

# Effects of Acyclic Retinoid on Growth, Cell Cycle Control, Epidermal Growth Factor Receptor Signaling, and Gene Expression in Human Squamous Cell Carcinoma Cells

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## ABSTRACT

We described recently the growth inhibitory effects of the novel compound acyclic retinoid (ACR) in human hepatoma cell lines (M. Suzui *et al.*, *Cancer Res.*, 62: 3997–4006, 2002). In this study we examined the cellular and molecular effects of ACR on human squamous cell carcinoma (SCC) cells. ACR inhibited growth of the esophageal SCC cell line HCE7, and the head and neck SCC cell lines YCU-N861 and YCU-H891, with IC<sub>50</sub> values of ~10, 25, and 40 μM, respectively. Detailed studies were then done with HCE7 cells. Treatment of these cells with 10 μM ACR caused an increase of cells in G<sub>0</sub>-G<sub>1</sub> and induced apoptosis. This was associated with two phases of molecular events. During phase 1, which occurred within 6–12 h, there was an increase in the retinoic acid receptor β (RARβ) and p21<sup>CIP1</sup> proteins, and their corresponding mRNAs, and a decrease in the hyperphosphorylated form of the retinoblastoma protein. During phase 2, which occurred at ~24 h, there was a decrease in the cellular level of transforming growth factor α, and the phosphorylated (*i.e.*, activated) forms of the epidermal growth factor receptor, Stat3, and extracellular signal-regulated kinase proteins, and a decrease in both cyclin D1 protein and mRNA. Reporter assays indicated that ACR inhibited the transcriptional activity of the cyclin D1, *c-fos*, and activator protein promoters. On the other hand, ACR markedly stimulated the activity of a retinoic acid response element-CAT reporter when the cells were cotransfected with a RARβ expression vector. A hypothetical model explaining these two phases is presented. The diverse effects

that we obtained with ACR suggest that this agent might be useful in the chemoprevention and/or therapy of human SCCs.

## INTRODUCTION

Retinoids, a group of structural and functional analogues of vitamin A, exert fundamental effects on the regulation of epithelial cell growth, differentiation, and development (1). Some retinoids display chemopreventive and chemotherapeutic effects for various human cancers, including squamous cell carcinoma (SCC; Refs. 2, 3). The prognosis in most patients with esophageal SCC is poor, because current modalities of therapy for this type of cancer usually offer low survival and cure rates. In clinical trials, 13-*cis*-retinoic acid has been shown to suppress or reverse epithelial carcinogenesis and to prevent second primary tumors in patients with SCC (4, 5). These findings provide a rationale for the use of retinoids in both the prevention and treatment of human SCCs. However, most of the currently available retinoids exert significant clinical toxicity especially during long-term exposure. Therefore, there is a need to develop more effective and less toxic retinoids for the prevention and treatment of SCC and other types of cancer.

Acyclic retinoid (ACR), a novel synthetic retinoid, inhibits the growth of hepatoma cells both *in vivo* and *in vitro* (6–10). Furthermore, in clinical studies the administration of ACR to patients that had been treated for hepatoma prevented the occurrence of secondary tumors of the liver (11, 12). It is also remarkable that this preventive effect of ACR was not associated with significant clinical toxicity (11, 12). ACR also induces apoptosis in human hepatoma cell lines (7–9). We reported recently that ACR causes an arrest of the cell cycle in G<sub>0</sub>-G<sub>1</sub>, increased cellular levels of the p21<sup>CIP1</sup> protein, and decreased levels of the cyclin D1 protein in a human hepatoma cell line (10). In view of the above-described findings, ACR may be a valuable agent in the chemoprevention and chemotherapy of not only hepatoma, but other types of human cancer.

Retinoids are thought to exert most of their effects by regulating gene expression primarily through two classes of nuclear receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs), both composed of three subtypes (α, β, and γ; Ref. 13). RARs and RXRs modulate the expression of target genes by interacting as either homodimers or heterodimers with the retinoic acid response element (RARE) located in the promoter regions of target genes (13). Among these retinoid receptors, RARβ is thought to be the most important with respect to suppression of cell growth and tumorigenicity. Indeed, RARβ expression is reduced progressively during the multistage development of esophageal SCC (14). The *in vitro* response of esophageal SCC cells to the growth inhibitory effects of all-

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*trans*-retinoic acid (ATRA) is associated with up-regulation of the expression of RAR $\beta$  (15). Transfection of a RAR $\beta$  expression vector into RAR $\beta$ -negative human esophageal SCC cells decreased tumor cell growth and induced apoptosis (16), and overexpression of exogenous RAR $\beta$  and induction of endogenous RAR $\beta$  by ATRA also play important roles in mediating the growth-inhibitory effects of ATRA in human lung SCC cells (17). These findings provide evidence that alteration in the cellular level of RAR $\beta$  play an important role in both the development and growth of human SCC cells. However, possible effects of ACR on the expression and activity of RAR $\beta$  and other retinoid receptors have not been studied previously in detail in SCC.

Human esophageal SCC display numerous genetic abnormalities including abnormalities in the expression of cell cycle control proteins (18, 19). In addition, amplification and/or overexpression of epidermal growth factor receptor (EGFR) and its ligand transforming growth factor  $\alpha$  (TGF- $\alpha$ ) are observed frequently in human esophageal SCC (20–22). We reported recently that ACR decreases the levels of expression of the cyclin D1 protein and mRNA, and inhibits the transcriptional activity of the cyclin D1 promoter in HepG2 human hepatoma cells (10). It has also been reported that treatment of a human hepatoma cell line with ACR resulted in a decrease in the cellular levels of TGF- $\alpha$  mRNA (8). Almost all of the previous studies on ACR have been confined to hepatoma cells and yet, as mentioned above, other retinoids have shown growth-inhibitory activity in SCC. Therefore, the purpose of this study was to examine in detail the molecular mechanisms by which ACR might exert growth-inhibitory effects on human SCC cells. For the reasons mentioned above, we have focused on the effects of ACR on cell growth, apoptosis, specific retinoid receptors, cell cycle control proteins, and the EGFR and related downstream signaling pathways in a human esophageal SCC cell line.

## MATERIALS AND METHODS

**Chemicals.** ACR (NIK333; Nikken Chemicals Co., Ltd., Tokyo, Japan) was provided by Dr. Hisataka Moriwaki (Gifu University School of Medicine, Gifu, Japan; Ref. 6).

**Cell Lines and Cell Culture.** The HCE7 human esophageal cancer cell line was provided by Dr. Curtis C. Harris (National Cancer Institute, Bethesda, MD; Ref. 18), and YCU-H891 and YCU-N861 human head and neck squamous cancer cell lines were provided by Dr. Mamoru Tsukuda (Yokohama City University, Yokohama, Japan; Ref. 23), and were maintained in RPMI-1640 containing 10% fetal bovine serum (RF-10; Invitrogen, San Diego, CA). Cells were treated with ACR and cultured in an incubator with humidified air at 37°C with 5% CO<sub>2</sub>. As an untreated solvent control, cells were treated with DMSO (Sigma Chemical Co., St. Louis, MO) at a final concentration of <0.1%.

**Cell Proliferation Assays.** Ten thousands cells of each cell lines were plated into six-well/35-mm diameter culture dishes and treated with the indicated concentrations of ACR for 48 h in RF-10. The numbers of cells were then determined using a Coulter Counter (Beckman Coulter Co., Fullerton, CA). Results were expressed as percentage of growth with

100% representing control cells treated with DMSO alone. To examine whether the growth-inhibitory effects of ACR were dependent on the treatment time, HCE7 cells were treated with 10  $\mu$ M ACR for the indicated time (0, 3, 6, 12, 24, and 48 h), and this medium was then removed. The cells were more incubated in RF-10 until the indicated time (0, 24, 48, 72, and 96 h) and counted using a Coulter Counter (Beckman Coulter Co.).

**Apoptosis Assays.** HCE7 cells were treated with DMSO or 10  $\mu$ M ACR for the indicated time (0, 24, and 48 h), and the cell lysates were used for apoptosis assays. To quantify the induction of apoptosis, a DNA fragmentation assay was performed using the Cell Death Detection ELISA<sup>P</sup>-LUS kit (Roche Diagnostics Co., Indianapolis, IN), according to the manufacturer's instructions. The level of DNA fragmentation found at 0 h was set to 1, and the increases were shown as fold activation.

**Cell Cycle Assays.** HCE7 cells were seeded into 10-cm dishes (5  $\times$  10<sup>5</sup> cells/dish) in RF-10 and cultured overnight to allow for cell attachment. They were then treated with DMSO or 10  $\mu$ M ACR for the indicated time (0, 24, and 48 h), harvested, fixed with 70% ethanol, centrifuged, resuspended in PBS containing 2 mg/ml RNase (Sigma), and stained with 400  $\mu$ M of 0.1 mg/ml propidium iodide (Sigma). Samples were then analyzed for DNA histograms and cell cycle phase distribution by flow cytometer using a FACSCalibur instrument (Becton Dickinson, Franklin Lakes, NJ), and the data were analyzed by using the CELL Quest computer program (Becton Dickinson), as described previously (10).

**Protein Extraction and Western Blot Analysis.** HCE7 cells were treated with ACR for the indicated time (0, 3, 6, 12, 24, and 48 h) and concentrations (0, 1, 10, and 30  $\mu$ M). Protein extracts were then prepared as described previously (10). Cell lysates (40–80  $\mu$ g/lane) were separated by SDS-PAGE using 7.5–15% polyacrylamide gels and transferred onto Immobilon-P transfer membranes (Millipore Co., Bedford, MA). The primary antibodies for cyclin D1, p16<sup>INK4A</sup>, p21<sup>CIP1</sup>, p27<sup>KIP1</sup>, cyclin-dependent kinase (cdk) 4, cdk6, hyperphosphorylated form of retinoblastoma protein (ppRb), and actin were as described previously (10). The RAR $\alpha$  (C-20), RAR $\beta$  (C-19), RAR $\gamma$  (C-19), RXR $\alpha$  (D-20), RXR $\beta$  (C-20), and RXR $\gamma$  (Y-20) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antimouse IgG or antirabbit IgG antibodies (Amersham-Pharmacia Biotech, Buckinghamshire, United Kingdom) were used as the secondary antibodies. Each membrane was developed using an ECL-enhanced chemiluminescence system (Amersham-Pharmacia Biotech). The intensities of blots were quantified using NIH Image software version 1.62.

Assays for the state of phosphorylation of EGFR, extracellular signal-regulated kinase (ERK), and Stat3 were performed using specific antibodies as described previously (23). To determine cellular levels of TGF- $\alpha$ , EGFR, ERK, Stat3, and the phosphorylated forms of these molecules, HCE7 cells were incubated in RPMI 1640 minus serum for 24 h and then treated with 10  $\mu$ M ACR for the indicated time course. Proteins were then extracted and Western blot analysis was performed as described above. TGF- $\alpha$  (Ab-1) antibody was purchased from Oncogene Research Products (San Diego, CA).

**RNA Extraction and Reverse Transcription-PCR (RT-PCR) Analysis.** Total RNA was isolated from frozen HCE7 cells using TRIzol reagent as recommended by the manufacturer (Invitrogen). cDNA was amplified from 1  $\mu$ g of total RNA using SuperScript one-step RT-PCR with the platinum Taq system (Invitrogen). We chose the 22-, 25-, 28-, and 28-cycle rounds of PCR to analyze the expression levels of cyclin D1, p21<sup>CIP1</sup>, RAR $\beta$ , and TGF- $\alpha$  mRNA, respectively, because a semiquantitative assessment indicated that under these conditions the reaction had not reached a plateau and was still in the log phase. Cyclin D1 and actin specific primer sets were used as described elsewhere (10). The sequences for RAR $\beta$ -, p21<sup>CIP1</sup>-, and TGF- $\alpha$ -specific primers were as follows: RABF1 (5'-AAA AAG ACC AAC AGC CTA CG-3') and RABR1 (5'-AGC CCT TAC ATC CCT CAC AG-3'), CIF4 (5'-CTG GGG ATG TCC GTC AGA AC-3') and CIR4 (5'-AAT CTG TCA TGC TGG TCT GC-3'), TAF2 (5'-CAG GCC TTG GAG AAC AGC AC-3') and TAR2 (5'-CTG GGC TCT TCA GAC CAC TG-3'), respectively. The primers were designed using published sequences (24–26). At the same time, amplified PCR products obtained with actin-specific primers served as internal controls. Each amplification cycle consisted of 0.5 min at 94°C for denaturation, 0.5 min at 55°C for primer annealing, and 1 min at 72°C for extension (10). After PCR amplification, the PCR fragments were stained with ethidium bromide and analyzed by agarose gel electrophoresis.

**Cyclin D1, *c-fos*, and Activator Protein (AP-1) Reporter Assays.** Reporter assays were performed as described previously (27). The cyclin D1 promoter luciferase reporter plasmid -1745CD1LUC was kindly provided by Dr. Richard G. Pestell (Georgetown University, Washington, DC; Ref. 28). The *c-fos* promoter luciferase reporter plasmid pFos-wt-luc was described previously (27). The pAP-1-luciferase plasmid was kindly provided by Dr. Jacalyn H. Pierce (National Cancer Institute, Bethesda, MD; Ref. 29). The pcDNA3 plasmid DNA (Invitrogen) was used as a control vector and used as needed to achieve the same total amount of plasmid DNA/transfection. Cells ( $2 \times 10^5$ ) were transfected using Lipofectin reagent (Invitrogen) in six-well/35-mm diameter plates. One  $\mu$ g of DNA of the indicated luciferase reporter plasmid was transfected into HCE7 cells in opti-MEM I medium (Life Technologies, Inc.). After 24 h, the medium was changed to RF-10 containing the indicated concentrations of ACR (1, 10, or 30  $\mu$ M) or DMSO. Cells were then incubated for 24 or 48 h, and luciferase activities were determined using a luciferase assay system (Program Co., Madison, WI). In all of these reporter assays the cells were also cotransfected with a cytomegalovirus- $\beta$ -galactosidase ( $\beta$ gal) reporter. Luciferase activities were normalized with respect to the  $\beta$ -gal activities, to correct for differences in transfection efficiency. Luciferase activity obtained with cells treated with 0.1% DMSO was used as the 100% value at each time point.

**RARE CAT-ELISA Assays.** The RARE-CAT reporter plasmid and retinoid receptor expression vectors (RAR $\alpha$ , RAR $\beta$ , RAR $\gamma$ , and RXR $\alpha$ ) were kindly provided by Dr. David A. Talmage (Columbia University, New York, NY; Ref. 30). Transient transfection assays using a RARE promoter reporter were performed as described above. Two  $\mu$ g of a RARE-

CAT plasmid and 1  $\mu$ g of each expression vector were cotransfected into HCE7 cells in six-well dishes in opti-MEM I medium (Invitrogen). After 24 h, the medium was changed to RF-10 containing 10  $\mu$ M ACR or DMSO. The cells were then incubated for 12 h, and RARE-CAT activity was measured by using an ELISA kit (CAT ELISA; Roche Diagnostics Co.) according to the manufacturer's instructions. The level of RARE-CAT activity found in control DMSO-treated cells was set to a value of 1, and changes observed in the treated cultures were expressed as fold activation.

**Reversibility Studies.** HCE7 cells were treated with 10  $\mu$ M ACR for 12 h, and this medium was then removed. The cells were then treated with or without 10  $\mu$ M ACR for an additional 36 h (total 48 h), and extracts were prepared for Western blot analysis or RT-PCR analysis as described above.

**Statistical Analysis.** Statistical analyses of the DNA fragmentation assays, cyclin D1, *c-fos*, and AP-1 reporter assays, and RARE-CAT ELISA assays were analyzed by either Student's or Welch's *t* test. The results were considered statistically significant if the *P* < 0.05.

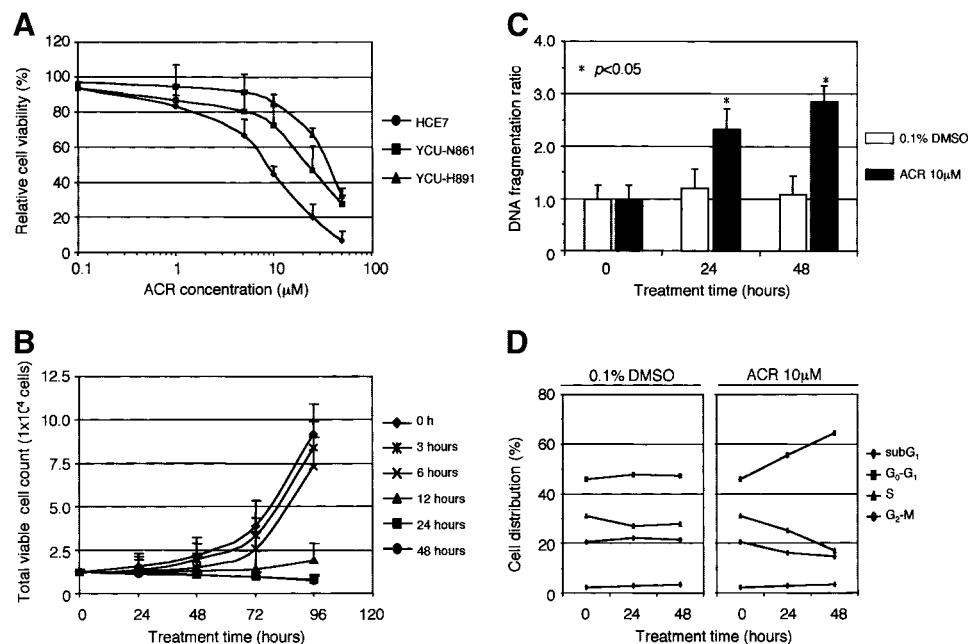
## RESULTS

**ACR Inhibits the Growth of Human SCC Cells.** We found that ACR inhibited growth of the HCE7, YCU-N861, and YCU-H891 cell lines, with IC<sub>50</sub> values of ~10, 25, and 40  $\mu$ M, respectively, when the cells were grown in RF-10 (Fig. 1A). Because the HCE7 cell line was the most sensitive to the growth-inhibitory effect of ACR, additional studies were then done with these cells. When we examined the time-dependent growth-inhibitory effects of ACR, we found that treatment for only 12 h was sufficient to cause irreversible growth inhibition of HCE7 cells (Fig. 1B).

**ACR Induces Apoptosis in HCE7 Cells.** ACR can induce apoptosis in human hepatoma cells (7–9). To determine whether the growth inhibition we observed with HCE7 cells was associated with induction of apoptosis we carried out DNA fragmentation assays (Fig. 1C). We found that when tested at 10  $\mu$ M ACR caused a time-dependent induction of apoptosis; thus, the DNA fragmentation ratio increased by 2.3-fold after 24 h and by 2.9-fold after 48 h (Fig. 1C). In contrast, no significant changes were observed in DMSO-treated cells during this time course (Fig. 1C).

**ACR Causes HCE7 Cells to Arrest in the G<sub>0</sub>-G<sub>1</sub> Phase of the Cell Cycle.** To determine whether this growth inhibition was also associated with specific changes in cell cycle distribution, cell cycle analysis was performed using DNA flow cytometry (Fig. 1D). Flow cytometric analysis indicated that when HCE7 cells were treated with 10  $\mu$ M ACR, the percentage of cells in G<sub>0</sub>-G<sub>1</sub> increased by 9.7% after 24 h and increased by 18.7% after 48 h, and this was associated with a concomitant decrease of cells in the S and G<sub>2</sub>-M phases of cell cycle (Fig. 1D). In contrast, no significant changes were observed in the DMSO-treated control cells during these time courses (Fig. 1D).

**ACR Induces p21<sup>CIP1</sup>, Decreases Cyclin D1, and Inhibits Hyperphosphorylation of Retinoblastoma Protein.** Because we found that treatment of HCE7 cells with 10  $\mu$ M ACR caused an increase of cells in the G<sub>0</sub>-G<sub>1</sub> phase of the



**Fig. 1** Inhibition of cell growth, induction of apoptosis, and progression of cell cycle by acyclic retinoid (ACR) in human squamous cell carcinoma (SCC) cell lines. **A**, dose-dependent effects of ACR in human SCC cell lines. The human esophageal SCC cell line HCE7, and the human head and neck SCC cell lines YCU-H891 and YCU-N861, were treated with the indicated concentrations of ACR for 48 h, and the total numbers of cells were then determined using a Coulter counter. **B**, time-dependent growth-inhibitory effects of ACR in the HCE7 cell line. HCE7 cells were treated with 10  $\mu\text{M}$  ACR for the indicated times and then the medium was replaced with fresh medium lacking ACR. The number of cells were then counted at the indicated times. Values are means of triplicate assays. **C**, induction of apoptosis by ACR in the HCE7 cell line. HCE7 cells were treated with 10  $\mu\text{M}$  ACR or DMSO for the indicated times, and cell lysates were examined for apoptosis using the DNA fragmentation assay. Each \* indicates a significant difference ( $P < 0.05$ ) between the control untreated cells and the ACR-treated cells. **D**, exponential cultures of HCE7 cells were treated with DMSO or 10  $\mu\text{M}$  ACR, and the cells were then analyzed by DNA flow cytometry at the indicated times. The distribution of cells in the sub-G<sub>1</sub>, G<sub>0</sub>-G<sub>1</sub>, S, and G<sub>2</sub>-M phases of the cell cycle were calculated and plotted. Bars, SD.

cell cycle during the subsequent 24–48 h (Fig. 1D), we examined the effects of ACR on cellular levels of the G<sub>1</sub> cell cycle control proteins cyclin D1, cdk4, cdk6, and the cdk inhibitors p16<sup>INK4a</sup>, p21<sup>CIP1</sup>, and p27<sup>KIP1</sup>, using Western blot analysis (Fig. 2A). We also examined the cellular levels of cyclin D1 and p21<sup>CIP1</sup> mRNAs using semiquantitative RT-PCR analysis (Fig. 2B). To examine time-dependent effects of ACR, HCE7 cells were treated with 10  $\mu\text{M}$  ACR, and extracts were prepared at 0, 3, 6, 12, 24, and 48 h after the addition of the drug. We found that there was a marked increase in the levels of expression of the p21<sup>CIP1</sup> protein during the subsequent 6–12 h (Fig. 2A). The expression levels of the cyclin D1 protein displayed a moderate decrease at 24 h and a marked decrease at 48 h (Fig. 2A). These specific changes of cyclin D1 and p21<sup>CIP1</sup> proteins were correlated with the changes of their corresponding genes (Fig. 2B). There were no significant changes in the expression levels of the p16<sup>INK4a</sup>, p27<sup>KIP1</sup>, cdk4, and cdk6 proteins (Fig. 2A).

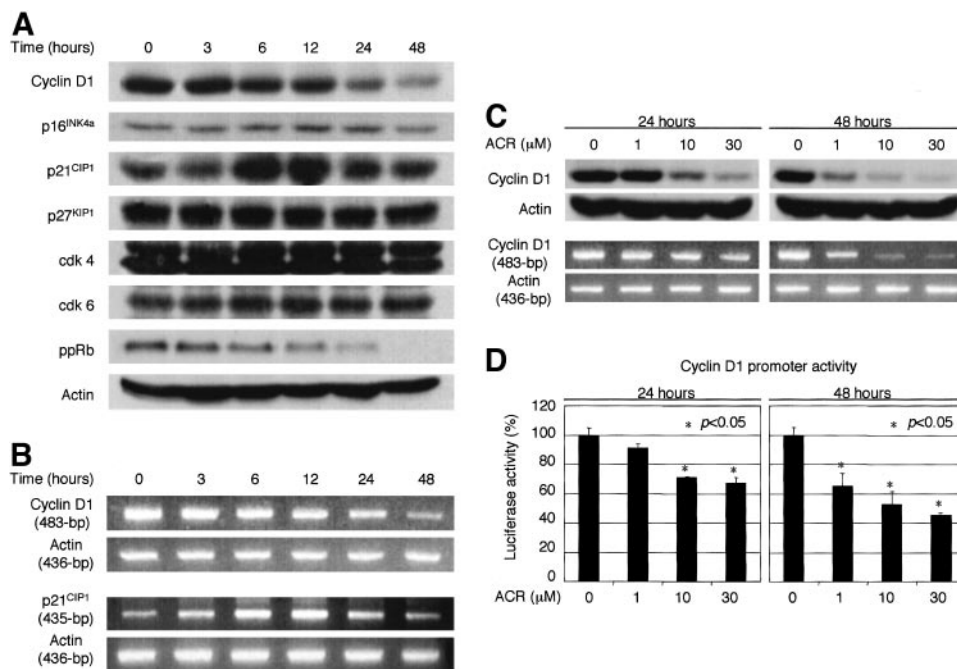
The tumor suppressor retinoblastoma protein inhibits the G<sub>1</sub> to S transition, but when it becomes hyperphosphorylated (designated ppRb) it no longer exerts this inhibitory effect (31, 32). Therefore, we also examined whether treatment with ACR alters the cellular level of the ppRb. We found that the level of the ppRb displayed a marked decrease after 6–12 h, and was

virtually undetectable at 24–48 h, after treatment with 10  $\mu\text{M}$  ACR (Fig. 2A).

To examine the dose-dependent effect of ACR on cyclin D1 expression, HCE7 cells were treated with 0, 1, 10, or 30  $\mu\text{M}$  ACR for 24 or 48 h (Fig. 2C). At 24 h the 1  $\mu\text{M}$  dose had no effect but by 48 h even this low dose of ACR caused a marked decrease in the cellular level of both the cyclin D1 protein and mRNA (Fig. 2C).

**ACR Inhibits Transcription from the Cyclin D1 Promoter.** In view of our finding that ACR caused a decrease in cellular levels of both the cyclin D1 protein and mRNA (Fig. 2, A–C), we examined whether these changes were associated with inhibition of *de novo* transcription of the *cyclin D1* gene by using transient transfection cyclin D1 promoter-luciferase reporter assays (Fig. 2D). After transfection of the luciferase reporter plasmid –1745CD1LUC, HCE7 cells were treated with increasing concentrations of ACR or DMSO for 24 or 48 h, and luciferase activity was then determined in the cell extracts. We found that treatment with ACR caused a dose- and time-dependent decrease in activity of the cyclin D1 promoter, especially in the assays done after 48 h. Thus, at 48 h 1  $\mu\text{M}$  ACR caused a 35% inhibition and 10  $\mu\text{M}$  caused a 48% inhibition of this activity (Fig. 2D). These results provide evidence that ACR causes a decrease in the cellular level of cyclin D1 mRNA and protein by inhib-



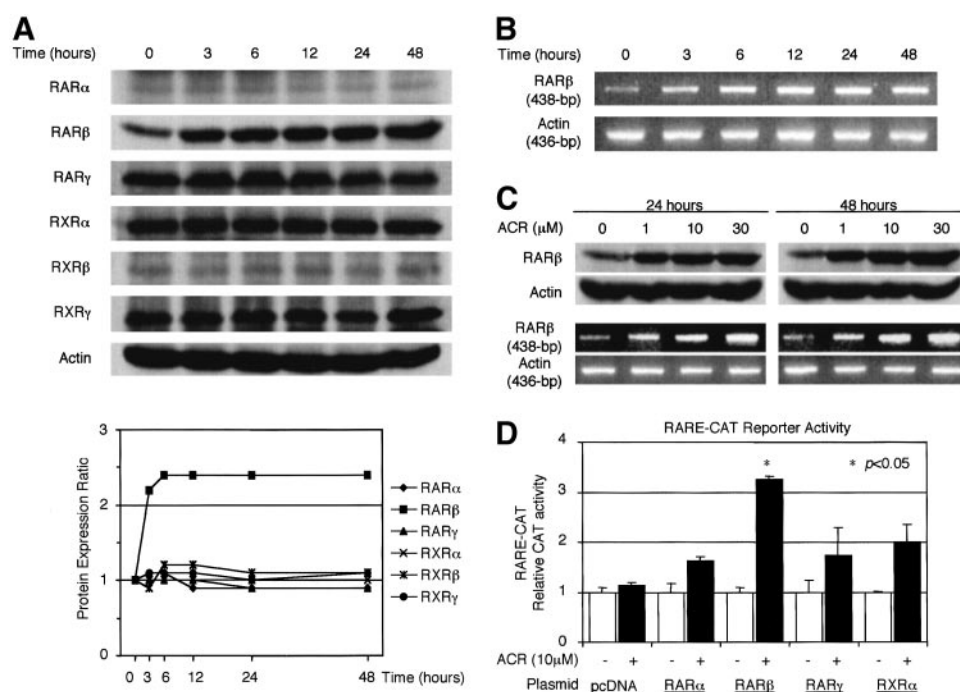


**Fig. 2** Effects of acyclic retinoid (ACR) on the expression of cell cycle control molecules. HCE7 cells were treated with 10  $\mu\text{M}$  ACR for the indicated times, and cell extracts were then examined by Western blot analysis using the respective antibodies (A) or examined by reverse transcription-PCR (RT-PCR) analysis using the cyclin D1 or p21<sup>CIP1</sup> specific primers (B), as described in "Materials and Methods." C, to examine the time- and dose-dependent effects of ACR on levels of expression of both the cyclin D1 protein and mRNA, HCE7 cells were treated with ACR for the indicated times and concentrations, and cell extracts were then examined by Western blot analysis or RT-PCR analysis for levels of cyclin D1. An antibody to actin and actin specific PCR products served as controls. D, effects of ACR on transcriptional activity of the cyclin D1 promoter. The cyclin D1 promoter luciferase reporter plasmid-1745CD1LUC was transfected into HCE7 cells, and the transfected cells were treated with ACR for the indicated times and concentrations. The extracts were then examined for luciferase activity. Each \* indicates a significant difference ( $P < 0.05$ ) between control untreated cells and ACR-treated cells. Bars, SD.

iting the transcriptional activity of the cyclin D1 promoter. The time course aspects of these effects are considered in the "Discussion" section.

**ACR Causes an Increase in the Cellular Level of Both the RAR $\beta$  Protein and mRNA.** Because retinoids are thought to exert most of their effects by regulating gene expression by binding to specific nuclear receptors (13), we examined the effects of ACR on cellular levels of the retinoid receptors RARs  $\alpha$ ,  $\beta$ , and  $\gamma$ , and RXRs  $\alpha$ ,  $\beta$ , and  $\gamma$  proteins using Western blot analysis (Fig. 3A). When we examined the time-dependent effect of 10  $\mu\text{M}$  ACR, we found that there was a marked and rapid increase in the levels of expression of the RAR $\beta$  protein at 3 h after the addition of the drug; the protein expression ratio increased by 2.2-fold after 3 h (Fig. 3A). However, there were no significant changes in the expression levels of the other molecules (Fig. 3A). When we used semiquantitative RT-PCR analysis to examine levels of RAR $\beta$  mRNA we found an increase in the RAR $\beta$  band intensity within 3 h after the addition of the drug, and this induction increased and persisted for up to 48 h (Fig. 3B). In addition, treatment of HCE7 cells with only 1  $\mu\text{M}$  ACR caused a marked increase in the level of expression of both the RAR $\beta$  protein and mRNA at 24 h (Fig. 3C). Thus, 1  $\mu\text{M}$  ACR can induce cellular levels of RAR $\beta$  to the same extent as 10  $\mu\text{M}$  ACR (Fig. 3C).

**ACR Stimulates the Activity of a RARE-CAT Construct When the Cells Are Cotransfected with a RAR $\beta$  Expression Vector.** RARs modulate the expression of target genes by interacting with the RARE element located in the promoter regions of target genes (13). In view of our finding that ACR caused a rapid and marked increase in the levels of expression of both the RAR $\beta$  protein and mRNA (Fig. 3, A–C), we examined whether ACR stimulates the transcriptional activity of a RARE-CAT reporter in transient transfection assays (Fig. 3D). In these studies, we also examined the effects of cotransfection with expression plasmids that encode the retinoid receptors RAR $\alpha$ , RAR $\beta$ , RAR $\gamma$ , and RXR $\alpha$  to determine whether ACR preferentially activates specific retinoid receptors. After transfection, the cells were incubated with or without 10  $\mu\text{M}$  ACR for 12 h, and extracts assayed for CAT activity using an ELISA assay as described in "Materials and Methods." In studies in which the cells were not cotransfected with an exogenous retinoid receptor, 10  $\mu\text{M}$  ACR caused an  $\sim 1.1$ -fold stimulation of reporter activity when compared with the DMSO-treated control cells (Fig. 3D). Among the retinoid receptors tested only RAR $\beta$  caused a significant enhancement of ACR stimulation of reporter activity (Fig. 3D). Therefore, it appears that ACR preferentially interacts with RAR $\beta$  in stimulating the RARE transcription element. The results we obtained with RAR $\beta$  in



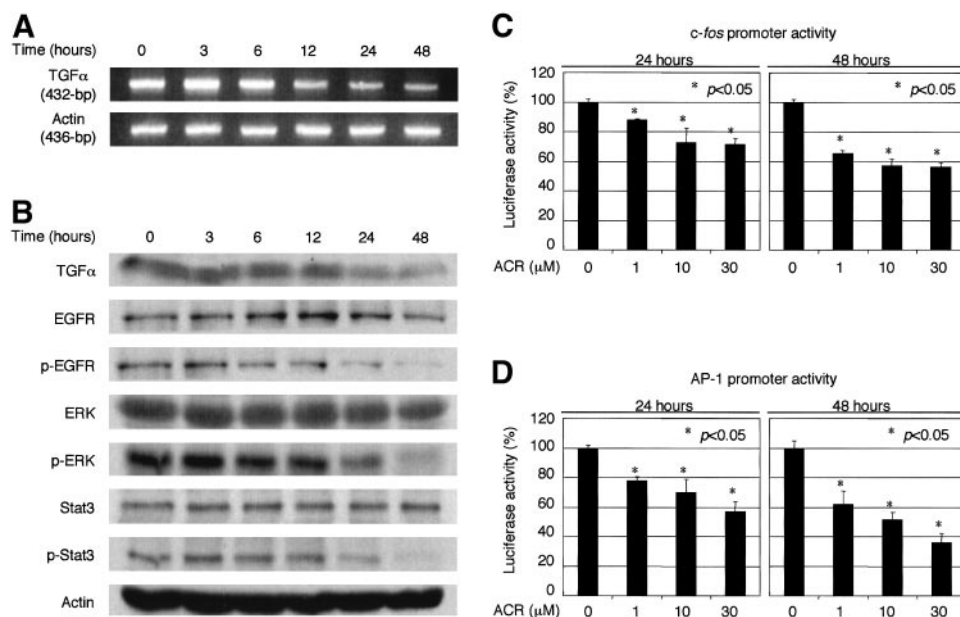
**Fig. 3** Effects of acyclic retinoid (ACR) on the expression of nuclear retinoid receptors. HCE7 cells were treated with 10  $\mu\text{M}$  ACR for the indicated times, and cell extracts were then examined by Western blot analysis using the respective antibodies (A) or examined by reverse transcription-PCR (RT-PCR) analysis using the RAR $\beta$  specific primer (B), as described in "Materials and Methods." The results obtained in A were quantitated by densitometry and are displayed in the *bottom panel* of A. C, HCE7 cells were treated with ACR for the indicated times and concentrations, and cell extracts were then examined for levels of RAR $\beta$  protein and mRNA, using Western blot or RT-PCR analysis, respectively. An antibody to actin and actin specific products served as controls. D, Effects of ACR on RARE-CAT reporter activity when HCE7 cells were cotransfected with various retinoid receptor expression vectors. The RARE-CAT reporter plasmid and the indicated retinoid receptor expression vectors were cotransfected into HCE7 cells, and the cells were then treated with 10  $\mu\text{M}$  ACR or DMSO for 12 h. RARE-CAT activities were measured by using an ELISA system, as described in "Materials and Methods." Each \* represents a significant difference ( $P < 0.05$ ) between control untreated cells and ACR-treated cells. Bars, SD.

these reporter assays (Fig. 3D) coupled with our findings that ACR preferentially induces the expression of RAR $\beta$  in HCE7 cells (Fig. 3, A–C) suggest that the biological effects of ACR in HCE7 cells are mediated mainly through RAR $\beta$ .

**ACR Causes a Decrease in the Cellular Level of TGF- $\alpha$  mRNA and Protein, and a Decrease in the Phosphorylated Forms of the EGFR, Stat3, and ERK Proteins.** Because the EGFR and its ligand TGF- $\alpha$  appear to play critical roles in the growth of human esophageal SCC (20–22), we examined the effects of ACR on expression of TGF- $\alpha$ /EGFR pathway (Fig. 4, A and B). Using semiquantitative RT-PCR analysis, we found that when HCE7 cells were treated with 10  $\mu\text{M}$  ACR there was a marked decrease in the cellular level of TGF- $\alpha$  mRNA at 12 h and an additional decline at 24 and 48 h (Fig. 4A). There was also a marked decrease in the cellular level of the TGF- $\alpha$  protein at 24 h after addition of the drug (Fig. 4B). We then examined the time-dependent effects of 10  $\mu\text{M}$  ACR on cellular levels of the EGFR protein and the downstream signaling proteins, ERK and Stat3, as well as cellular levels of the corresponding phospho-proteins. We found that although ACR did not have an appreciable effect on the levels of any of these proteins at 24 and 48 h, there was a marked decrease in the levels of the phosphorylated (*i.e.*, activated) forms of the EGFR protein, the ERK protein, and the Stat3 protein (Fig. 4B).

**ACR Inhibits the Transcriptional Activity of the *c-fos* and AP-1 Promoters.** AP-1 is one of the important downstream effectors of ERK and Stat3 (33, 34). In view of our finding that ACR inhibited activation of the TGF- $\alpha$ /EGFR pathway (Fig. 4, A and B), we examined the effects of ACR on the transcriptional activity of the *c-fos* and AP-1 promoters, using transient transfection luciferase reporter assays (Fig. 4, C and D). After transfection with the *c-fos* or AP-1 luciferase reporter plasmid, HCE7 cells were treated with increasing concentrations of ACR for 24 or 48 h, and luciferase activities were determined in cell extracts. Treatment with ACR caused a dose- and time-dependent decrease in activity of both the *c-fos* (Fig. 4C) and AP-1 (Fig. 4D) luciferase reporters. These effects were greater at the 48 h than the 24 h time point and were greater with the AP-1 reporter than the *c-fos* reporter (Fig. 4, C and D). Significant inhibitory effects were seen with only 1  $\mu\text{M}$  ACR (Fig. 4, C and D). At 48 h 10  $\mu\text{M}$  ACR, the IC<sub>50</sub> concentration for growth inhibition caused about 50% inhibition of AP-1 activity (Fig. 4D). Therefore, treatment of HCE7 cells with concentrations of ACR that are in the same range as those that inhibit growth also inhibit the transcriptional activities of both the *c-fos* and AP-1 promoters.

**Treatment with ACR for 12 h Causes Sufficient Molecular Changes to Inhibit the Growth of HCE7 Cells.** Because we found that treatment of HCE7 cells with 10  $\mu\text{M}$  ACR



**Fig. 4** Effects of acyclic retinoid (ACR) on activation of the transforming growth factor (TGF) $\alpha$ /epidermal growth factor receptor (EGFR) signaling pathway. HCE7 cells were treated with 10  $\mu$ M ACR for the indicated times, and cell extracts were then examined by reverse transcription-PCR using TGF- $\alpha$  specific primer (A) or examined by Western blot analysis using the respective antibodies (B), as described in "Materials and Methods." Actin-specific PCR products and an antibody for actin served as controls. Effects of ACR on transcriptional activity of the *c-fos* (C) and activator protein 1 (AP; D) promoters. Either *c-fos* (C) or AP-1 (D) promoter luciferase reporter plasmids were transfected into HCE7 cells, and cells were treated with ACR for the indicated times and concentrations. Cell extracts were then examined for luciferase activity. Each \* indicates a significant difference ( $P < 0.05$ ) between control untreated cells and ACR-treated cells. Bars, SD.

for 12 h caused an irreversible growth inhibition (Fig. 1B), we examined whether treatment for only 12 h was sufficient to alter the expression of RAR $\beta$ , cyclin D1, ppRb, and suppression of the TGF- $\alpha$ /EGFR pathway (Fig. 5). HCE7 cells were treated with 10  $\mu$ M ACR for 12 h. The medium was then removed and replaced with fresh medium containing or lacking 10  $\mu$ M ACR, and the cells were then incubated for an additional 36 h (total 48 h), and extracts were prepared. We found that there was a marked increase in the levels of expression of both the RAR $\beta$  protein and mRNA (Fig. 5A), and a significant decrease in the levels of expression of both the cyclin D1 protein and mRNA, and in the ppRb protein (Fig. 5B), in both the cells that were or were not exposed to ACR during the 12–48 h interval. Activation of the TGF- $\alpha$ /EGFR pathway was also suppressed to the same extent in both types of cell cultures (Fig. 5C). These findings suggest that treatment of HCE7 cells with ACR for only 12 h is sufficient to induce a cascade of changes in gene expression that persists for at least an additional 36 h. Presumably this explains why treatment with ACR for only 12 h is also sufficient to cause irreversible inhibition of cell growth (Fig. 1B).

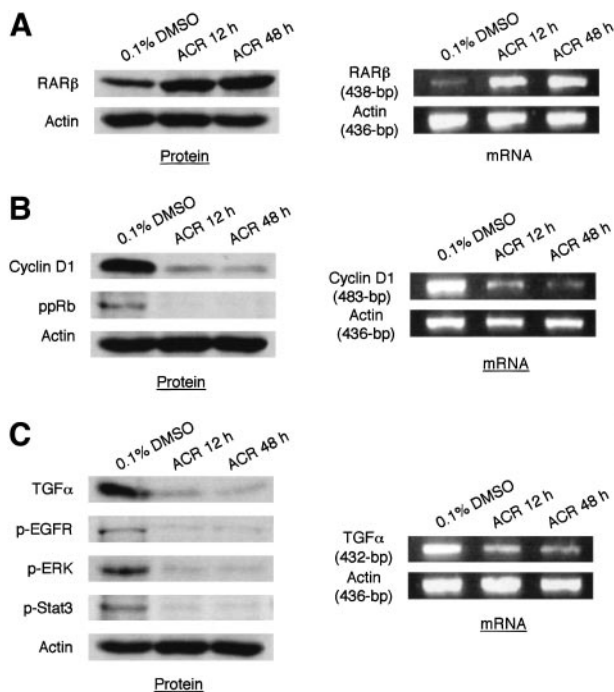
## DISCUSSION

Most of the previous experimental studies on the novel synthetic retinoid ACR have been done on human hepatoma cell lines (7–10). Because of the clinical interest in using retinoids in the chemoprevention and therapy of SCCs of the aerodigestive system, in the present study we examined the effects of ACR on human SCC cell lines. We found that ACR inhibits growth

of the esophageal SCC cell line HCE7 with an IC<sub>50</sub> value of  $\sim$ 10  $\mu$ M (Fig. 1A), which is similar to that for human hepatoma cells (10). Treatment of the HCE7 cells with ACR caused an increase of cells in the G<sub>0</sub>-G<sub>1</sub> phase (Fig. 1D) and induced apoptosis (Fig. 1C). Temporal studies indicated that these cellular effects were associated with two phases of molecular events. During phase 1, which occurred within 6–12 h after the addition of ACR, there was an increase in the levels of expression of the RAR $\beta$  and p21<sup>CIP1</sup> proteins and mRNAs, and a decrease in the ppRb. During phase 2, which occurred at about 24 h, there was a decrease in the cellular level of TGF- $\alpha$ , and the activated forms of the EGFR, ERK, and Stat3 proteins, and also a decrease in cellular levels of both cyclin D1 protein and mRNA.

We will first discuss the events that we observed in phase 1. Nuclear retinoid receptors are ligand-dependent transcription factors, which bind to RARE elements in the promoter regions of retinoid responsive target genes (13). The RAR $\beta$  gene contains a RARE in its promoter, which explains why ACR (Fig. 3, A and B) and other retinoids (35, 36) can cause a fairly rapid induction of RAR $\beta$ . RAR $\beta$  expression is suppressed in several types of human malignancies including esophageal SCC (14). Xu *et al.* (15) found that there is a loss of RAR $\beta$  expression in about 70% of human esophageal SCC, and that the growth inhibition and induction of apoptosis in human esophageal SCC cell lines with ATRA is associated with up-regulation of the expression of RAR $\beta$ . In this study, we found that treatment of HCE7 cells with ACR caused a marked and rapid increase in cellular levels of the RAR $\beta$  protein and mRNA, which occurred





**Fig. 5** Time-dependent effects of acyclic retinoid (ACR) on the expression of retinoid acid receptor (RAR) $\beta$  and cell cycle control molecules, and on activation of the transforming growth factor (TGF) $\alpha$ /epidermal growth factor receptor (EGFR) pathway. HCE7 cells were treated with 10  $\mu$ M ACR for 12 h, and this medium was then removed. The cells were then treated with fresh medium containing 10  $\mu$ M ACR (ACR 48 h) or fresh medium without ACR (ACR 12 h) for an additional 36 h (total 48 h), and extracts were prepared for Western blot analysis or reverse transcription-PCR analysis. The control cells were treated with final concentration of 0.1% DMSO for 48 h (0.1% DMSO). The respective antibodies or specific primers for RAR $\beta$  (A), cyclin D1 and hyperphosphorylated form of protein retinoblastoma (ppRb; B), and TGF $\alpha$ , phosphorylated (p)-EGFR, phosphorylated extracellular signal-regulated kinase (p-ERK), and phosphorylated (p)-Stat3 (C) are described in "Materials and Methods." Actin-specific PCR products and an antibody for actin served as controls.

within 3 h of the addition of ACR (Fig. 3, A and B). We also found that within 12 h after the addition of ACR there was a marked stimulation of RARE-CAT reporter activity when the HCE7 cells were cotransfected with a RAR $\beta$  expression vector (Fig. 3D). Taken together, these findings suggest that the early induction of RAR $\beta$  by ACR occurs at the level of transcription and, as with other retinoids, plays an important role in the subsequent growth-inhibitory activity seen with ACR (Fig. 1, A and B). The fact that ACR specifically increased RAR $\beta$  and that in cotransfection studies RAR $\beta$  exerted a greater stimulation of RARE-CAT activity than other nuclear receptors (Fig. 3) suggests that ACR acts as a ligand mainly through RAR $\beta$ , although other retinoid receptors may also play a role. Our findings are consistent with other evidence that among the retinoid receptors RAR $\beta$  appears to be the most important with respect to induction of apoptosis (17, 37).

Within 6–12 h of treating HCE7 cells with ACR there was also a marked increase in cellular levels of the cdk inhibitor p21<sup>CIP1</sup> protein and its related mRNA (Fig. 2, A and B). Previ-

ous studies indicate that ATRA induces transcription of the p21<sup>CIP1</sup> gene by enhancing binding of the retinoid receptor complex to the RARE present in the promoter region of the p21<sup>CIP1</sup> gene (38). Therefore, it is likely that ACR activates transcription of the p21<sup>CIP1</sup> gene through a similar mechanism. Presumably, the early induction of RAR $\beta$  enhances this process. Within 6–12 h after the addition of ACR, we also observed a reduction in the ppRb (Fig. 2A). The retinoblastoma protein plays a key role as a negative regulator of the G<sub>1</sub>-S transition of the cell cycle; activation of cyclin D-cdk4 or -cdk6 complexes results in hyperphosphorylation of retinoblastoma protein, thus relieving this inhibitory activity, and the p21<sup>CIP1</sup> protein can bind to these complexes and inhibit their kinase activity (31, 32). In addition, p21<sup>CIP1</sup> can bind to and directly inhibit the activity of E2F (39). Thus, the early induction of p21<sup>CIP1</sup> by ACR in HCE7 cells could explain the arrest in G<sub>1</sub> that we observed (Fig. 1D). These results are consistent with those of our previous study indicating that ACR also induces a rapid increase in the cellular level of p21<sup>CIP1</sup> and a decrease in the level of ppRb in a human hepatoma cell line (10).

We will now discuss the events that occur in what we describe as phase 2 in the response of HCE7 cells to treatment with ACR. The carcinogenic process that leads to the development of esophageal SCC is associated frequently with amplification and/or overexpression of cyclin D1 (18, 19). In this study, we found that the level of expression of the cyclin D1 protein in HCE7 cells decreased at about 24 h after the addition of ACR (Fig. 2A). A previous study demonstrated that treatment of bronchial epithelial cells with ATRA led to a decrease in cyclin D1 protein and that this was due to post-transcriptional proteolysis of the cyclin D1 protein (40). However, we found that in HCE7 cells ACR caused a decrease in the cellular level of cyclin D1 mRNA in a time- and dose-dependent manner (Fig. 2, B and C). Furthermore, ACR also caused a time- and dose-dependent inhibition of the transcriptional activity of the cyclin D1 promoter (Fig. 2D). We have reported that ACR caused similar effects related to cyclin D1 in the HepG2 human hepatoma cell line, and the ACR-induced decrease in cyclin D1 protein was not blocked by the proteasome inhibitor *N*-acetyl-Leu-Leu-nor-leu-al (10). When human breast carcinoma cells were treated with the synthetic retinoid *N*-(4-hydroxyphenyl) retinamide there was also a decrease in the cellular level of cyclin D1, and this effect correlated with transcriptional repression rather than enhanced proteolysis of the cyclin D1 protein (41). In this respect, the effects of ACR on cyclin D1 resemble those of *N*-(4-hydroxyphenyl) retinamide.

Increased expression of the EGFR and its ligand TGF- $\alpha$  occur frequently in human esophageal SCC, thus providing an autocrine growth-stimulatory mechanism (20–22). In the present study we found that within 12 h after the addition of ACR to HCE7 cells, there was a significant decrease in the cellular level of TGF- $\alpha$  mRNA (Fig. 4A) and at 24 h there was a decrease in the cellular level of the phosphorylated (*i.e.*, activated) form of the EGFR (Fig. 4B). Thus, treatment with ACR presumably disrupts this autocrine mechanism. Previous studies indicated that treatment with ATRA reduced the levels of TGF- $\alpha$  and EGFR mRNA in human SCC cell lines by inhibiting the transcription of these two genes (42), and the RXR-selective retinoid LGD1069 also inhibited both TGF- $\alpha$  and EGFR expression



in human SCC cell lines (43). Furthermore, in bronchial epithelial cells retinol inhibited TGF- $\alpha$  promoter activity in the presence of RAR $\beta$  (44). Our results with ACR are also consistent with evidence that this compound decreases TGF- $\alpha$  expression in human hepatoma cells (8).

Mitogen-activated protein kinase cascades and the transcription factor Stat3 are major downstream effectors of the TGF- $\alpha$ /epidermal growth factor mitogenic signaling pathway (45, 46), and constitutive activation of Stat3 is associated with TGF- $\alpha$ /EGFR autocrine stimulation in human SCC both *in vitro* and *in vivo* (45, 47). Therefore, we examined whether ACR affects the levels of expression and state of activation of the EGFR, ERK, and Stat3 proteins in HCE7 cells. We found that ACR causes a decrease in the cellular levels of phosphorylated (*i.e.*, activated) forms of the EGFR, ERK, and Stat3 proteins at about 24 h after the addition of ACR to these cells (Fig. 4B). The results we obtained with ERK are consistent with a previous study indicating that other retinoids suppress EGFR-associated cell proliferation by inhibiting EGFR-dependent ERK activation (48). Because the changes that we ascribe to phase 2 in the response of HCE7 cells to ACR occur only after a delay of about 12–24 h, we postulate that they are mediated by the accumulation of one or more newly transcribed and translated gene products that repress the expression of TGF- $\alpha$  and other yet-to-be-identified genes. This could lead to inhibition of the activation of several signaling molecules, including EGFR, Stat3, and ERK. It is of interest that treatment of HCE7 cells with 10  $\mu$ M ACR for only 12 h was sufficient to induce irreversible growth inhibition (Fig. 1B) and also the molecular changes that occur at later time points (Fig. 5). These findings support our hypothesis that when cells are treated with ACR the molecular changes that occur during phase 1 (within 6–12 h) play a critical role in initiating the changes that occur during phase 2 (at about 24 h).

Previous studies indicate that treatment of cells with various retinoids can lead to inhibition of the activity of the transcription factor AP-1, a complex composed of dimers of members of the Jun and Fos family of DNA binding proteins (49). Various mechanisms have been proposed to explain this inhibitory effect on AP-1 activity. These include: (a) induction of expression of phosphatase 1, which decreases cellular levels of the activated form of c-Jun NH<sub>2</sub>-terminal kinase, phospho-c-Jun NH<sub>2</sub>-terminal kinase (50); (b) alterations in the assembly of transcription factors and cofactors on AP-1 regulated promoters (51); and (c) inhibition of AP-1 activity by RAR $\beta$  itself, *i.e.*, in a ligand-independent mechanism, perhaps through the recruitment of a receptor corepressor (52). It is of interest that overexpression of RAR $\beta$  in MDA-MB-231 breast cancer cells (52) and in squamous carcinoma cells (37) inhibits cell proliferation in a ligand-independent manner. In the latter studies, this was associated with increased acetylation of histone H4, but the precise mechanism responsible for this effect is not known. Thus, the induction of RAR $\beta$  by ACR during phase 1 could result in inhibition of AP-1 activity. This could then lead to disruption of the autocrine TGF- $\alpha$ /EGFR pathway and the loss of activation of ERK and Stat3. In addition, because transcription of the *cyclin D1* gene is regulated by ERK, Stat3, and AP-1 activity (33, 34, 53–55), the latter effects could account for the

decreased expression of cyclin D1 mRNA and protein during phase 2. Consistent with this formulation is our finding that treatment of HCE7 cells with ACR led to inhibition of the transcriptional activity of the cyclin D1, *c-fos*, and AP-1 promoters (Fig. 2D; Fig. 4, C and D). This effect was considerably greater at 48 h than at 24 h, perhaps because of the time required for cells to express the putative inhibitor of AP-1 activity.

In conclusion, this article provides the first evidence that the novel compound ACR inhibits growth and markedly alters the expression of several key signaling molecules in human SCC cells. It also indicates that these effects appear to be mediated mainly through RAR $\beta$ . Thus, despite its unusual structure, ACR appears to function in cells as a typical retinoid compound. However, because its administration appears to be associated with little or no toxicity (11, 12), ACR may be useful when used alone or in combination with other agents in the prevention and/or treatment of SCC of the esophagus, head, and neck.

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