All-trans Retinoic Acid Inhibits Vascular Smooth Muscle Cell Proliferation Targeting Multiple Genes for Cyclins and Cyclin-Dependent Kinases

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Retinoids have been shown to promote vascular smooth muscle cell differentiation, although the underlying mechanism is unclear. In fact, treatment of rat aortic smooth muscle cells with all-trans retinoic acid (ATRA) has been shown to markedly elevate the mRNA and protein levels of smooth muscle α-actin. Considering that an exit from the cell cycle is a prerequisite for cell differentiation, we examined the effect of ATRA on cellular events during the progression from G0 to S phase. Pretreatment with ATRA dose-dependently inhibited DNA synthesis induced by basic fibroblast growth factor. However, ATRA did not inhibit transient activation of mitogen-activated protein kinase (MAPK) in response to mitogenic stimulation. And ATRA consistently failed to influence the phosphorylation of MAPK kinase (MEK) and the expression of MAPK-specific dual phosphatase (MKP-1). ATRA did not interfere with other early mitogenic signals either, such as the phosphorylation of FGF-1 receptor or the induction of immediate early genes c-fos, c-jun, and c-myc. In contrast, ATRA strongly suppressed the pRb kinase activities of the cyclin-dependent kinases (Cdns) Cdk4, Cdk6, and Cdk2. ATRA did not influence the expressions of cip/Kip family Cdk inhibitors or those of cyclins D1 and D2, whereas it strongly inhibited the expressions of cyclins D3 and E, Cdk4, Cdk6, and Cdk2. These results suggest that ATRA targets multiple genes essential for entry into the cell cycle and for the subsequent progression to G1 phase, but without interrupting early mitogenic signals upstream of MAPK.

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Key Words: all-trans retinoic acid, vascular smooth muscle cell, cell cycle, cyclin, cyclin-dependent kinase

Introduction

Vascular smooth muscle cells (VSMCs) in the arterial media are fully differentiated to play their physiological roles as regulators of vascular wall tension. In culture and also in atherosclerotic and restenotic lesions, however, their phenotypes have been converted to dedifferentiated immature ones which generally lose the expressions of VSMC-specific contractile proteins such as smooth muscle α-actin (SM α-actin), myosin heavy chain (MHC) isoforms, desmin, calponin, and SM22α (1). Dedifferentiated VSMCs migrate into the intima, where they proliferate and synthesize extracellular matrices, thereby contributing to the formation of neointima (2). Therefore, to prevent VSMC hyperplasia in vivo, substances that promote VSMC differentiation may be

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more effective than those that only inhibit VSMC proliferation. A number of substances have been shown to inhibit VSMC proliferation, but only a few of these have also been shown to promote differentiation.

Retinoids, derivatives of vitamin A, inhibit proliferation and induce differentiation in several cell systems (3), and are actually used in the treatment of several disorders, such as malignant neoplasms (4). In VSMCs, all-trans retinoic acid (ATRA), a naturally occurring retinoid, inhibits proliferation induced by serum, platelet-derived growth factor (PDGF), and endothelin-1 (5-7), and also inhibits cell migration (5, 8). ATRA also has been shown to promote VSMC differentiation, i.e., this compound increases the protein level of SM α-actin in conjunction with a marked elevation in protein kinase C-α activity in rat VSMCs (9). ATRA also induces other markers for VSMC differentiation, such as the L-type calcium channel in a rat VSMC line (10) and MHC isoforms, desmin, and smoothelin in porcine VSMCs (11). Therefore, ATRA shows promise as a possible therapeutic agent against atherosclerotic vascular diseases. Indeed, in vivo administration of ATRA has been shown to prevent intimal thickening after arterial injury (12, 13).

Induction of differentiation is generally accompanied by a cessation of proliferation. Recently, such cell cycle regulators as cyclins, cyclin-dependent kinases (Cdks), and Cdk inhibitor proteins have been suggested to be involved in signaling pathways as regulators of cell differentiation (14). Since the expression levels of contractile proteins increase in cultured VSMCs whose proliferation has been inhibited by cell-cell contact or serum deprivation (15, 16), it is considered that an exit from the cell cycle is required for the induction of VSMC differentiation. Therefore, we studied the effect of ATRA on cellular events that occur between the G0 and S phases. We found that ATRA inhibits VSMC proliferation by preventing the expression of multiple genes for cyclins and Cdks essential for entry into the cell cycle and for the subsequent progression to G1 phase, but without interrupting mitogen-activated protein kinase (MAPK)-dependent pathways. The mechanism for ATRA-mediated cell cycle arrest is therefore unique and may be related to the differentiation-promoting action of ATRA.

**Materials and Methods**

**Chemicals**

ATRA (Sigma Chemical Co., St. Louis, USA) was dissolved in MeSO4, stored at -70°C, and diluted with a culture medium immediately before use. Experiments were performed under reduced lighting conditions to minimize photoisomerization of ATRA. Other common chemicals were of reagent grade.

**Cell Culture**

VSMCs were obtained from the aortas of 8-week-old male Sprague-Dawley rats via an explant. Briefly, aortas dissected from anesthetized rats were washed in phosphate-buffered saline (PBS) and opened longitudinally. The media was iso-cultured, maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, USA), 100 units/ml penicillin, and 100 μg/ml streptomycin. The medium was replaced every other day. Cells were harvested before reaching confluence with an EDTA (0.05%)/trypsin (0.02%) solution and passed at a ratio of 1:5. Cells in the third to fifth passages were used for experiments.

**DNA Synthesis Assay**

DNA synthesis was measured by the level of thymidine (Tdr) incorporation. VSMCs seeded on 24-multiwell plates (1,000 cells/well) were grown to 70-80% confluence. After Go-synchronization by serum starvation, cells were pretreated with ATRA or its vehicle (0.01% MeSO4) for 48 h, and thereafter they were stimulated with 10 ng/ml of human recombinant basic fibroblast growth factor (bFGF; PeproTech, Rocky Hill, USA) and 0.5% FBS. Cells were labeled with 37 kBq/ml [6-3H]Tdr (0.74-1.1 TBq/mmol; Amersham Pharmacia Biotech, Buckinghamshire, UK) for 12 h. They were then washed three times with PBS and extracted with 5% trichloroacetic acid. After removing the acid, cells were solubilized with 0.5 mM NaOH and neutralized with 1 mM HCl. Radioactivity was determined using a liquid scintillation counter (LS5801; Beckman Instruments, Berkeley, USA).

**Immunoprecipitation, Immunoblotting, and Cdk Kinase Assay**

These experiments were performed as described previously (17). When whole cell lysates were used for immunoblotting, cells were lysed by mixing them with a 2×Laemmli's loading buffer (1% SDS, 5% 2-mercaptoethanol, 62.5 mM Tris/HCl [pH 6.8], 10% glycerol, and 0.02% bromophenol blue). The lysates were sonicated for 15 s, boiled for 3 min, and centrifuged at 16,000×g for 20 min to remove insoluble pellets. The supernatants were then applied to SDS-PAGE.

**Northern Blotting**

Total cellular RNAs were extracted with Nicon (Wako Pure Chemicals, Osaka, Japan). Northern blotting was performed as described previously (17). Briefly, equal amounts of RNAs (10μg/ lane) were electrophoresed and transferred to nylon filters. After fixation by ultraviolet irradiation, the filters were hybridized with [32P]-labeled cDNAs in 50% for-
Fig. 1. Effect of ATRA on the expression of SM α-actin. (a) Go-synchronized rat VSMCs were incubated in DMEM containing 0.1% BSA in the presence of a vehicle (−) or ATRA (1 μM) (+). The medium was changed every other day. Cells were lysed on the indicated day after the addition of ATRA. The whole cell lysates were fractionated by SDS-PAGE (7.5%) and analyzed by immunoblotting with an anti-SM α-actin monoclonal antibody (Boehringer Mannheim) and an anti-α-tubulin monoclonal antibody (Oncogene Research Products, Cambridge, USA). (b) Go-synchronized rat VSMCs were incubated in DMEM containing 0.1% BSA in the presence of various concentrations of ATRA for 48 h and then lysed to prepare the whole cell lysates. Proteins were analyzed by immunoblotting for SM α-actin and a-tubulin as described above. (c) Cells pretreated with a vehicle (−) or ATRA (1 μM) (+) as described above were lysed on the indicated day after the addition of ATRA to extract total cellular RNAs. Equal amounts of RNAs (10 μg/lane) were analyzed by Northern blotting for SM α-actin and GAPDH. (d) Cells pretreated with various concentrations of ATRA for 48 h as described above were lysed to extract total cellular RNAs. Equal amounts of RNAs (10 μg/lane) were analyzed by Northern blotting for SM α-actin and GAPDH.

Statistical Analysis

Results were expressed as the means ± SD. Statistical significance was assessed by the Student’s t-test for unpaired values. A p value of less than 0.05 was considered to indicate statistical significance. Experiments were repeated at least three times.

Results

Pretreatment with ATRA Induced SM α Actin

We first examined whether ATRA would stimulate the expression of SM α-actin under our experimental conditions as demonstrated in a previous report (9). We synchronized cells in the G0 phase by serum starvation and thereafter incubated them with ATRA in a serum-free medium. ATRA pretreat-
Fig. 2. Effect of ATRA on DNA synthesis. Rat VSMCs grown to 70–80% confluence in a 24-multiwell plate were synchronized in Go by serum starvation, and treated with various concentrations of ATRA in DMEM containing 0.1% BSA for 48 h. Cells were then cultured in DMEM containing 0.1% BSA (no stimulation: NS) or stimulated with DMEM containing 0.5% FBS and 10 ng/ml of bFGF in the presence of the same concentrations of ATRA given as pretreatment. DNA synthesis was assessed by the level of [3H]thymidine (TdR) incorporation. Each column represents the mean ± SD (n=5). **p<0.01 vs. growth-stimulated vehicle-treated cells.

Pretreatment with ATRA inhibited DNA Synthesis

We next examined the effect of ATRA on cell proliferation. DNA synthesis started about 12 h after stimulation of Go-synchronized cells with bFGF (10 ng/ml) and FBS (0.5%)(data not shown). When cells were pretreated with ATRA for 48 h in a serum-free medium prior to mitogenic stimulation, DNA synthesis was strongly suppressed. The inhibition depended on the dose of ATRA given as pretreatment and the maximal effect was again obtained at 1 µmol/l, where the inhibition was over 80% (Fig. 2). Similar results were observed when cells were stimulated with bFGF or serum alone, or with bFGF in the presence of insulin, transferrin, or selenium, indicating that the antiproliferative effect of ATRA was universal and not dependent on the mitogen species (data not shown).

ATRA at concentrations up to 1 µmol/l did not induce cytotoxicity, since it did not change cell shape, impair trypan blue exclusion, or increase the level of lactate dehydrogenase

Fig. 3. Effect of ATRA on early mitogenic signals. Rat VSMCs pretreated with a vehicle (0.01% MeSO4) or ATRA (1 µM) were stimulated with 0.5% FBS and 10 ng/ml bFGF as described in the legend for Fig. 2, and harvested for immunoblotting or Northern blotting. (a) Cell lysates prepared at the indicated times were immunoprecipitated with a polyclonal antibody to FGF-1 receptor (Santa Cruz Biotechnology, Santa Cruz, USA). Proteins fractionated by SDS-PAGE (7.5%) were blotted with an antibody to FGF-1 receptor or an anti-phosphotyrosine antibody (PY-20; Transduction Laboratories, Lexington, USA) to detect total or phosphorylated FGF-1 receptor, respectively. Arrows indicate specific bands. (b) Whole cell lysates prepared at the indicated times were fractionated by SDS-PAGE (10%) and blotted with polyclonal antibodies to total MAPK, phosphorylated MAPK, phosphorylated MEK (New England Biolabs, Beverly, USA), and MKP-1 (Santa Cruz Biotechnology). (c) Total cellular RNAs were extracted at the indicated times. Equal amounts of RNAs (10 µg/lane) were analyzed by Northern blotting for c-fos, c-jun, c-myc, and GAPDH.
**Fig. 4.** Effect of ATRA on Cdk activities. (a) Rat VSMCs pretreated with a vehicle (−) or ATRA (1 μM) (+) were stimulated with 0.5% FBS and 10 ng/ml bFGF as described in the legend for Fig. 2. Cells were lysed 12 h after stimulation and immunoprecipitated with anti-Cdk2, Cdk4, or Cdk6 polyclonal antibodies (Santa Cruz Biotechnology). The kinase assay was performed using glutathione S-transferase-fused pRb fragment (GST-Rb) as the substrate. The assay mixtures were fractionated by SDS-PAGE (10%) and visualized with autoradiography. Bars indicate specific bands. (b) Radioactivities of the bands were quantified using a bioimage analyzer (Fuji Photo Film Co.) and the kinase activities are shown as the fold-increase against the value in unstimulated vehicle-treated cells. Data represent the means±SD (n=3). *p<0.05, **p<0.01 vs. the activity in growth-stimulated vehicle-treated cells.

**Fig. 5.** Effect of ATRA on expressions of the Cdk inhibitors. Rat VSMCs pretreated with a vehicle (−) or ATRA (1 μM) (+) were stimulated with 0.5% FBS and 10 ng/ml bFGF as described in the legend for Fig. 2, and lysed at the indicated times. To detect the expression of p21, cell lysates prepared at the indicated times were immunoprecipitated with a goat polyclonal antibody to p21 (Santa Cruz Biotechnology). Proteins were fractionated by SDS-PAGE (12.5%) and blotted with a rabbit polyclonal antibody to p21 (Santa Cruz Biotechnology). To detect p27 and p57, whole cell lysates prepared at the indicated times were fractionated by SDS-PAGE (12.5% for p27 and 10% for p57) and blotted with polyclonal antibodies to p27 and p57 (Santa Cruz Biotechnology). Immunoblots for α-tubulin are also shown.

in the culture medium (data not shown). The fact that ATRA increased the expression level of SM α-actin also indicated that it was not cytotoxic (Fig. 1). Moreover, ATRA did not induce DNA fragmentation or increase hypodiploid cells in flow cytometry, indicating that it did not inhibit cell proliferation by inducing apoptosis (data not shown).

**Effect of ATRA on Early Mitogenic Signals**

To determine the mechanism underlying the ATRA-mediated antiproliferative effect, we studied the effect of ATRA on early mitogenic signals. Downregulation of growth factor receptors has been proposed as a mechanism for retinoid-induced growth inhibition in human trophoblastic and myeloma cells (18, 19). In VSMCs in the present study, however, ATRA did not influence the expression level of FGF-1 receptor, a membrane surface receptor for bFGF; moreover, it did not inhibit tyrosine phosphorylation on the receptor (Fig. 3a). We then examined the effect of ATRA on the MAPK-dependent signaling pathway, since this pathway is thought to play an essential role in the transition from the G0 to G1 phase (20). MAPK is activated by the MAPK kinase (MEK)-induced phosphorylation and inactivated by the MAPK-spe-
Fig. 6. The effect of ATRA on the protein expressions of cyclins and Cdns. (a) Rat VSMCs pretreated with a vehicle (−) or ATRA (1 μM) (+) were stimulated with 0.5% FBS and 10 ng/ml bFGF as described in the legend for Fig. 2, and lysed at the indicated times. Proteins were fractionated by SDS-PAGE (10%) and probed with polyclonal antibodies to the indicated cyclins and Cdns (Santa Cruz Biotechnology) except for a monoclonal antibody to cyclin D3 (Santa Cruz Biotechnology). (b) Cells pretreated with various concentrations of ATRA for 48 h were stimulated with 0.5% FBS and 10 ng/ml of bFGF and lysed 18 h after stimulation. (c) The intensities of the specific bands were quantified using a bioimage analyzer and are shown as the fold-increase against vehicle-treated cells. Data represent the means±SD (n = 3). *p<0.05, **p<0.01 vs. the expression level in vehicle-treated cells.

ATRA Inhibited Cdk Activities without Inducing Cdk Inhibitors

Gi-phase progression is mediated by Cdns that phosphorylate pRb (2J). Cdk4 and Cdk6 are activated by coupling with D-type cyclins in the early Gi phase, and subsequently Cdk2 is activated by cyclin E in the late Gi phase. In VSMCs, the activities of Cdk4 and Cdk6 began to increase immediately after mitogenic stimulation and reached maximal levels after 12 h (data not shown). Cdk2 activity started to increase 8 h after stimulation and continued to increase up to 24 h (data not shown). Increases in the activities of these Cdns were
Fig. 7. Effect of ATRA on the mRNA expressions of cyclins and Cdns. (a) Rat VSMCs pretreated with a vehicle (−) or ATRA (1 μM) (+) were stimulated with 0.5% FBS and 10 ng/ml bFGF as described in the legend for Fig. 2, and lysed at the indicated times to extract total cellular RNAs. Equal amounts of RNAs (10 μg/lane) were analyzed by Northern blotting for cyclins, Cdns, and GAPDH. (b) Cells pretreated with various concentrations of ATRA for 48 h were stimulated with 0.5% FBS and 10 ng/ml bFGF. Total cellular RNAs were extracted 18 h after stimulation, and equal amounts of RNAs (10 μg/lane) were analyzed by Northern blotting for cyclins, Cdns, and GAPDH.

Table 1. Summary of the Effect of ATRA on G1 Cyclins and Cdns

<table>
<thead>
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→: no change
↓: decrease
–: not done

5–7 fold when measured 12 h after mitogenic stimulation; however, pretreatment with ATRA significantly suppressed these activities (Fig. 4). To determine how ATRA inhibited Cdk activities, we examined the effect of ATRA on the expression of Cip/Kip family Cdk inhibitor proteins, since retinoids have been shown to enhance their expressions (22–24). Immunoblotting showed that the expression levels of p21 and p27 were elevated after mitogenic stimulation, but were not enhanced by pretreatment with ATRA (Fig. 5). ATRA actually decreased the expression levels of p21 after 18–24 h. p57 was expressed throughout the G0/G1 phase without significant changes in its expression level, and it was not altered by pretreatment with ATRA (Fig. 5).

Effect of ATRA on the Expression of Cyclins and Cdns

Finally, we examined whether ATRA influenced the expression of cyclins or Cdns. The expression levels of cyclins D1, D2, D3, and E were all low in quiescent cells, and increased from the early G1 phase in response to mitogenic stimulation (Fig. 6a). Cdk4, Cdk6, and Cdk2 were expressed in G0 cells, and their levels significantly increased 3–6 h after mitogenic stimulation, with the increases being sustained up to 24 h (Fig. 6a). However, pretreatment with ATRA inhibited the expression levels of cyclins D3 and E and all of the Cdns tested (Fig. 6a). These inhibitory effects were significant and dose-dependent (Figs. 6b and 6c). However, ATRA did not reduce the protein levels of cyclins D1 or D2.

The expression of mRNAs for cyclins and Cdns were also induced after mitogenic stimulation (Fig. 7a). Consistent with the effect of ATRA on protein expressions, ATRA suppressed the induction of cyclins D3 and E, Cdk4, Cdk6, and Cdk2. The dose-dependencies were also consistent with those of protein expressions (Fig. 7b).
Discussion

In the present study, we showed that ATRA inhibits the expressions of multiple genes for cell cycle regulator proteins (Table 1). However, ATRA did not influence the early events in mitogenesis, such as the phosphorylation of FGF-1 receptor, activation of the MAPK pathway, or expression of immediate early genes. Our results are consistent with those in a previous report by Miano et al. (6) in that ATRA inhibited PDGF-induced rat VSMC proliferation without inhibiting the expression of immediate early genes such as c-fos, c-jun, and erg-1, which are considered to be mediated by the MAPK-dependent pathway. However, contradictory results were reported by Chen and Gardner, who found that the inhibition in endothelin-1-induced proliferation in rat VSMCs was correlated with decreased kinase activity of ERK2 (p42\textsuperscript{mapk}), but not c-Jun N-terminal kinase (7). Although the reason for this discrepancy is unclear, it may be due to the different conditions for ATRA treatment or growth stimulation. However, considering that the expression of cyclin D1 is positively regulated by the MAPK-dependent pathway (25, 26), the inability of ATRA to inhibit MAPK is consistent with its inability to inhibit cyclin D1 expression. In addition, the absence of its effect on c-myc was consistent with previous reports in other cell systems (27, 28), although there has also been a single conflicting report (29).

The activities of Cdk4, Cdk6, and Cdk2 were all reduced by ATRA. Since G1-phase progression is mediated by Cdkks that phosphorylate pRb (21), it is reasonable to assume that the mechanisms responsible for the ATRA-induced cell cycle arrest involve reduced Cdk activities. However, ATRA did not induce the expression of the Cdk inhibitors p21, p27, or p57, in spite of the fact that the induction of Cip/Kip proteins has been proposed to be the mechanism for retinoid-mediated growth inhibition (22–24). Interestingly, the expression of p21 was inhibited after the release from ATRA pretreatment. This appeared to be paradoxical in light of the role of p21 as a Cdk inhibitor. Recently, however, the Cip/Kip proteins have been thought to play a role as assembly factors that promote binding of Cdk4/6 to D-type cyclins and thereby contribute to G1-phase progression (30). Therefore, reduced expression of p21 could contribute to Cdk inhibition. The inhibition of p21 expression by retinoids has also been reported in some other cell species (31–34).

ATRA suppressed the gene expressions of cyclins D3 and E, Cdk4, Cdk6, and Cdk2. Inhibition of cyclin and Cdk expression by ATRA has also been shown in other cell systems whose growth is inhibited by ATRA (24, 28, 31–38), although a reduction in Cdk6 level has not previously been reported. Seewaldt et al. (28) showed that ATRA reduced the protein levels of cyclin D1, cyclin E, and Cdk4 without any significant changes in the levels of c-myc, p53, p21, or p27 in normal human mammary epithelial cells. Zhou et al. (33) reported that in human breast cancer cells, ATRA reduced the protein levels of cyclins D1 and D3, Cdk2, and Cdk4, but not those of cyclin E and Cdk6, whose expressions were strongly suppressed by ATRA in the present study. Taken together, these results indicate that the target molecules for ATRA may vary among cell species.

The effect of ATRA on cyclin E may be secondary to the G1 arrest induced by the compound, because cyclin E expression is amplified in the late G1 phase by a positive feedback loop that consists of Cdk2, E2F, pRb, and cyclin E itself (21). However, D-type cyclins can be primary targets for ATRA. Interestingly, the effect of ATRA have been shown to differ among subspecies of the D-type cyclins. Some investigators have shown that ATRA reduced cyclin D1 expression (28, 33, 34, 37), whereas this effect of ATRA was not found in the present or in previous studies (27, 32, 38). Cyclin D3 is abundant in mammary tissues and is required for the G1/S transition in a fibroblast cell line (39). Retinoid-mediated inhibition of cyclin D3 expression has also been reported in other cell lines (24, 32, 33). A recent study suggested that cyclin D3 is involved in the antiproliferative effect of ATRA (40). Overexpression of cyclin D3, which has been identified as a gene whose expression was reduced after ATRA-induced differentiation of F9 teratocarcinoma cells, overcomes the growth arrest induced by ATRA. Therefore, the reduction in the expression level of cyclin D3 may be directly involved in the antiproliferative effect by ATRA.

Although post-translational modifications such as ubiquitination have been suggested to be the mechanisms for ATRA-induced reduction in the protein levels of cyclin D1 (37), Cdk2, and Cdk4 (34), our results suggest that ATRA inhibits cyclin and Cdk expression at mRNA levels. ATRA-induced mRNA reduction has also been reported for cyclin E in HL-60 (38) and Cdk2 in MCF-7 cells (35). Retinoids transactivate or transrepress several genes through ligand-inducible transcription factor nuclear receptors (41). Retinoids have been shown to inhibit cell proliferation by suppressing the activity of AP-1 transcription factor (42, 43). ATRA also inhibits the activity of AP-1 in VSMCs (6). Since an AP-1 binding consensus resides in the promoters of cyclin D3 (44) and Cdk2 (45), AP-1 may have been involved in the ATRA-induced inhibition in the expressions of mRNA for cyclin D3 and Cdk2 observed in the present study. In addition, another transcription factor, E2F, which regulates the transcription of several genes implicated in control of the cell cycle progression (46), has also been suggested to be involved in retinoid-induced signal transduction. ATRA inhibits the expression and the transcriptional activity of E2F with concomitant reductions in the expression levels of the E2F-regulated genes B-myb, cyclin A, and cyclin E in human bronchial epithelial cells (47). Similar reductions in E2F1 activity and in expression of the E2F-regulated gene products, cyclin A, p34\textsuperscript{cos2}, and pRb, were also observed in ATRA-treated MCF-7 cells (32). This may account for the mechanisms by which ATRA inhibits cyclin E.

ATRA stimulated the expression of SM \(\alpha\)-actin, indicat-
ing that it promoted VSMC differentiation under our experimental conditions. An exit from the cell cycle, i.e., the prevention of the G0/G1 transition, has been considered to be a prerequisite for the induction of cell differentiation in a variety of model systems (14), although the biochemical background remains undetermined. Therefore, cell cycle regulator proteins have been proposed to regulate differentiation as well as proliferation, at least during the early stage in the cell cycle (14). Recently, in fact, forced expression of cyclin D1 was shown to inhibit the ability of MyoD to transactivate muscle-specific genes in skeletal myoblasts (48). This effect may be due to the cyclin D1-induced nuclear translocation of Cdk4 and the subsequent direct interaction between MyoD and Cdk4 (49). Furthermore, a decrease in the levels of Cdns similar to those in the present study has been observed during differentiation processes not mediated by retinoids. Thus erythroid differentiation in hexamethylen bisacetamide-treated murine erythroblastic leukemia cells has been shown to be accompanied with a downregulation of Cdk4 (50). And intestinal cell differentiation induced by cell-cell contact in a human colon cancer cell line has been associated with a decrease in the protein levels of Cdk2, Cdk4, and D-type (D1, D2, D3) and E-type cyclins (51). A reduction in the protein levels of Cdk4 and Cdk6 has also been observed in 1,25-dihydroxyvitamin D3-mediated monocytic differentiation in HL-60 cells (52). These studies suggest that cell differentiation is directly regulated by the interaction between Cdns and D-type cyclins. Therefore, the ATRA-induced reduction in cyclin D3, Cdk4, and Cdk6 expressions observed in the present study may not only account for the mechanism by which G0/G1 transition is prevented, but may also suggest that the antiproliferative effect of ATRA is closely linked to its ability to promote VSMC differentiation. Further studies are needed to elucidate the mechanisms and molecular targets involved in the action of ATRA on VSMC differentiation and to develop new therapeutic strategies against vascular diseases.

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