Inhibition of Aldose Reductase Prevents Growth Factor–Induced G1-S Phase Transition through the AKT/Phosphoinositide 3-Kinase/E2F-1 Pathway in Human Colon Cancer Cells

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Abstract

Colon cancer is the leading cause of cancer death in both men and women worldwide. The deregulated cell cycle control or decreased apoptosis of normal epithelial cells leading to uncontrolled proliferation is one of the major features of tumor progression. We have previously shown that aldose reductase (AR), a NADPH-dependent aldo-keto reductase, has been shown to be involved in growth factor–induced proliferation of colon cancer cells. Herein, we report that inhibition of AR prevents epidermal growth factor (EGF)– and basic fibroblast growth factor (bFGF)–induced HT29 cell proliferation by accumulating cells at G1 phase of cell cycle. Similar results were observed in SW480 and HCT-116 colon cancer cells. Treatment of HT29 cells with AR inhibitor, sorbinil or zopolrestat, prevented the EGF- and bFGF-induced DNA binding activity of E2F-1 and phosphorylation of retinoblastoma protein. Inhibition of AR also prevented EGF- and bFGF-induced phosphorylation of cyclin-dependent kinase (cdk)-2 and expression of G1-S transition regulatory proteins such as cyclin D1, cdk4, proliferating cell nuclear antigen, cyclin E, and c-myc. More importantly, inhibition of AR prevented the EGF- and bFGF-induced activation of phosphoinositide 3-kinase/AKT and reactive oxygen species generation in colon cancer cells. Further, inhibition of AR also prevented the tumor growth of human colon cancer cells in nude mouse xenografts. Collectively, these results show that AR mediates EGF- and bFGF-induced colon cancer cell proliferation by activating or expressing G1-S phase proteins such as E2F-1, cdk4, and cyclins through the reactive oxygen species/phosphoinositide 3-kinase/AKT pathway, indicating the use of AR inhibitors in the prevention of colon carcinogenesis. Mol Cancer Ther; 9(4); 813–24.

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adenocarcinomas and metastasis (9–13). Further, various natural and synthetic drugs prevent colon cancer cell growth by inhibiting the expression of important cell cycle proteins such as E2F-1, cdk2, cyclin E, cyclin A, etc. (7, 13–15).

It has been shown that various growth factors such as transforming growth factor, insulin-like growth factor II, hepatocyte growth factor, epidermal growth factor (EGF), vascular endothelial growth factor, basic fibroblast growth factor (bFGF), and platelet-derived growth factor (PDGF) and their receptors are overexpressed in colon cancer epithelial cells obtained from colon cancer patients (16, 17). In addition, multiple reports show that growth factors are known to promote tumor progression by up-regulating important G1-S phase cell cycle proteins such as cyclin D1, cyclin E, cdk2, PCNA, and transcription factors including E2F-1 and c-myc by activating phosphoinositide 3-kinase (PI3K)/AKT (18–20). Therefore, elucidation of the mechanisms of colon cancer cell proliferation in the presence of growth factors is important for understanding the mechanisms of colon carcinogenesis, which is potentially important in developing drugs against abnormal cell proliferation.

Our recent studies with human colon cancer cells (Caco-2) suggest that the polyol pathway enzyme aldose reductase (AR; AKR1B1 in human) is a regulator of reactive oxygen species (ROS) signals induced by growth factors such as bFGF and PDGF and cytokines such as tumor necrosis factor-α (16, 21–23). AR reduces one of the most abundant and toxic lipid aldehydes, hydroxy-trans-2-nonenal (HNE), to 1,4-dihydroxyxnonene (DHN) and its glutathione conjugate, GS-HNE, to GS-DHN. We have shown earlier that inhibition of AR could prevent protein kinase C, NF-κB, and activator protein-1 activation and the increase in cell growth caused by HNE and GS-DHN, but not by GS-HNE, suggesting that the already reduced form of GS-DHN is insensitive to AR inhibition and could be the main mediator of oxidative stress–induced NF-κB activation (16, 22, 23). We have shown earlier that inhibition of AR prevents growth factor–, chemokine–, cytokine–, and hyperglycemia-induced cellular signals (16, 21–24). Furthermore, we have shown that AR inhibition as well as ablation by siRNA could prevent the bFGF- and PDGF-induced activation of NF-κB, expression of cyclooxygenase-2 (Cox-2), and production of prostaglandin E2 (PGE2) in colon cancer cells (16). Most remarkably, in a nude mouse xenograft model, we have shown that AR siRNA prevents the growth of human colon adenocarcinoma cells (SW480; ref. 16). However, the molecular mechanism of AR in modulating colon cancer cell growth and tumor progression remains unclear. We therefore determined the involvement of AR in the modulation of cell cycle progression and expression of cell cycle–related proteins such as E2F-1, cyclins, and cdk2 through the AKT/P13K pathway during colon carcinogenesis. Our results with human colon cancer cells indicate that inhibition of AR prevents growth factor–induced G1-S phase transition of cell cycle by inhibiting the expression of E2F-1 and related signals.

Materials and Methods

Materials
McCoy's 5A medium, RPMI 1640, PBS, penicillin/streptomycin solution, trypsin, and fetal bovine serum (FBS) were purchased from Invitrogen. Antibodies against E2F-1, cyclin D1, cyclin A, cdk2, phospho-Rb (pRb), phospho-cdk2, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and consensus oligonucleotide for E2F-1 (5'-ATTTAGTCGCCCGCCCTTCTCAA-3') were obtained from Santa Cruz Biotechnology, Inc. Antibodies against PCNA, AKT, and phospho-AKT were purchased from Cell Signal, Inc. Sorbinil and zopolrestat were gifts from Pfizer. MTT, EGF, bFGF, and all other analytic grade reagents were obtained from Sigma.

Cell culture
The human colon cancer cell lines HT29, SW480, and HCT-116 were obtained from the American Type Culture Collection. HT29 and HCT-116 cells were maintained and grown in McCoy's 5A medium supplemented with 10% FBS and 1% penicillin/streptomycin. Human colon adenocarcinoma SW480 cells were cultured at 37°C in a humidified atmosphere of 5% CO2 in RPMI 1640 supplemented with 10% (v/v) heat-inactivated FBS, 1% (v/v) penicillin/streptomycin solution, 2 mmol/L L-glutamine, 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, 4.5 g/L glucose, and 1.5 g/L sodium bicarbonate.

Measurement of cytotoxicity
HT29, HCT-116, and SW480 cells were grown to confluence in McCoy's and RPMI 1640, respectively, and trypsinized. Cells were plated in a 96-well plate at 2,500 per well. Subconfluent cells were growth arrested in 0.1% FBS with or without the AR inhibitor sorbinil or zopolrestat (20 μmol/L). After 24 h, EGF (5 ng/mL) or bFGF (10 ng/mL) was added to the medium and the cells were incubated for another 24 h. Cells incubated with the AR inhibitors alone served as control. Cell viability was determined by MTT assay as described earlier (16).

Cell cycle analysis
Cell cycle analysis was done as described earlier (16). Briefly, HT29 cells were grown in six-well plates at a density of 1.5 × 10⁵ per well. HT29 cells were preincubated with sorbinil (20 μmol/L) or carrier in DMEM containing 0.1% FBS for 24 h, followed by stimulation with EGF (5 ng/mL) or bFGF (10 ng/mL) for another 24 h. For cellular DNA staining, HT29 cells were suspended in 250 μL of solution A containing polyethyleneglycol (30 mg/mL), propidium iodide (0.05 mg/mL), Triton X-100 (1 μL/mL), sodium citrate (4 mmol/L), and RNase A (10 μg/mL) and incubated at 37°C. After 20 min of incubation, solution B containing 400 mmol/L NaCl instead of 4 mmol/L sodium citrate in solution A was added and incubated overnight at 4°C. For cell cycle analysis in nude mouse xenografts, tumor sections (50 μm) were cut from paraffin.
blocks, placed in Eppendorf tubes, dewaxed in xylene, and rehydrated sequentially into PBS. The sections were incubated with 1 mg/mL of collagenase and trypsin for 1 h at 37°C. Cell clumps were dispersed by passing through a 23-G needle and RNA content was removed by incubating with RNase (100 μg/mL) for 30 min at 37°C. The nuclei were stained with propidium iodide (50 μg/mL). Cell cycle analysis was done with a minimum of 10,000 events per analysis by using a FACScan flow cytometer (Becton, Dickinson and Co.).

**Western blot analysis**
To examine the expression of E2F-1, cyclin D1, cyclin E, c-myc, cdk2, cdk4, PCNA, phospho-Rb, phospho-cdk2, p-AKT, total AKT, and GAPDH proteins, Western blot analysis was carried out. Equal amounts of protein from cell extracts were subjected to 12% SDS-PAGE followed by transfer of proteins onto nitrocellulose filters and probing with the indicated antibodies. The antigen-antibody complex was detected by enhanced chemiluminescence (Pierce).

**Reverse transcription-PCR analysis**
Total RNA was isolated from HT29 cells by using an RNeasy micro isolation kit (Qiagen) as described earlier. Total RNA sample (1.5 μg) was reverse transcribed with Omniscript and Sensiscript reverse transcriptase one-step reverse transcription-PCR system with HotStarTaq DNA polymerase (Qiagen) at 55°C for 30 min followed by PCR amplification. The oligonucleotide primer sequences were as follows: 5’-CAAGAAGTCGGAAGGACATCATCC-3’ (sense) and 5’-AGATATCTACGGAGCTTGAC-3’ (antisense) for E2F-1, 5’-GTGTCTACGGAGCTTG-3’ (sense) and 5’-ACGTCAGCCTCCACACTCTT-3’ (antisense) for cyclin D1, 5’-GGGGATCTTGCTCCATGAGAGACA-3’ (sense) and 5’-GGGGATCTTGCTCCATTGAC-3’ (antisense) for c-myc, and 5’-CTTTGGAGACCTTCAACACC-3’ and 5’-CTTTGGAGACCTTCAACACC-3’ for β-actin. PCR was carried out in a GeneAmp 2700 thermocycler (Applied Biosystems) under the following conditions: initial denaturation at 95°C for 15 min and 35 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 1 min and then 72°C for 5 min for final extension. PCR products were electrophoresed in 2% agarose-1× TAE gels containing 0.5 μg/mL ethidium bromide. Bands were quantified using Kodak Image Station 2000R loaded with Kodak one-dimensional image analysis software and the average fold change intensities were calculated.

**Electrophoretic mobility shift assay**
Cytosolic and nuclear extracts were prepared as described earlier (23). Consensus oligonucleotide for E2F-1 was 5’-end labeled using T4 polynucleotide kinase. The assay procedure was as described before (23). Briefly, nuclear extracts prepared from control and treated cells were incubated with the labeled oligonucleotide for E2F-1 for 15 min at 37°C and the DNA-protein complex formed was resolved on 6.5% native polyacrylamide gel. After electrophoresis, the gels were dried using a vacuum gel dryer and were autoradiographed on Eastman Kodak Co. X-ray films. The radiolabeled bands were quantified by using Kodak Image Station 2000R.

**Luciferase reporter assay**
The E2F-1 luciferase activity was determined as described elsewhere (25). Briefly, HT29 cells were plated in 96-well plates at a density of 1 × 10^4 per well. After 24 h, cells were transiently cotransfected with 1 μg of pGL2-E2F1-Luc plasmid and 0.1 μg of pRL-CMV plasmid using FuGENE 6 transfection reagent for 6 h followed by serum starvation overnight in the presence or absence of sorbinil (20 μmol/L). The E2F1-luciferase reporter construct containing the −728/+77 region of the E2F-1 gene promoter was a generous gift from Dr. Stephen Safe (Texas A&M University, College Station, TX; ref. 26). HT29 cells were then stimulated with EGF (5 ng/mL) or bFGF (10 ng/mL) for 24 h and dual luciferase activity (Promega) was measured by using a luminometer. The luciferase activity was normalized against the ratio of firefly luciferase to renilla luciferase units and expressed as relative luciferase activity.

**Immunoprecipitation of PI3K and determination of PI3K activity**
PI3K activity in HT29 cells was determined using a competitive ELISA kit (Echelon Biosciences, Inc.) as per manufacturer’s instructions. The total cell lysates of HT29 cells were made using lysis buffer (buffer A + 1% NP40 and 1 mmol/L phenylmethylsulfonyl fluoride). The supernatant was incubated with anti-PI3K p85-α antibody (5 μL antibody/sample) for 1 h at 4°C followed by protein A-agarose chromatography. The PI3K activity in the immunoprecipitated beads was measured by incubating with phosphatidylinositol(4,5)-biphosphate [PI(4,5) P2] substrate (100 pmol) in kinase reaction buffer [4 mmol/L MgCl2, 20 mmol/L Tris (pH 7.4), 10 mmol/L NaCl, and 25 μmol/L/ATP] for 2 h. The supernatant was incubated with PI-[3,4,5]P3 detector protein for 1 h, followed by transfer to a PI-[3,4,5]P3-coated detection plate for competitive binding. After washing with Tris buffer [150 mmol/L NaCl, 10 mmol/L Tris (pH 7.5), and 0.05% (v/v) Tween 20], a peroxidase-linked secondary detector was added and developed with 3,3,5,5-tetramethylbenzidine solution. The PI-[3,4,5]P3 detector protein binding to the plate was determined by measuring the absorbance at 450 nm. The colorimetric signal is inversely proportional to the amount of PI-[3,4,5]P3 produced by PI3K.

**Nude mouse xenografts**
The effect of AR inhibition on tumor growth of human colon cancer cells (SW480) in nude mouse xenografts was studied as described earlier (16). Briefly, 5- to 6-week-old athymic nude nu/nu mice (Charles River) were injected s.c. with 1 × 10^6 SW480 human colon adenocarcinoma...
cells in 100 μL of PBS. Animals were treated with AR inhibitor, fidarestat (50 mg/kg body weight/d), in drinking water when the tumor surface area exceeded 30 mm² (day 21). The in vivo tumor volumes were measured in two dimensions using calipers (16). All animal experiments were carried out in accordance with a protocol approved by the Institutional Animal Care and Use Committee.

**Immunohistochemical analysis**

SW480 tumor xenograft samples were perfusion fixed with 4% paraformaldehyde and stored in 70% ethanol. Paraffin-embedded tumor sections (5 μm) were stained with antibodies against E2F-1, cyclin D1, AR, cleaved caspase-3, and c-myc using DakoCytomation LSAB+ System-HRP kit.

**Determinations of ROS**

ROS were quantified as described earlier (16). Briefly, HT29 cells were grown in a 24-well plate at a density of 1.5 × 10⁴ per well. At 70% to 80% confluence, serum-starved HT29 cells without or with sorbinil (20 μmol/L) were treated with the ROS-sensitive fluorophore 2’,7’-dichlorofluorescein diacetate for 15 min. Subsequently, HT29 cells were exposed to EGF (5 ng/mL) or bFGF (10 ng/mL) for 60 min and fluorescence was measured with a CytoFluorII fluorescence plate reader (PerSeptive Biosystems) at excitation of 485 nm and emission of 528 nm.

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**Figure 1.** Inhibition of AR prevents EGF- and bFGF-induced proliferation and cell cycle in human colon cancer cells. Growth-arrested HT29 (A), SW480 (B), or HCT-116 (C) cells were preincubated with sorbinil or zopolrestat (20 μmol/L) or carrier for 24 h, followed by stimulation with EGF (5 ng/mL) or bFGF (10 ng/mL) for another 24 h. Cell viability was determined by MTT assay. D, cell cycle analysis was done with a minimum of 10,000 events per analysis by using a FACSComp flow cytometer as described in Materials and Methods. Columns, mean (n = 4); bars, SE. #, P < 0.001, compared with control; **, P < 0.01, compared with cells treated with growth factors.
S-phase entry of cells, we next examined the effect of colon cancer cells DNA binding activity and expression of E2F-1 in Inhibition of AR prevents EGF- and bFGF-induced S phase of cell cycle. Thus, accumulation of cells at the G2-M phase suggests and inhibition of AR significantly (>60%) prevented it. entry of cells into the synthesis (S) phase of cell cycle, treatment of HT29 cells with EGF and bFGF induced vented by inhibiting AR. As shown in Fig. 1D and Table 1, we next determined which phase of cell cycle is pre- proliferation of cancer cells is regulated by cell cycle, HT29, SW480, or HCT-116 cell proliferation. Because however, sorbinil or zopolrestat alone did not affect was significantly attenuated (>60%) by AR inhibitors. The results shown in Fig. 1A to C show that treatment of HT29, SW480, or HCT-116 (27). First, we measured the expression of AR in colon cancer cells treated without or with growth factors in the absence and presence of AR inhibitor sorbinil. Our results indicate that both EGF and bFGF induced AR expression in all the cancer cells and inhibition of AR prevented it (data not shown). The results shown in Fig. 1A to C show that treatment of HT29, SW480, or HCT-116 cells with EGF (5 ng/mL) and bFGF (10 ng/mL) for 24 hours significantly (>40%) stimulated the growth. The increased growth of these cells was significantly attenuated (>60%) by AR inhibitors. However, sorbinil or zopolrestat alone did not affect HT29, SW480, or HCT-116 cell proliferation. Because proliferation of cancer cells is regulated by cell cycle, we next determined which phase of cell cycle is prevented by inhibiting AR. As shown in Fig. 1D and Table 1, treatment of HT29 cells with EGF and bFGF induced entry of cells into the synthesis (S) phase of cell cycle, and inhibition of AR significantly (>60%) prevented it. Thus, accumulation of cells at the G2-M phase suggests that inhibition of AR prevents entry of cells from G1 to S phase of cell cycle.

### Inhibition of AR prevents EGF- and bFGF-induced DNA binding activity and expression of E2F-1 in colon cancer cells

To further understand how AR inhibition prevents S-phase entry of cells, we next examined the effect of AR inhibition on the DNA binding activity of one of the important G1-S phase transition cell cycle regulatory transcription factors, E2F-1. As an autoregulatory transcription factor, E2F-1 binds to promoters of various cell cycle regulatory enzymes, cyclins, cdks, and c-myc, including its own E2F-1 promoter, and upregulates the expression of genes (28, 29). The DNA binding activity was determined by electrophoretic mobility shift assay using an oligonucleotide containing an E2F-1 binding site as a probe. As shown in Fig. 2A, stimulation of HT29 cells with EGF and bFGF caused a pronounced activation of E2F-1. In contrast, preincubation of HT29 cells with AR inhibitors significantly prevented the DNA binding activity of E2F-1. We further confirmed the effect of AR inhibition on the transcriptional activity of E2F-1 by luciferase reporter gene assay. As shown in Fig. 2B, inhibition of AR significantly prevented EGF- or bFGF-induced transcriptional activation of E2F-1. Treatment of HT29 cells with sorbinil alone did not affect the basal level of E2F-1 activation. These results indicate that inhibition of AR could prevent the DNA binding activity of E2F-1, which consequently inhibits the expression of genes required for G1-S phase transition. Because E2F proteins stimulate their own activity directly by binding to their gene promoters, we determined whether AR inhibition could prevent growth factor–induced de novo synthesis of E2F-1. Treatment of HT29, SW480, and HCT-116 cells with EGF or bFGF increased the expression of E2F-1, and inhibition of AR prevented the expression of E2F-1 significantly (Fig. 2C). Collectively, these results indicate that inhibition of AR prevented G1-S phase transition in colon cancer cells by inhibiting the transcriptional activity and expression of E2F-1.

### Inhibition of AR prevents EGF-induced phosphorylation of Rb

In quiescent cells, the unphosphorylated form of Rb functions as a cell cycle repressor by binding with E2F-1 to inhibit E2F-mediated gene transcription. Growth factor–induced hyperphosphorylation of Rb releases active E2F-1, which in turn upregulates the various genes required for G1-S phase cell cycle progression and proliferation.

![Table 1](image.png)
To determine the effect of AR inhibition on Rb phosphorylation, HT29 cells were serum starved with or without sorbinil for 24 hours and then stimulated with EGF as a function of time. The results showed that EGF induced the phosphorylation of Rb in HT29 cells. As shown in Fig. 3A, maximum phosphorylation was observed at 6 hours. The increase in phosphorylation of Rb protein was significantly inhibited by AR inhibition. These results suggest that inhibition of AR suppresses growth factor–induced E2F-1 activation/expression by blocking the phosphorylation of Rb.

**Inhibition of AR prevents EGF- and bFGF-induced expression of cyclin D1, cdk4, and PCNA in colon cancer cells**

We next examined the effect of AR inhibition on growth factor–induced expression of the heterodimer complex of cyclin D1 and cdk4, which is known to initiate phosphorylation of Rb during the mid-G1 phase (7). As shown in Fig. 3B, cyclin D1 and cdk4 levels in the basal state were low, and after stimulation with either EGF or bFGF, there was a marked increase in the expression of cyclin D1 and cdk4 proteins. Pretreatment...
of HT29 cells with AR inhibitor prevented significantly (60–80%) the expression of cyclin D1 and cdk4. Inhibition of AR also significantly prevented the expression of EGF- and bFGF-induced PCNA. These observations suggest that AR inhibition prevented the expression of G1-phase cell cycle regulatory proteins thereby inhibiting Rb phosphorylation and the release of active E2F-1.

Inhibition of AR prevents EGF- and bFGF-induced phosphorylation of cdk2 and the expression of 
cyclin E and c-myc in colon cancer cells

Because during late G1 phase, the maintenance of Rb in phosphorylated state is ensured by activated cdk2/cyclin E heterodimer complex, we next measured the effect of AR inhibition on phosphorylation of cdk2 and expression of cyclin E. We found that stimulation of HT29 cells with EGF induced phosphorylation of cdk2 as a function of time (Fig. 3A). Maximum phosphorylation was observed at 24 hours and inhibition of AR significantly prevented growth factor–induced phosphorylation of cdk2. Further, treatment of HT29 cells with either EGF or bFGF increased the expression of cyclin E, an effect that was significantly prevented by inhibiting AR (Fig. 3B). These results suggest that inhibition of AR could prevent EGF-induced phosphorylation of Rb by possibly modulating the cyclin E/cdk2 complex. To investigate the role of c-myc, an important transcription factor that regulates the expression of various cell cycle proteins such as cyclins, cdk6, and E2F family of proteins (32), we next measured the effect of AR inhibition on growth factor–induced c-myc expression. As shown in Fig. 3B, inhibition of AR prevented EGF- or bFGF-induced increase in the expression of c-myc in HT29 cells.

Inhibition of AR prevents EGF- and bFGF-induced 
E2F-1, cyclin D1, and c-myc mRNA expression in colon cancer cells

We next determined the effect of AR inhibition on important cell cycle proteins such as E2F-1, cyclin D1, and c-myc expression at the mRNA level. As shown in Fig. 4A, treatment of HT29 cells with EGF or bFGF for 4 hours significantly increased the expression of E2F-1, cyclin D1, and c-myc mRNA, and inhibition of AR prevented mRNA expression. These results were correlated with protein levels of E2F-1, cyclin D1, and c-myc.

Inhibition of AR prevents PI3K activity and 
phosphorylation of AKT in HT29 cells

Because PI3K/AKT activation in G1 phase has been shown to be required for c-myc stabilization and S-phase entry of cells (33), we next measured the effect of AR inhibition on the EGF- and bFGF-induced activation of PI3K and AKT. As shown in Fig. 4B, EGF and bFGF stimulated PI3K activity approximately 2.5- and 2.0-fold, respectively, as compared with control, and inhibition of AR significantly (~64 and 72%) prevented PI3K activity. Similarly, inhibition of AR significantly prevented EGF- or bFGF-induced phosphorylation of AKT (Fig. 4C). Collectively, these results suggest that inhibition of AR could prevent EGF- or
bFGF-induced activation of the PI3K/AKT pathway. Because ROS generated during oxidative stress is known to activate the PI3K/AKT pathway leading to activation of E2F-1 and c-myc (34), we next determined whether inhibition of AR could affect growth factor–induced changes in ROS generation. As shown in Fig. 4D, inhibition of AR by sorbinil prevented ROS generation as measured by dichlorofluorescein fluorescence, suggesting that inhibition of AR could prevent c-myc expression by inhibiting ROS generation in HT29 cells.

**Inhibition of AR prevents the progression of colon cancer SW480 cell growth and the expression of E2F-1, cyclin D1, and c-myc in nude mouse xenografts**

We next confirmed the results of in vitro studies in in vivo nude mouse xenografts of human adenocarcinoma SW480 cells. SW480 cells were injected s.c. and allowed to grow in nu/nu nude mice to ~30 mm² over a period of 21 days, followed by treatment of the animals with the AR inhibitor fidarastat (50 mg/kg body weight/d) in drinking water. At different days, in vivo tumor volumes were measured in two dimensions using calipers and percentage increase in tumor size was calculated by taking 30 mm² as starting zero percent (Fig. 5A). Animals that received AR inhibitor, fidarastat, clearly show the significant arrest of tumor progression, whereas uncontrolled growth was observed in the SW480 alone–injected mice. The photographs of animals and tumors that were taken at end of the experiment also clearly show the arrest of tumor growth (Fig. 5B). Because inhibition of AR prevented tumor growth in nude mouse xenografts, we next determined the effect of AR...
inhibition on the expression of important cell cycle proteins such as E2F-1, cyclin D1, and c-myc in tumor sections. As shown in Fig. 5C, immunostaining of tumor sections against antibodies to E2F-1, cyclin D1, and c-myc showed increased expression of these proteins in control mice compared with fidarestat-treated mice, suggesting that AR inhibition prevents the expression of E2F-1, cyclin D1, and c-myc. Further, inhibition of AR also prevented the expression of AR protein in xenograft tissues (Fig. 5C). However, inhibition of AR did not cause
significant increase in caspase-3 activity, suggesting that AR inhibition could be tumoristatic but not tumoricidal (Fig. 5C). Cell cycle analysis in xenograft sections shows that inhibition of AR significantly (>60%) arrested the entry of cells at G1 to S phase (Fig. 6A). Collectively, these results further confirm that AR inhibition prevents tumor progression by cell cycle arrest at the G1 phase.

Discussion

The deregulated cell cycle control and decreased apoptosis of normal epithelial cells leading to uncontrolled proliferation are the major cause of carcinogenesis (1, 35). The main initiators of uncontrolled proliferation in carcinogenesis include (a) cells that suffered irreparable DNA damage due to increased free radicals, which cause activation of specific nucleases and damage DNA, RNA, proteins, and lipids; (b) loss of extracellular stimulation, which regulates cell growth and upregulation of growth factors and their receptors; and (c) autosomal dominant inheritance of cancer genes among the multiple family members (16, 20, 36). In addition, it has been shown that chronic inflammatory diseases such as hepatitis, gastritis, and ulcerative colitis elevate the risk of colon cancer (22, 37). Upregulation of cytokines, growth factors, and their receptors has been observed in colon cancer cells obtained from colon cancer patients (16, 20).

Overexpression of inflammatory cytokines and growth factors causes uncontrolled production of ROS in autocrine and paracrine fashion. ROS further enhance the expression of inflammatory genes such as Cox-2 and iNOS for the production of PGE2 and NO, respectively (16, 17). These inflammatory molecules, in turn, activate various genes involved in the expansion of normal epithelial cells to dysplasia (precancer) and cancer (38). We have previously shown that FGF- and PDGF-induced expression of Cox-2 and PGE2 in Caco-2 cells is prevented by inhibiting AR. Further, we have shown that AR siRNA could prevent FGF- and PDGF-induced Caco-2 cell proliferation in vitro and the growth of human adenocarcinoma cells (SW480) in nude mouse xenografts (16). The current investigations were intended to understand the molecular mechanism(s) by which AR inhibitors prevent entry of cancer cells from G1 to S phase in the presence of growth-modulating agents (Fig. 6B).

Various reports show that E2F-1 is essential for regulating cell cycle progression, particularly the G1-S transition (5, 6). E2F proteins (E2F-1 to E2F-6) form a dimer with one of the subunits of DP (DP-1 to DP-3) proteins and control the transcription of several important genes that are directly involved in DNA replication and cell proliferation and G1-to-S phase transition (5, 6, 39). Those gene products include key enzymes such as DNA polymerase and thymidine kinase, as well as cell cycle regulators such as E2F-1, c-myc, cyclins, cdkS, and PCNA (4, 5). Our results show that inhibition of AR prevented E2F-1, cyclin, cdk, and PCNA expression and blocked the entry of HT29 cells to S phase on stimulation by growth factors. The retinoblastoma protein is a critical protein expressed during G1-to-S phase transition (6, 7). The hypophosphorylated form of Rb binds to E2F-1 and prevents its activation (40). During the mid-G1 to late G1 phase of cell cycle, hyperphosphorylated Rb releases the transcription factor E2F-1 to transcribe the genes required for DNA synthesis (7, 28). In this report, we show that the inhibition of AR prevents time-dependent phosphorylation of Rb on stimulation with growth factors. This suggests that AR inhibition prevents G1-S phase transition by blocking the release of E2F-1 from pRb. Deregulated expression of E2F-1 is associated with tumor progression and metastasis of various cancers including breast, colorectal, and small-cell lung carcinomas (11, 41). Johnson et al. (42) in primary
rat embryo fibroblasts reported that transformation of fibroblasts with E2F-1 gene in conjunction with ras oncogene showed more oncogenic capacity in tumor formation in the nude mouse xenograft model compared with ras oncogene alone. In addition, hepatocarcinoma cells transformed with gankyrin, an oncogenic protein that binds to pRB protein and releases active E2F-1, showed increased tumorigenicity in nude mice (43). These results indicate that activation of E2F-1 gene is required for tumor formation, and drugs that inhibit E2F-1 will have therapeutic potential in treating various cancers including colorectal cancer.

The molecular mechanisms that regulate cell proliferation are complex. Several studies suggest that the heterodimer complex of cyclin E/cdk2 plays a pivotal role in the late G1-S phase transition by phosphorylating Rb (7). In the absence of this second phosphorylation event, pRB would likely remain activated and bound to E2F-1 (7). In the absence of this second phosphorylation event, pRB would likely remain activated and bound to E2F-1 (7). In the absence of this second phosphorylation event, pRB would likely remain activated and bound to E2F-1 (7). In the absence of this second phosphorylation event, pRB would likely remain activated and bound to E2F-1 (7).

E2F-1 transcriptional and cdk2 kinase activities play an additive role in induction of S phase (7, 10). Inhibition of cyclin E/cdk2 does not block S-phase induction in Rb−/− cells where E2F-1 activity is deregulated, suggesting that cyclin E/cdk2 activity is specific to phosphorylate Rb (44). Interestingly, the antimitogenic effect of AR inhibitors indicates that inhibition of AR could prevent growth factor-induced sequential phosphorylation of Rb by cyclin D1/cdk4 and cyclin E/cdk2. The antiproliferative results with AR inhibition are consistent with the effects observed with oligopeptides designed against E2F-1 (45) and the cdk2 inhibitor SU9516 (7) in inhibiting E2F activity and cdk2 phosphorylation.

Recent studies have shown that stimulation of growth factor receptors such as EGF receptor, PDGF receptor, and IRS-1 induces the generation of ROS (17, 46). The ROS, in turn, could cause production of toxic lipid peroxidation products, such as GS-DHN, catalyzed by AR, may be the mediator of carcinogenic signals that activate NF-κB-dependent expression of growth factors and cytokines that promote colon cancer growth. Hence, inhibition of AR could be chemopreventive as well as chemostatic.

In summary, our data indicate that inhibition of AR prevents growth factor–induced proliferation of colon cancer cells by inhibition of ROS generation, activation of AKT/P3K, expression of c-myc, and inhibition of E2F-1 activation/expression by reducing cyclin D1/cdk4 and cyclin E/cdk2 activities. Based on these findings, we speculate that AR could mediate colon cancer cell proliferation through G1–to-S phase transition. Understanding this mechanism is potentially important in developing therapeutic strategies to ameliorate or prevent colon carcinogenesis by inhibition of AR.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Inhibition of Aldose Reductase Prevents Growth Factor–Induced G₁-S Phase Transition through the AKT/Phosphoinositide 3-Kinase/E2F-1 Pathway in Human Colon Cancer Cells

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