
(26) Betticher DC, Heighway J, Hasleton PS, Altermatt HJ, Ryder WD, Cerny T, et al. Prognostic significance of CCND1 (cyclin D1) overexpression in primary resected non-small-cell lung cancer. Br J Cancer 1996;73:294–300.
(27) Betticher DC, Heighway J, Thatcher N, Hasleton PS. Abnormal expression of CCND1 and RB1 in resection margin epithelia of lung cancer patients. Br J Cancer 1997;75:1761–8.
(28) Petersen I, Bujard M, Petersen S, Wolf G, Goeze A, Schwendel A, et al. Patterns of chromosomal imbalances in adenocarcinoma and squamous cell carcinoma of the lung. Cancer Res 1997;57:2331–5.
(29) Bockmuhl U, Schwendel A, Dietel M, Petersen I. Distinct patterns of chromosomal alterations in high- and low-grade head and neck squamous cell carcinomas. Cancer Res 1996;56:5325–9.
(30) Lotan R, Xu XC, Lippman SM, Ro JY, Lee JS, Lee JJ, Hong WK. Suppression of retinoic acid receptor-β in premalignant oral lesions and its up-regulation by isotretinoin. N Engl J Med 1995;332:1405–10.
(31) Xu XC, Sneige N, Liu X, Nandagiri R, Lee JJ, Lukmanji F, et al. Progressive decrease in nuclear retinoic acid receptor β messenger RNA level during breast carcinogenesis. Cancer Res 1997;57:4992–6.
(32) Mangelsdorf DJ, Evans RM. The RXR heterodimers and orphan receptors. Cell 1995;83:841–50.

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Supported in part by Public Health Service grants R01CA54494-06 (E. Dmitrovsky), T32CA09512 and K12CA01712 (J. Langenfeld), and T32CA09685 (J. O. Boyle), National Cancer Institute, National Institutes of Health, Department of Health and Human Services; by American Cancer Society grant RPG-90-019-08-DDC (E. Dmitrovsky); by the Byrne Fund (E. Dmitrovsky); by the Oracle Chemoprevention Research Fund (E. Dmitrovsky); and by the American Society of Clinical Oncology Young Investigator Award (J. O. Boyle).

We thank Dr. Curtis Harris, Laboratory of Human Carcinogenesis, National Cancer Institute, for the gift of the BEAS-2B cell line.

Manuscript received June 12, 1998; revised December 2, 1998; accepted December 21, 1998.

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