Growth Inhibition of Human Hepatoma Cells by Acyclic Retinoid Is Associated with Induction of p21\(^{CIP1}\) and Inhibition of Expression of Cyclin D1\(^1\)

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**ABSTRACT**

Acyclic retinoid (ACR), a novel synthetic retinoid, can prevent the recurrence of human hepatoma after surgical resection of primary tumors, but the molecular mechanisms by which ACR exerts antitumor effects are not known. In this study, we found that ACR inhibited the growth of three human hepatoma cell lines. In HepG2 cells, this inhibition was associated with an arrest of the cell cycle in G\(_0\)-G\(_1\), increased cellular levels of p21\(^{CIP1}\), decreased levels of the hyperphosphorylated form of the retinoblastoma protein, and decreased levels of cyclin D1, but no significant changes were seen in the levels of the p16\(^{INK4a}\), p27\(^{kip1}\), cyclin-dependent kinase 4, cyclin-dependent kinase 6, glycogen synthase kinase 3\(\beta\), or \(\beta\)-catenin proteins. ACR also caused a decrease in the level of cyclin D1 mRNA. Cotreatment of HepG2 human hepatoma cells with the proteasome inhibitor N-acetyl-Leu-Leu-norleu-al did not prevent the ACR-induced decrease in cyclin D1 protein, in contrast to the protective effect of N-acetyl-Leu-Leu-norleu-al on the cyclin D1 protein in cells treated with all-trans-retinoic acid. In transient transfection reporter assays, ACR, but not all-trans-retinoic acid, inhibited transcription from the cyclin D1 promoter. As reported previously in colon carcinoma cells, we found that in hepatoma cells, cyclin D1 promoter activity is markedly stimulated by the \(\beta\)-catenin/T-cell factor pathway. Nevertheless, even in the presence of excess \(\beta\)-catenin, ACR markedly inhibited the transcriptional activity of the cyclin D1 promoter. This is the first systematic study of the inhibitory effects of ACR, or any other retinoid compound, on \(\beta\)-catenin/T-cell factor-stimulated cyclin D1 promoter activity in human tumor cells. These novel effects of ACR provide further evidence that ACR may be a valuable agent in the chemoprevention and therapy of hepatoma and possibly other human malignancies.

**INTRODUCTION**

Hepatomas (hepatocellular carcinomas) are a relatively common malignancy, ranking fifth in frequency on a worldwide basis and causing more than one million deaths annually (1, 2). Persistent infection with hepatitis B or hepatitis C viruses accounts for 80% of the cases of hepatoma (1). Most of the cases of hepatoma occur in developing countries in Eastern Asia and in Middle and West Africa (1). However, hepatoma incidence has been increasing in Japan, the United Kingdom, France, and the United States (3). In the United States, approximately 3.9 million people are infected with hepatitis C virus (3) and there has been a progressive increase in the number of hepatoma cases over the past two decades (3). Unfortunately, most of the cases of hepatoma are not curable because extensive resection is not possible, there is extensive liver dysfunction caused by cirrhosis, and/or the disease is rarely identified at an early stage. Furthermore, at the present time there is no effective chemotherapy for cases with advanced disease.

Vitamin A and its derivatives are termed retinoids. Both natural and synthetic retinoids have been used for the treatment of patients with breast carcinomas, head and neck squamous cell carcinomas, and acute leukemia (4). ATRA,\(^3\) 13-cis-retinoic acid, and HPR have been used as chemopreventive agents for carcinoma in the aerodigestive tract and for the treatment of leukoplakia (5–8). Treatment with 13-cis-retinoic acid has been demonstrated to reduce the occurrence of second primary carcinomas in patients with previously resected head and neck carcinomas (9). Clinical trials using 9-cis-RA are under way in several types of human solid tumors (10).

In 1981, Muto et al. (11) synthesized a novel retinoid, named ACR, that binds to the cellular retinoic acid-binding protein. This unique agent inhibited chemically induced hepatocarcinogenesis in rats (12) and spontaneously occurring hepatoma in mice (12). It also inhibited growth and induced apoptosis in human hepatoma cell lines (13, 14). In 1996, Muto et al. (15) reported that oral administration of ACR for 12 months significantly reduced the recurrence of primary hepatoma in patients who had their initial lesions resected. In these studies, ACR did not cause the typical toxic effects seen with conventional retinoids (15–17). Although the above-described results obtained with ACR in experimental and clinical studies are promising, the molecular mechanisms by which this agent causes tumor suppression are not known.

Cyclins are key molecules in cell cycle control because of their specific and periodic expression during cell cycle progression. The D-type cyclins (cyclins D1, D2, and D3) complex with cdk4 and cdk6 and thereby regulate transition from the G\(_1\) phase into the S phase by phosphorylation and inactivation of pRb (18). The activities of these cyclin D/cdk complexes are negatively regulated by the cdk inhibitors p16\(^{INK4a}\), p27\(^{kip1}\), and p21\(^{CIP1}\) (18). Amplification and/or overexpression of the cyclin D1 (bcl1, PRAD1, and CCND1) gene has been found in 11–13% of human hepatomas (19, 20). Cyclin D1 is also frequently overexpressed in a variety of other human carcinomas (18). These findings suggest that aberrant expression of cyclin D1 may play an important role in the development of human hepatoma and other carcinomas. Indeed, overexpression of cyclin D1 is sufficient to initiate hepatocarcinogenesis in transgenic mice (21). Thus, cyclin D1 can function as an oncogene in the liver. Therefore it is a potential target for hepatoma prevention and therapy.

Recent studies indicate that transcription of the cyclin D1 gene is stimulated by activation of the \(\beta\)-catenin/TCF/lymphoid enhancer-binding factor signaling pathway in human colon carcinoma (22, 23). Because \(\beta\)-catenin frequently accumulates in colon carcinoma cells as a result of mutations in the APC gene, the above-mentioned findings explain why cyclin D1 is often overexpressed in human colon carci-
noma (24, 25). Mutations in the APC gene have not been seen in experimental or human hepatomas (26, 27). However, β-catenin is normally targeted for proteolytic degradation by phosphorylation of specific sites by GSK-3β (28, 29), and mutations in the β-catenin gene that prevent this phosphorylation have been found in a wide variety of human carcinomas, including hepatoma (30–32), and also in carcinogen-induced mouse hepatoma (33–35). An increased expression of β-catenin has recently been found to be associated with a poor prognosis in patients with hepatoma (36). However, the role of abnormalities in the β-catenin signaling pathway in controlling cyclin D1 expression in human hepatoma cells has not been examined previously.

The purpose of this study was to examine the molecular mechanisms by which ACR causes inhibition of cell growth in human hepatoma cells, focusing on the possibility that it might act, at least in part, by suppressing the expression of cyclin D1, and to also examine in parallel studies the role of β-catenin in the expression of cyclin D1 in human hepatoma cells.

**MATERIALS AND METHODS**

**Chemicals.** ACR (Fig. 1) was provided by Dr. H. Moriwaki (11, 12). ATRA and LLnL were purchased from Sigma (St. Louis, MO).

**Cell Lines and Treatment with Chemicals.** Three human hepatoma cell lines, HepG2, Hep3B, and Huh7 (37, 38) were maintained in DMEM (Life Technologies, Inc., Rockville, MD) supplemented with 10% FBS (Life Technologies, Inc.). Cells were treated with ACR or ATRA and cultured in an incubator with humidified air at 37°C with 5% CO₂. As an untreated solvent control, cells were treated with DMSO (Sigma) at a final concentration of <0.1%.

**Cell Proliferation Assays.** Cell proliferation was measured by both a colony formation assay and a MTT assay. Three human hepatoma cell lines were plated into 6-well 35-mm-diameter culture plates (1 × 10⁵ cells/well) in DMEM plus 10% FBS and cultured overnight to allow for cell attachment. As shown in Figs. 8 and 9, we used cyclin D1 promoter-luciferase (CD1LUC) reporters that contain 1745 bp of the cyclin D1 promoter (−1745CD1LUC) and several truncated (−22CD1LUC) and mutant constructs of the cyclin D1 promoter (−22CD1LUC and −12CD1LUC) and mutant constructs of the cyclin D1 promoter (−39CD1LUC). The −39CD1LUC reporter contains intact SP1, TCF, CRE, and NF-κB sites (42). The −66CD1LUC reporter contains intact CRE, NF-κB and −66CD1LUC sites (42). The −66CD1LUC reporter contains intact CRE and NF-κB sites (42). The −66CD1LUC reporter contains intact CRE and NF-κB sites (42). The −66CD1LUC reporter contains intact CRE and NF-κB sites (42). The −66CD1LUC reporter contains intact CRE and NF-κB sites (42).

**Flow Cytometry Analysis.** HepG2 cells were plated onto 10-cm dishes (1 × 10⁵ cells/dish) in DMEM containing 10% FBS and grown overnight to allow for cell attachment. They were then treated with 20 μM ACR for 24 or 48 h, harvested, fixed with 70% ethanol, centrifuged, and resuspended in 400 μL of PBS containing 2 mg/ml RNase (Sigma), and stained with 400 μL of 0.1 mg/ml propidium iodide (Sigma). The cell suspension was filtered through a 60-μm Spectra/Mesh nylon filter (Spectrum Medical Industries). Samples of 10,000–20,000 cells were then analyzed for DNA histograms and cell cycle phase distributions by flow cytometer using a FACSCalibur instrument (Becton Dickinson, Franklin Lakes, NJ), and the data were analyzed by a CELLQuest computer program (Becton Dickinson), as described previously (39).

**Western Blot Analysis.** HepG2 cells were lysed with modified radioimmunoprecipitation assay buffer [150 mM NaCl, 1% NP40, 0.1% SDS, 50 mM Tris-HCl (pH 8.0), 0.5% deoxycholic acid, 1 mM EDTA, 1 mM DTDT, and 25% glycercrol]. Cell lysates (50 μg/lane) were separated by SDS-PAGE and transferred onto Immobilon-P transfer membranes (Millipore Corp., Bedford, MA). Immunoblots using monoclonal or polyclonal antibodies were then prepared by established methods (40). The primary antibodies used in this study were cyclin D1 (06-137; Upstate Biotechnology Inc., Lake Placid, NY), p16INK4a (13251A; BD Transduction Laboratories, Lexington, KY), p21³⁵⁹⁴ (Ab-1; Oncogene Research Products, Darmstadt, Germany), p27³⁵⁹⁴ (K25020; BD Transduction Laboratories), cdk4 (06-139; Upstate Biotechnology), cdk6 (MS-398P; Neo Markers, Fremont, CA), GSK-3β (G22320-050; BD Transduction Laboratories), β-catenin (C19220; BD Transduction Laboratories), actin (A2066; Sigma), and pPRb (ser780; M045-3; Medical and Biological Laboratories, Nagoya, Japan). Antimouse IgG or antirabbit IgG antibodies (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) were used as the secondary antibodies. Each membrane was developed using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

**Transient Transfection Assays.** Reporter assays were performed as described elsewhere (41). HepG2 cells were plated into 6-well 35-mm-diameter plates (0.5 × 10⁵ cells/well) in DMEM plus 10% FBS and cultured overnight to allow for cell attachment. As shown in Figs. 8 and 9, we used cyclin D1 promoter-luciferase (CD1LUC) reporters that contain 1745 bp of the cyclin D1 promoter (−1745CD1LUC) and several truncated (−963CD1LUC, −261CD1LUC, −163CD1LUC, −66CD1LUC, and −22CD1LUC) and mutant constructs of the cyclin D1 promoter (42). The −163CD1LUC reporter contains intact SP1, TCF, CRE, and NF-κB sites (42). The −66CD1LUC reporter contains intact CRE and NF-κB sites (42). The −66CD1LUC reporter contains intact CRE and NF-κB sites (42).

**RT-PCR Analysis.** These assays were performed as described previously (46, 47). Total RNA was isolated from frozen HepG2 cells using Trizol reagent as recommended by the manufacturer (Life Technologies, Inc.). cDNA was amplified from 1 μg of total RNA using a SuperScript one-step RT-PCR with platinum Taq system (Life Technologies, Inc.). PCR was conducted for 22–30 cycles in a thermal controller (Programmable Thermal Controller; MJ Research Inc., Watertown, MA). Primers used for amplification were as follows:
cyclin D1-specific primer set, CD1F (5′-CTG-GCC-ATG-AAC-TAC-CTGGAGA-3′) and CD1R (5′-GTC-ACA-CTT-GAT-CAC-TCTCC-3′). β-A actin-specific PCR products from the same RNA samples were amplified and served as internal controls. Primers FBA (5′-CCA-GGC-ACC-AGG-GGCTGTA-3′) and RBA (5′-CGG-CGA-GCC-AGG-TCC-AGA-AGG-3′) were used for amplification of β-actin. These primers were designed using the database on the published sequences (48–50). 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. In all of the amplification procedures, we included reverse transcriptase-free control assays consisting of the amplification mixture, the RNA sample, and distilled water in place of reverse transcriptase to check for possible contamination of the RNA samples with DNA. After PCR amplification, the fragments were stained with ethidium bromide and analyzed by agarose gel electrophoresis.

**Northern Blot Analysis.** These assays were also performed as described elsewhere (40, 46). Total RNA was isolated as mentioned above. An aliquot (20 μg/lane) of each RNA sample was separated on a formaldehyde-agarose gel and then blotted onto a nylon membrane (Hybond-N; Amersham Pharmacia Biotech). rRNA was used as a loading control. A cyclin D1 cDNA probe was synthesized using primers CD1F and CD1R as described in the RT-PCR procedure, the RNA sample, and distilled water in place of reverse transcriptase to check for possible contamination of the RNA samples with DNA. After PCR amplification, the fragments were stained with ethidium bromide and analyzed by agarose gel electrophoresis.

**RESULTS**

**ACR Inhibits the Growth of Human Hepatoma Cells.** ACR exerted a marked and dose-dependent inhibition of growth of the Hep3B, HepG2, and Huh7 human hepatoma cell lines, with IC₅₀ values of about 15, 8, and 1 μM, respectively, when the cells were grown in DMEM containing 1% FBS (Fig. 2A). ACR is known to bind strongly to the cellular retinoic acid-binding protein (11), and the serum content in the growth medium can alter the effective concentrations of this compound (13). To examine a possible relationship between serum concentration and the growth-inhibitory effects of ACR, HepG2 cells were treated with different concentrations of ACR in DMEM containing 1%, 2%, or 10% FBS for 48 h, and then cell growth was measured by MTT assays. The IC₅₀ value of the HepG2 cell line was about 3-fold higher in DMEM containing 10% FBS than in DMEM containing 1% FBS (Fig. 2B). Thus the IC₅₀ value for the HepG2 cells was about 30 μM. Additional studies were then done with the HepG2 cells, as described below.

**ACR Causes Hepatoma Cells to Arrest in the G₁ Phase of the Cell Cycle.** Cell cycle analysis was performed to examine whether ACR-treated cells arrest in a specific phase of the cell cycle. Flow cytometric analysis indicated that when HepG2 cells were treated with 20 μM ACR in DMEM containing 10% FBS, the percentage of cells in G₁ increased by 7% after 24 h and increased by 13% after 48 h, and this was associated with a concomitant decrease of cells in the S and G₂-M phases of the cell cycle (Fig. 3). Only when HepG2 cells were treated with higher concentrations (>60 μM) of ACR for more than 48 h did they begin to detach from the culture plate and display evidence of apoptosis by an increase in the sub-G₁ population of DNA (data not shown). Nakamura et al. (13, 14) found that ACR-induced apoptosis in Huh7 human hepatoma cells and obtained evidence that this was due to down-regulation of transforming growth factor α.

**Treatment with ACR Induces p21/WAF1, Decreases Cyclin D1, and Inhibits Hyperphosphorylation of pRb.** Because we found that ACR induced a G₁ arrest in the cell cycle (Fig. 3), we performed Western blot analyses to determine whether treatment of hepatoma cells with ACR alters cellular levels of the G₁ cell cycle control proteins cyclin D1, cdk4, and cdk6 and the cell cycle inhibitor proteins p16INK4a, p21/WAF1, p27/KIP1. We also examined the levels of expression of the GSK-3β and β-catenin proteins in hepatoma cells treated with ACR because these proteins are known to regulate the transcription of cyclin D1 in colon carcinoma cells (51). In addition, GSK-3β can affect cellular levels of cyclin D1 by directly phosphorylating the cyclin D1 protein on Thr²⁸³ (52). HepG2 cells were treated with 5, 20, or 30 μM ACR in DMEM containing 10% FBS, and extracts were prepared at 24, 48, and 72 h and examined by Western blot analysis for the levels of expression of the above-mentioned proteins (Fig. 4A). We found that when the cells were treated with 5, 20, or 30 μM ACR within 24 h, there was a marked increase in the p21/WAF1 protein, and this increase persisted at the 48 and 72 h time points. In the ACR-treated cells, the level of cyclin D1 protein was not decreased at 24 h, but at 48 h, it displayed a moderate decrease (about 50% decrease), and at 72 h, it displayed a marked decrease, with all three doses of ACR (Fig. 4A). There were no significant effects of these concentrations of ACR on the levels of the cdk4, cdk6, p16INK4a, p27/KIP1, GSK-3β, or β-catenin proteins (Fig. 4A).
The protein encoded by the \( Rb \) gene, pRb, normally plays a key role as a negative regulator of the G\(_1\)-S transition of the cell cycle by binding the transcription factor E2F and preventing it from activating the transcription of genes required for the S phase (18). Therefore, we investigated whether treatment of hepatoma cells with ACR also alters cellular levels of ppRb because the cyclin D1/ cdk4 complex specifically phosphorylates Ser\(^{780} \) in pRb (53, 54). We used an anti-phospho-pRb antibody (MBL) that recognizes only pRB that is phosphorylated on Ser\(^{780} \). Treatment of HepG2 cells with 5, 20, or 30 \( \mu \)M ACR for 24 h caused a marked decrease in the level of ppRb, and this effect persisted at 48 h and was even greater at 72 h (Fig. 4B). In view of the above-described changes in p21\(^{CIP1} \), cyclin D1, and ppRb, we did a more detailed time course study of these proteins in HepG2 cells treated with 20 \( \mu \)M ACR for 0, 3, 6, 12, 24, and 48 h (Fig. 4C). We found that there was an increase in the p21\(^{CIP1} \) protein and a decrease in the ppRb protein within 3 h of treatment with ACR. On the other hand, the decrease in cyclin D1 did not occur until after 24 h, which is consistent with the results obtained in Fig. 4A. We also found that ACR markedly increased p21\(^{CIP1} \) mRNA within 3 h by a p53-independent pathway.\(^4\)

**ACR Causes a Decrease in the Cellular Level of Cyclin D1 mRNA.** In view of our finding that ACR caused a decrease in cellular levels of the cyclin D1 protein (Fig. 4, A and B), we examined whether this was associated with decreased levels of expression of cyclin D1 mRNA by using both a semiquantitative RT-PCR assay and Northern blot analysis (Fig. 5). HepG2 cells were treated with 20 or 40 \( \mu \)M ACR for 0, 3, 6, 12, 24, and 48 h, and PCR products were generated during both log- and plateau-phase reactions by conducting 22-, 25- and 30-cycle rounds of PCR using actin as an internal control, as described in “Materials and Methods.” We chose the 22-cycle rounds of PCR for data analysis because a semiquantitative assessment indicated that the reaction had not reached a plateau and was still in the log phase (data not shown), and the intensities of the cyclin D1-related bands with the 22-cycle rounds of PCR products reflected the corresponding mRNA levels detected by Northern blot analysis (Fig. 5B). The results obtained with 22 cycles are therefore shown in Fig. 5A. In cells treated with 20 \( \mu \)M ACR, a decrease in the cyclin D1 band intensity was apparent after 24 h. With 40 \( \mu \)M ACR, the decrease was apparent at 12 h, a marked decrease was seen at 24 h, and no band was detected at 48 h. Similar results were obtained when we conducted 22-cycle rounds with another primer set that generates a 726-bp fragment of cyclin D1 (data not shown). Northern blot analysis of RNA samples prepared from HepG2 cells treated with 20 \( \mu \)M ACR also indicated a significant decrease in the level of cyclin D1 mRNA within 24 h (Fig. 5B). Because the decrease in cyclin D1 protein in ACR-treated HepG2 cells was only apparent at about 48 h (Fig. 4, A and C), the decrease in cyclin D1 mRNA appears to precede, by about 24 h, the decrease in cyclin D1 protein.

**ACR Does Not Induce Proteolysis of the Cyclin D1 Protein.** There is evidence that ATRA triggers G\(_1\) arrest of the cell cycle via a ubiquitin-dependent mechanism of proteolysis of the cyclin D1 protein (55–57). Because we found that ACR causes a decrease in the

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**Fig. 3.** Effects of ACR on cell cycle progression. Exponentially growing cultures of HepG2 were treated with 0.1% DMSO (Control) or 20 \( \mu \)M ACR in DMEM/10% FBS. After treatment for 24 or 48 h, the cells were analyzed by DNA flow cytometry.

**Fig. 4.** Effects of treating HepG2 cells with ACR on the expression of various proteins. A and B, HepG2 cells were treated with increasing concentrations of ACR (5, 20, and 30 \( \mu \)M) for 24, 48, and 72 h in DMEM/10% FBS. Untreated control cells were treated with 0.1% DMSO alone. Cell extracts were then examined by Western blot analysis for the indicated proteins, using the respective antibodies, as described in “Materials and Methods.” C, HepG2 cells were treated with 20 \( \mu \)M ACR for 0, 3, 6, 12, 24, and 48 h, and extracts were examined by Western blot analysis.
transiently transfected HepG2 cells were treated with either 30 μM ACR or 30 μM ATRA. The latter concentration of ATRA was chosen because, like ACR, we found that it markedly inhibited the proliferation of HepG2 cells (data not shown). As shown in Fig. 6B, 30 μM ATRA did not inhibit cyclin D1 promoter activity, whereas in parallel assays, 30 μM ACR resulted in a significant decrease in cyclin D1 promoter activity (P < 0.006 by Welch’s t-test). These results suggest that treatment of hepatoma cells with ACR leads to a decrease in cellular levels of cyclin D1 mRNA and protein by inhibiting the transcriptional activity of the cyclin D1 promoter and that the natural retinoid ATRA lacks the latter activity.

\( \beta \)-Catenin Stimulates Cyclin D1 Promoter Activity through TCF in HepG2 Cells, and This Activity Is Inhibited by ACR. In human colon carcinoma cells, transcription from the cyclin D1 promoter is stimulated by \( \beta \)-catenin through its interaction with the TCF transcription factor (22). However, the role of the \( \beta \)-catenin/TCF signaling pathway in the de novo transcription of cyclin D1 in human hepatoma cells has not been examined previously. To determine whether \( \beta \)-catenin stimulates the transcription of cyclin D1 in human hepatoma cells, the −1745CD1LUC reporter was transfected into HepG2 cells together with wild-type or mutant \( \beta \)-catenin. We found that wild-type \( \beta \)-catenin activated (up to about 11-fold) the cyclin D1 reporter, and even greater stimulation was obtained with the mutant cellular level of the cyclin D1 protein in HepG2 cells (Fig. 4, A and C), we examined the effects of the proteasome inhibitor LLnL (Sigma). We found that cotreatment of HepG2 cells with 100 μM LLnL did not prevent the decline in the cyclin D1 protein seen in HepG2 cells treated with 20 μM ACR (Fig. 5C), whereas LLnL treatment did prevent the decline in the cyclin D1 protein in the same cell line treated with 20 μM ATRA (Fig. 5C). The results obtained with ACR are also in contrast to the ability of LLnL to block the decrease in cyclin D1 protein caused by ATRA in bronchial epithelial cells (55–57). Our findings, together with those of the RT-PCR assays and Northern blot assays (Fig. 5, A and B), suggest that ACR causes a decrease in the levels of expression of the cyclin D1 protein not through proteolysis but by causing a decrease in the level of cyclin D1 mRNA.

ACR Inhibits Transcription from the Cyclin D1 Promoter. To examine whether the decreased levels of cyclin D1 mRNA in ACR-treated HepG2 cells might be due to inhibition of de novo transcription of the cyclin D1 gene, we examined the effects of ACR on the transcriptional activity of the cyclin D1 promoter in transient transfection luciferase reporter assays using a cyclin D1 reporter, −1745CD1LUC (42). After transfection of this reporter into HepG2 cells, the cells were treated with increasing concentrations (0, 1, 5, 20, and 30 μM) of ACR for 24 h, and luciferase activity was then determined in cell extracts. We found that treatment with ACR caused a dose-dependent decrease in the activity of the cyclin D1 promoter, with about 65% inhibition at 30 μM ACR (Fig. 6A). Similar effects were seen in the other two hepatoma cell lines Hep3B and HuH7 (data not shown). For the reasons discussed above, it was of interest to also examine the effects of ATRA in the same cell system. Therefore, the
β-catenin (Fig. 7). To determine whether this activation depends on the transcription factor TCF, we then cotransfected either wild-type or mutant β-catenin with a dominant negative mutant of human TCF4E (22), and we found that cyclin D1 reporter activity was totally blocked with the dominant negative TCF4E (Fig. 7). These results indicate that in human hepatoma cells, as in colon carcinoma cells (22), β-catenin stimulates the transcription of cyclin D1 through the transcription factor TCF.

We then did transient transfection luciferase reporter assays with a series of truncations in the cyclin D1 promoter (42), in both the absence and presence of exogenous wild-type β-catenin, to map the regions of the cyclin D1 promoter that are stimulated by β-catenin in HepG2 cells. Specific response elements in the cyclin D1 promoter are eliminated in these shorter constructs (Fig. 8 and Ref. 42). In this study, luciferase activity obtained with the full-length −1745CD1LUC construct plus wild-type β-catenin was defined as 100%. We found that in both the absence and presence of wild-type β-catenin, high reporter activity was retained with the −963CD1LUC, −261CD1LUC, and −163CD1LUC constructs, but this activity was dramatically decreased with the −66CD1LUC and −22CD1LUC constructs, both of which lack the TCF response element (Fig. 8). With all of those reporter constructs, wild-type β-catenin activated the cyclin D1 reporter, and this activation was totally blocked with a dominant negative TCF. The decreases in reporter activity between −163CD1LUC and −66CD1LUC were statistically significant by Student’s t-test ($P < 0.0001$). Therefore, strong activation of the cyclin D1 promoter by β-catenin requires the −163 region, presumably because it contains the TCF response element. However, it is of interest that the residual reporter activity of the −66 construct was still stimulated by wild-type β-catenin and inhibited by the dominant negative TCF (Fig. 8, bottom four rows). These results suggest that the −66 region of the cyclin D1 promoter still contains TCF-like responsive sites, which is consistent with previous findings of Tetsu and McCormick (22).

In the above-mentioned studies with ACR (Fig. 6), we found that ACR inhibited the activity of the −1745CD1LUC reporter when tested in the absence of stimulation by exogenous β-catenin. Therefore, it was of interest to determine whether ACR also inhibited cyclin D1 promoter activity under conditions in which it was markedly stimulated by exogenous β-catenin. Using the −1745CD1LUC re-

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**Fig. 7.** The role of β-catenin and TCF in stimulating cyclin D1 promoter activity in HepG2 cells. One μg of the −1745CD1LUC reporter gene was transfected into HepG2 cells grown in DMEM/10% FBS. The cells were also cotransfected with wild-type (WT) or mutant (MT) β-catenin or with dominant negative (DN) TCF4E plasmids (1.0 μg/assay), as described in the figure. Luciferase activity was assayed in triplicate at 24 h after transfection and normalized for β-gal activity as described in the Fig. 6 legend. pcDNA3 plasmid DNA was added, as needed, to achieve the same total amount of plasmid DNA in each assay.

**Fig. 8.** Analysis of the region of the cyclin D1 promoter required for β-catenin stimulation. The −1745CD1LUC reporter and a series of mutants in the cyclin D1 promoter (−963CD1LUC, −261CD1LUC, −163CD1LUC, −66CD1LUC, and −22CD1LUC; 1.0 μg/assay) were transfected into HepG2 cells with or without wild-type β-catenin and a dominant negative (DN) TCF (1.0 μg each/assay) in DMEM/10% FBS, as shown in the figure. Luciferase activity obtained with the −1745CD1LUC reporter plus wild-type β-catenin was defined as 100%.
porter and exogenous stimulation with β-catenin, we found that 30 μM
ACR still markedly inhibited reporter activity (Fig. 9), even though, as
shown in Fig. 7, the total reporter activity in the presence of β-catenin
was about 13-fold greater than that in the absence of exogenous
β-catenin. In the studies described in Fig. 8, we found that in the
absence of ACR, the truncated −163CD1LUC construct retained full
β-catenin-stimulated reporter activity. Therefore, we examined
whether ACR exerts its inhibitory effects on β-catenin-stimulated
cyclin D1 promoter activity through one or more specific elements
present in the −163 construct by assaying reporter activity with the
−163 construct and a series of truncated and mutant derivatives of the
latter construct (see Fig. 8). These studies were done in the presence
of exogenous β-catenin and in the absence or presence of 30 μM ACR.
We found that ACR caused a statistically significant decrease in
luciferase activity, not only with the −163 construct that contains the
TCF response element but also with truncated reporters that lack this
element or with constructs that lack or are mutated in the SP1, CRE,
and NF-κB response elements (Fig. 9). Taken together, these results
suggest that although ACR can inhibit the transcriptional activity of
the cyclin D1 promoter in both the absence and presence of exogenous
stimulation by β-catenin, this inhibition is not confined to the function
of the β-catenin/TCF pathway, nor does it appear to depend on intact
SP1, CRE, or NF-κB elements in the proximal region of the cyclin D1
promoter.

**DISCUSSION**

As described in the “Introduction,” the compound ACR is of
considerable interest because it has been shown to prevent the recur-
rence of human hepatoma after surgical resection of primary tumors,
and also, in contrast to several other retinoid compounds, it has no
significant toxic effects (15–17). Therefore, it was of interest to
examine the cellular and molecular effects of ACR on human hepa-
toma cells. In our initial studies, we found that ACR inhibited the
growth of three hepatoma cell lines, Hep3B, HepG2, and Huh7, with
IC50 values of 15, 8, and 1 μM, respectively, when the cells were
grown in DMEM plus 1% FBS. Higher concentrations of ACR were
required when hepatoma cells were grown in DMEM plus 10% FBS.
Similar effects have been seen by Nakamura et al. (13) with Huh7
cells, presumably because of the binding of ACR to serum proteins.
We then chose the well-characterized HepG2 cell line for more
detailed studies. We found that growth inhibition induced by ACR
was associated with arrest of the cell cycle in G0–G1. Within 3 h of
the addition of ACR to the cell culture, there was a marked increase in
cellular levels of the cell cycle inhibitor protein p21CIP1 and a de-
crease in ppRb. There was also a decrease in the level of the cell cycle
control protein cyclin D1, but curiously, this did not occur until after
about 24 h of treatment with ACR (Fig. 4). We did not observe
significant changes in cellular levels of the cell cycle inhibitor pro-
teins p16INK4a or p27KIP1, cdk4 or cdk6, GSK-3β, or β-catenin in the
ACR-treated cells (Fig. 4).

The early increase in p21CIP1 could, by inhibiting cyclin D1/cdk4 or
cdk6 kinase activity, explain, at least in part, the early decrease in the
level of ppRb and thus the increase of cells in G1 that was seen within
24 h of ACR treatment (Fig. 3). The subsequent decrease in cyclin D1
may cooperate with the early induction of p21CIP1 to arrest cells in G1
and thereby further contribute to ACR-induced growth inhibition. It is

![Fig. 9. Effects of ACR on cyclin D1 promoter activity when stimulated by excess β-catenin, and studies with a series of truncated and mutant constructs. HepG2 cells were transfected with the −174CD1LUC reporter or with a series of truncated or mutant cyclin D1 luciferase reporters (−163CD1LUC, −66CD1LUC, −66Sp1CD1LUC, −66CREmut1CD1LUC,
−66CREmut2CD1LUC, and −66Np63mutCD1LUC) plus the wild-type β-catenin plasmid. The cells were then treated with 30 μM ACR for 24 h in DMEM/10% FBS. The ACR-induced decreases in activity obtained with all seven reporters were statistically significant by either Student’s or Welch’s t-test (each P was <0.004).](cancerres.aacrjournals.org)
of interest that treatment of TMK-1 human gastric carcinoma cells with 9-cis-R retinoid acid also induced the p21CIP1 protein within 3 h (58). Because the promoter region of the p21CIP1 gene has a retinoic acid response element located near position −1200, ACR might directly activate transcription of the p21CIP1 gene through this response element. In fact, we observed that ACR markedly induces p21CIP1 mRNA within 3 h. The induction of p21CIP1 is regulated through p53-dependent and p53-independent mechanisms (59), and HepG2 cells have wild-type p53 (60). On the other hand, an early and dramatic rise in the level of p21CIP1 occurs via a p53-independent manner in HL-60 human polymyelocytic leukemia cells treated with ATRA (61, 62), and a novel synthetic retinoid, 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid, causes a p53-independent G1 arrest and apoptosis in the MDA-MB-231 and MCF-7 human breast carcinoma cell lines (63). We believe that induction of p21CIP1 by ACR in HepG2 cells also occurs via a p53-independent mechanism because the ACR-treated cells do not display changes in the levels of the p53 protein or mRNA; however, this requires additional studies.

As mentioned above, although treatment of HepG2 cells with ACR led to a decrease in cellular levels of the cyclin D1 protein, this did not occur until 24 h after the addition of the drug to the cells. Therefore, it was of interest to examine the underlying mechanism. Previous studies indicated that the decline in cyclin D1 seen in human bronchial epithelial cells treated with ATRA was due to ubiquitin-mediated proteolysis because it was blocked by the inhibitor LLnL (55–57). However, LLnL did not block the decrease in cyclin D1 produced by ACR in HepG2 cells (Fig. 5C). Both RT-PCR analysis and Northern blot analysis indicated that the ACR-treated HepG2 cells displayed decreased levels of cyclin D1 mRNA within 12–24 h of the addition of the drug (Fig. 5, A and B). However, in parallel studies, ATRA did not alter cellular levels of cyclin D1 mRNA (data not shown). Furthermore, ACR inhibited the transcriptional activity of a cyclin D1 promoter in transient transfection luciferase reporter assays, but this effect was also not seen with ATRA (Fig. 6B). Therefore, in contrast to ATRA, which reduces cellular levels of cyclin D1 via proteolysis, ACR appears to do so by inhibiting de novo transcription of cyclin D1 mRNA. In this respect, the action of ACR resembles that of HPR, a synthetic retinoid, because when human breast carcinoma cells were treated with HPR, there was also a decrease in the cellular levels of cyclin D1, and this effect correlated with transcriptional repression rather than enhanced proteolysis of the cyclin D1 protein (64). We postulate that the delay of about 12–24 h in the decline of cyclin D1 mRNA in HepG2 cells after the addition of ACR reflects the time required for ACR to induce gene product(s) that directly or indirectly inhibit the transcription of cyclin D1. A similar mechanism has been previously postulated to explain the ability of ATRA to inhibit polyoma middle T-induced c-fos expression (65).

While analyzing the effects of ACR on the transcriptional activity of the cyclin D1 promoter in transient transfection luciferase reporter assays, we found that in human hepatoma cells, as established previously in human colon and breast carcinoma cells (22, 66), β-catenin markedly stimulates cyclin D1 promoter activity through the TCF transcription factor (Fig. 7). The reduction in activity of the cyclin D1 promoter upon deletion of the TCF site at position −81 that we observed in the current study (Fig. 8) is consistent with a large body of evidence that TCF proteins can serve as transactivator proteins in higher organisms (67, 68). It is well known, for example, that in SW480 human colon carcinoma cells, the inactivating mutation of APC that increases cytoplasmic free β-catenin enhances transcription through this TCF site of the cyclin D1 promoter (23). Various lymphoid enhancer-binding factor and TCF family members can function as either activators or repressors (67, 68). Most of the evidence for repression comes from studies of lower organisms, including Drosophila and Caenorhabditis elegans. Sampson et al. (69) and Soriano et al. (70) have shown that the HBP1 and PS1 proteins repress cyclin D1 promoter through the TCF site in mammalian cells. In these circumstances, the single TCF site at −81 was the site of repression, and deletion of the putative TCF sites was without effect (70). Therefore, the loss of basal activity upon deletion of the TCF site that we found in the current study is entirely consistent with a number of studies indicating that the TCF site of the cyclin D1 promoter functions as a TCF-dependent enhancer.

In the present study, we found that the stimulation of cyclin D1 promoter activity by excess β-catenin was also inhibited by ACR (Fig. 9). However, this inhibitory effect of ACR may not be confined to the action of TCF because ACR still inhibited reporter activity when we used various truncated and mutated forms of the cyclin D1 promoter (Fig. 9). Perhaps these inhibitory effects reflect the action of the above-hypothesized ACR-induced inhibitor of transcription, but the precise mechanisms remain to be determined. At the same time, our findings are not due to a nonspecific inhibition of transcription because ACR did not inhibit the transcriptional activity of the CMV promoter in CMV-β-gal reporter assays (data not shown), and, as shown in Fig. 4A, ACR did not inhibit the expression of several other proteins or cause a decrease in the level of actin mRNA (Fig. 5A). However, ACR does inhibit c-fos promoter activity, indicating that its inhibitory effects on transcription are not restricted to the cyclin D1 promoter. Our finding that ACR inhibits β-catenin/TCF-mediated cyclin D1 promoter activity in human hepatoma cell lines may provide an additional rationale for the clinical use of this agent because of recent evidence that an increased level of expression of the β-catenin protein is associated with poor prognosis in hepatoma patients (36).

Previous studies indicate that the PLC/PRF/5 and Huh7 human hepatoma cell lines constitutively express retinoid X receptor α mRNA and that ACR induced up-regulation of RARβ mRNA in both of these cell lines (71). In addition, ATRA induced increased levels of the RARβ protein in immortalized human bronchial epithelial cells and in human head and neck squamous cell carcinoma cell lines (5, 57, 72, 73). In recent unpublished studies, we found that when HepG2 cells were treated with ACR, there was a significant increase in RARβ mRNA and protein within 3 h. These findings suggest that the growth-inhibitory effects of ACR may be mediated, at least in part, through RARβ, but additional studies are required to determine whether specific retinoid receptors mediate the effects of ACR seen in the present study.

As mentioned in the “Introduction,” overexpression of cyclin D1 appears to play a critical role in human hepatomas and in several other types of human carcinomas. The demonstration in the present study that ACR inhibits the expression of cyclin D1 and exerts other inhibitory effects in human hepatoma cells should further encourage the use of this novel drug in the chemoprevention and treatment of hepatoma and other types of human carcinomas.

We should emphasize that most of the present studies were done with 30 μM ACR, and yet in clinical studies in which ACR was administered to patients at a dose of 600 mg/day, the serum level was only about 0.2 μM (17). However, it is possible that in vivo tumors are exposed to higher effective concentrations of ACR because of the more prolonged exposure that occurs in vivo and/or greater uptake by the tumor tissue. A similar caveat applies to in vitro studies with other retinoids including ATRA, 9-cis-R, and HPR, because in cell culture studies they have been used in the range of 1–10 μM to inhibit proliferation, depending on the cell line and growth conditions (55–58, 64, 74), and yet in patients who received 300–400 mg/day ATRA, the ATRA plasma concentration was only about 0.15 μM (74). Nev-


Growth Inhibition of Human Hepatoma Cells by Acyclic Retinoid Is Associated with Induction of p21CIP1 and Inhibition of Expression of Cyclin D1

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