Deoxycholic Acid Activates β -Catenin Signaling Pathway and Increases Colon Cell Cancer Growth and Invasiveness

Rama Pai,* Andrzej S. Tarnawski, and Teresa Tran

Medical Service, Department of Veterans Affairs Medical Center, Long Beach, California; and the Department of Medicine, University of California, Irvine, California 92717

Submitted December 16, 2003; Revised February 19, 2004; Accepted February 20, 2004
Monitoring Editor: Carl-Henrik Heldin

Colorectal cancer is often lethal when invasion and/or metastasis occur. Tumor progression to the metastatic phenotype is mainly dependent on tumor cell invasiveness. Secondary bile acids, particularly deoxycholic acid (DCA), are implicated in promoting colon cancer growth and progression. Whether DCA modulates β -catenin and promotes colon cancer cell growth and invasiveness remains unknown. Because β -catenin and its target genes urokinase-type plasminogen activator receptor (uPAR) and cyclin D1 are overexpressed in colon cancers, and are linked to cancer growth, invasion, and metastasis, we investigated whether DCA activates β -catenin signaling and promotes colon cancer cell growth and invasiveness. Our results show that low concentrations of DCA (5 and 50 μ M) significantly increase tyrosine phosphorylation of β -catenin, induce urokinase-type plasminogen activator, uPAR, and cyclin D1 expression and enhance colon cancer cell proliferation and invasiveness. These events are associated with a substantial loss of E-cadherin binding to β -catenin. Inhibition of β -catenin with small interfering RNA significantly reduced DCA-induced uPAR and cyclin D1 expression. Blocking uPAR with a neutralizing antibody significantly suppressed DCA-induced colon cancer cell proliferation and invasiveness. These findings provide evidence for a novel mechanism underlying the oncogenic effects of secondary bile acids.

INTRODUCTION

Primary bile acids (cholic and chenodeoxycholic acid) are hydrophobic derivatives of cholesterol synthesized in the liver and secreted with bile into the duodenum where they play important role in digestion and absorption of dietary lipids. Most of the bile acids are reabsorbed in the ileum and only small quantities reach the colon where they are converted by enteric bacteria to secondary bile acids such as deoxycholic acid and lithocholic acid (Hoffman, 1989). These secondary bile acids, particularly deoxycholic acid (DCA), are implicated as endogenous colon cancer promoters.

Epidemiological studies have demonstrated that consumption of a high fat/low fiber diet leads to elevated fecal concentrations of secondary bile acids, which is associated with an increased incidence of colorectal cancer (Armstrong and Doll, 1975; Rogers *et al.*, 1993). Clinical studies have shown that patients with colorectal cancer have higher levels of bile acids in colonic lumen, and studies in animals further substantiate potential role of bile acids as tumor promoters. However, the molecular mechanisms of tumor-promoting actions of bile acids remain unclear (Cohen and Raicht, 1981; Breuer and Goebell, 1985; Korpela *et al.*, 1988; Reddy, 1992; Bayerdorffer *et al.*, 1995).

Article published online ahead of print. Mol. Biol. Cell 10.1091/mbc.E03-12-0894. Article and publication date are available at www.molbiolcell.org/cgi/doi/10.1091/mbc.E03-12-0894.

* Corresponding author. E-mail address: rpai@uci.edu.

Abbreviations used: APC, adenomatous polyposis coli; DCA, deoxycholic acid; GSK, glycogen synthase kinase; TCF, T cell factor; uPA, urokinase-type plasminogen activator; uPAR, urokinase-type plasminogen activator receptor.

The loss of normal regulation of cellular growth and death are characteristics of neoplastic transformation. Mutations in oncogenes and tumor suppressors deregulate normal cellular pathways that are important for tissue homeostasis. The adenomatous polyposis coli (APC) tumor-suppressor gene is mutated in ~80% of sporadic colorectal carcinomas (Jacoby *et al.*, 1995; Kinzler and Vogelstein, 1996). Interaction of APC protein with β -catenin has been shown to be important for the tumor suppressive function of APC (Polakis, 1997). β -Catenin is a key component of adherens junctions that link the actin cytoskeleton to members of the cadherin family of transmembrane cell-cell adhesion receptors (Ben-Ze'v and Geiger, 1998). Overexpression of β -catenin and down-regulation of E-cadherin in poorly differentiated, highly invasive cancers implicate E-cadherin and β -catenin in the progression of cancer (Brabletz *et al.*, 2001). In contrast to normal colonic cells where the levels of free cytosolic β -catenin are low, high levels of β -catenin accumulate in various types of cancers (Kinzler and Vogelstein, 1996; Morin, 1999; Polakis, 2000). Tyrosine phosphorylation of β -catenin has been suggested to promote metastatic potential and tumor invasiveness by stabilizing β -catenin and promoting its binding of T cell factor (TCF)/lymphocyte enhancer factor 1 DNA transcription factors (Alexander *et al.*, 2002). The latter complex functions as a transcriptional activator and plays a key role in regulating cancer cell proliferation and metastasis (Jacoby *et al.*, 1995; Peifer, 1997). Transcriptional targets of β -catenin/TCF include cyclin D1, urokinase plasminogen activator receptor (uPAR), matrix metalloproteinase 7, cyclooxygenase-2, gastrin, and CD44 (Alexander *et al.*, 2002). Whether DCA induces tyrosine phosphorylation of β -catenin and its transcriptional activation in colon cancer cells remains unknown.

The urokinase plasminogen activator (uPA) interacts with its specific membrane receptor (uPAR) and converts the proenzyme plasminogen to plasmin, which is capable of degrading extracellular matrix directly or indirectly through activation of matrix metalloproteinases (Ellis *et al.*, 1992). Increasing evidence indicates that expression of uPAR plays a significant role in tumorigenesis and in the development of invasive phenotype in a variety of tumors (Andreasen *et al.*, 1997; Yu *et al.*, 1997; Comoglio and Trusolino, 2002). Previous studies have demonstrated uPAR overexpression in colon cancer cells forming invasive foci and a positive correlation between uPAR overexpression and reduced patients survival (Pyke *et al.*, 1991; Ganesh *et al.*, 1994). Despite the fact that deoxycholic acid is implicated in promoting colon cancer progression, its effect on uPA and uPAR expression and colon cancer cell invasiveness is not known.

In this study, we examined, using colon cancer cell lines, the effect of DCA on 1) tyrosine phosphorylation of β -catenin; 2) E-cadherin- β -catenin binding; 3) mRNA and protein expressions of uPA, uPAR, and cyclin D1; and 4) cell proliferation and invasiveness. Furthermore, we investigated using β -catenin small interfering RNA (siRNA) whether β -catenin targets uPAR and cyclin D1 and examined the role of uPAR in DCA-mediated colon cancer cell proliferation and invasiveness.

MATERIALS AND METHODS

Cell Culture

The human colon cancer cell lines SW480 and LoVo (American Type Culture Collection, Rockville, MD) were grown according to the supplier's instructions. All the studies were performed within 10 passages after obtaining the cell lines. Serum-starved cells were incubated with DCA (5–100 μ M; Sigma-Aldrich, St. Louis, MO) for 30–120 min to assess tyrosine phosphorylation and nuclear accumulation; 2 h for uPA, uPAR, and cyclin D1 mRNA expression; 3–24 h for uPA, uPAR, and cyclin D1 protein expression; 24 h for cell proliferation; and 15 d for colony formation assay in the presence or absence of uPAR neutralizing antibody (5 μ g/ml; R&D Systems, Minneapolis, MN).

Preparation of Nuclear Proteins

Nuclear extracts from cultured cells were prepared following the procedure described previously (Andrews and Faller, 1991). Protein concentrations were determined by using a BCA protein assay kit (Pierce Chemical, Rockford, IL). Equal amounts of denatured nuclear protein (30 μ g) were resolved on 7.5% SDS-PAGE and immunoblotted following the procedure detailed below.

Immunoprecipitation and Western Blot Analysis

Immunoprecipitation and Western blot analysis was performed following the method described previously (Pai *et al.*, 2002). Cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 0.25% sodium deoxycholate, 1 mM NaF, 1 mM sodium orthovanadate, 5 μ g/ml leupeptin, 5 μ g/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride) and clarified by centrifugation. Equal amounts of proteins were incubated with specific antibody immobilized onto protein A-Sepharose for 2 h at 4°C with gentle rotation. Beads were washed extensively with lysis buffer and immunocomplexes were eluted in 2 $\times$ Laemmli buffer, boiled, and microcentrifuged. Proteins were resolved on SDS-PAGE, transferred to nitrocellulose membrane, and incubated with specific primary antibodies. After washing and incubating with secondary antibodies, immunoreactive proteins were visualized by the ECL detection system (Amersham Biosciences, Piscataway, NJ). The antibodies used for immunoprecipitation and immunoblotting were β -catenin monoclonal antibodies from BD Biosciences (San Diego, CA), anti-phosphotyrosine (PY99), anti-uPA, anti-uPAR, anti-E-cadherin, and anti-cyclinD1 antibodies from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-phospho-glycogen synthase kinase (GSK)-3 β and GSK-3 β antibodies from Cell Signaling Technologies (Beverly, MA). Where indicated, the membranes were stripped (Reblot-Plus; Chemicon International, Temecula, CA) and reprobed with another antibody. The densities of specific protein bands were analyzed using video image analysis system (Image-1/FL; Universal Imaging, Westchester, PA). Quantitative analyses of tyrosine phosphorylation of β -catenin were performed by determining the ratio between total protein and the phosphorylation by using data from three separate experiments performed in triplicate. The uPA, uPAR, and cyclin D1 protein expression levels were quantified after normalizing to the total β -actin levels. All the

immunoprecipitation and Western blot analysis studies were performed in triplicate and repeated on three different occasions.

Determination of uPA and uPAR mRNA Expression

Total RNA was isolated using the RNeasy commercial kit (Ambion, Austin, TX) following the manufacturer's protocol. Reverse transcription-polymerase chain reaction (RT-PCR) was performed using a GeneAmp RNA PCR kit and a DNA thermal cycler (Applied Biosystems, Foster City, CA). The primers used in this study were based on previously published primer sequences (Fibbi *et al.*, 2001; Poch *et al.*, 2001; Tabengwa *et al.*, 2001). The primer sequences were as follows: uPA sense 5'-AAATGCTGTGTGCTGTGAC-C-3' and antisense 5'-CCCTGCCCTGAAGTCGTTAGTG-3'; uPAR sense 5'-TGCAGTGTA-AGACCAACGGGG-A-3' and antisense 5'-AATAG-GTGACAG-CCCGGCCAGAGT-3'; and cyclin D1 sense 5'-CTGGAGCCCGT-GAAAAAGAGC-3' and antisense 5'-CTGGAGAGGAAGCGTGTGAGG-3'. The annealing temperature was 60°C for uPA and uPAR and 55°C for cyclin D1, and the number of cycles was 28, 35, and 30 cycles, respectively. Human specific β -actin primers (BD Biosciences Clontech, Palo Alto, CA) were used as positive controls. Ten-microliter aliquots of the products were subjected to electrophoresis on a 1.25% agarose gel, and DNA was visualized by ethidium bromide staining. Location of the products and their sizes were determined by using a 100-base pair ladder (Invitrogen, Carlsbad, CA). The gel was photographed under UV illumination.

siRNA Studies

Synthetic siRNAs for β -catenin and nonspecific control pools were purchased from Upstate Biotechnology (Lake Placid, NY). Colon cancer (SW480) cells were grown in six-well plates to attain ~30–50% confluence. Transfection of the RNA oligonucleotides was performed using Oligofectamine (Invitrogen) to result in a final RNA concentration of 50 nM. Cells were treated either with Oligofectamine (mock transfection), siRNA, or nonspecific RNA pool. A day after transfection, cells were serum starved for the next 24 h and then treated with or without DCA (5 μ M) for 3 h. Cells were lysed in lysis buffer, and Western blot analysis was performed using β -catenin, uPAR, and cyclin D1 antibodies following the procedure described above. In separate experiments, cells were grown on collagen-coated coverslips and transfected with fluorescein isothiocyanate-labeled control oligonucleotides (QIAGEN, Valencia, CA) by using Oligofectamine. Transfection efficiency was calculated by determining the ratio of fluorescing cells to total number of cells per field. Five fields were counted per slide, and the experiment was performed in triplicate. Potential cytotoxic effect of various transfections was determined by assessing lactose dehydrogenase (LDH) release into the cell culture medium using a Cytoscan LDH-cytotoxicity assay kit (Bioworld, Dublin, OH).

Cell Proliferation Assay

The effect of DCA on cell proliferation was studied by determining the incorporation of [3 H]thymidine into cellular DNA. Colon cancer cells (SW480 and LoVo) were grown in 96-well culture plates to attain 70–75% confluence. Cells were serum starved for 24 h and treated with either 1) medium only, 2) DCA (5–100 μ M) for 24 h, 3) uPAR neutralizing antibody (5 μ g/ml), or 4) uPAR neutralizing antibody (5 μ g/ml; 4 h) followed by DCA (5 μ M) treatment for 24 h. Two hours before termination of the experiment, 0.5 μ Ci of [methyl- 3 H]thymidine was added to each well. After incubation, the cells were washed three times with phosphate-buffered saline, collected on glass wool fibers filters by using a multiple sample cell harvester, and the radioactivity was measured.

Colony Formation Assay

Colony formation assay was performed following the protocol described previously (Sheng *et al.*, 2001). In brief, colon cancer (SW480 and LoVo) cells (1×10^4) were suspended in 0.5 ml of 1:2 diluted Matrigel (Collaborative Biotech, Bedford, MA). The cell/Matrigel mixture was plated into 24-well polystyrene plates and incubated at 37°C. The uPAR neutralizing antibody (5 μ g/ml) with or without DCA (5 and 50 μ M) was then added in fresh medium (0.5 ml) every 2 d. After incubating for 15 d, images were captured using a camera attached to an inverted Nikon (TE300) microscope. Total number of colonies in each field was counted under a microscope (magnification 200 $\times$). Five fields were counted per well, and four wells were used for each treatment.

Cell Invasion Assay

Studies were carried out in Transwell chambers as described previously (Sheng *et al.*, 2001). Colon cancer cells (SW480 and LoVo) were serum starved for 24 h. Transwell chambers, equipped with 8- μ m Matrigel-coated filters (24-well format) (BD Biosciences, Franklin Lakes, NJ) were rehydrated, and 4×10^4 cells in 400 μ l of serum-free medium in presence or absence of uPAR antibody (5 μ g/ml, 4 h) was seeded in the upper chamber. Serum-free medium or medium-containing DCA (5 μ M) was used in the lower chamber. After 24-h incubation at 37°C, the cells on the upper surface of the filter were mechanically removed with a cotton swab. The filters were fixed and stained using a Diff-Quick staining kit (Dade Behring, Newark, DE). The cells on the

Figure 1. Dose- and time-response studies of DCA on tyrosine phosphorylation of β -catenin in colon cancer cells. Serum-starved colon cancer (SW480 and LoVo) cells were treated with either medium only (control) (Ct) or DCA in various concentrations (5–100 μ M) to varying time intervals (30 min–2 h). Tyrosine phosphorylation of β -catenin (P- β -cat) was determined by immunoprecipitation (β -catenin monoclonal antibody) and immunoblotting with anti-phosphotyrosine antibody. Total β -catenin in immunoprecipitates was determined by reprobing the same blot with anti- β -catenin antibody. Top, representative blots from three separate experiments performed in triplicate. Bottom, quantitative analysis of tyrosine phosphorylation of β -catenin by determining the ratio between β -catenin protein and tyrosine phosphorylation levels from three separate experiments (mean values $\pm$ SE).

lower surface were counted under a microscope (magnification 100 $\times$). Five fields were counted per each filter, and four wells were used for each treatment.

Statistical Analysis

Student's two-tailed *t* test was used to compare data between two groups. One-way analysis of variance and Bonferroni's correction were used to compare data between three or more groups. Values are expressed as mean $\pm$ SE. *P* values < 0.05 were considered statistically significant.

RESULTS

DCA Increases Tyrosine Phosphorylation of β -Catenin and Elevates Nuclear β -Catenin Levels in Colon Cancer Cells

Dose- and time-dependent studies with DCA demonstrated that treatment of colon cancer cells (SW480 and LoVo) with low concentrations of DCA (5 or 50 μ M) significantly increase tyrosine phosphorylation of β -catenin at 30 min (Figure 1). In contrast, higher concentration of DCA (100 μ M) and longer incubation time (1–2 h) reduced tyrosine phosphorylation of β -catenin (Figure 1). This increase in tyrosine phosphorylation was accompanied by a significant increase in nuclear β -catenin level versus control (Figure 2). To determine whether this effect was due to reduced GSK-3 β phosphorylation (negative regulator of β -catenin), we assessed GSK-3 β phosphorylation levels in response to DCA (5 and 50 μ M, 30 min). DCA did not cause any significant change in GSK-3 β phosphorylation levels (our unpublished data) indicating that in colon cancer cells, DCA-induced β -catenin stabilization and subsequent translocation into the nucleus is induced mostly by its tyrosine phosphorylation rather than by down-regulation of GSK-3 β activation.

DCA Causes Loss of E-Cadherin Binding to β -Catenin in Colon Cancer Cells

Because overexpression of β -catenin and down-regulation of E-cadherin is a hallmark of invasive cancers, next we investigated whether DCA affects E-cadherin binding to β -catenin. Stripping and reprobing of blots used for tyrosine phosphorylation of β -catenin in response to DCA by using anti-E-cadherin antibody demonstrated that treatment with DCA

(5 or 50 μ M, 30 min) causes a substantial loss of E-cadherin binding to β -catenin in colon cancer cells (SW480 and LoVo) (Figure 3) versus control. Similar results were obtained when β -catenin was immunoprecipitated, and immunoblot analysis was performed using E-cadherin antibody. These results indicate that phosphorylated β -catenin no longer associates with E-cadherin, resulting in loss of cell adhesion and acquisition of invasive phenotype.

DCA Induces uPA, uPAR, and Cyclin D1 mRNA and Protein Expression in Colon Cancer Cells

Because uPAR and cyclin D1 is implicated in enhancing cell invasiveness and proliferation, we next sought to determine

Figure 2. DCA increases nuclear β -catenin levels. Colon cancer cells (SW480 and LoVo) were treated with either medium only (control) or DCA (5 and 50 μ M for 30 min). Nuclear extracts were subjected to immunoblotting using anti- β -catenin antibody. Top, representative blots from three separate experiments performed in triplicate. Bottom, quantitative analysis of nuclear β -catenin levels showing relative densities of bands from three separate experiments (mean values $\pm$ SE).

Figure 3. DCA causes loss of E-cadherin association with β -catenin. Blots that were used to determine tyrosine phosphorylation of β -catenin in response to DCA were stripped and reprobed by using anti-E-cadherin antibody and subsequently reprobed with anti- β -catenin antibody. Top, representative blots from three separate experiments performed in triplicate. Bottom, quantitative analysis of E-cadherin bound to β -catenin after various treatments by determining the ratio between E-cadherin and total β -catenin levels from three separate experiments (mean values $\pm$ SE).

whether DCA induces uPA, uPAR, and cyclin D1 mRNA and protein expression in colon cancer cells. RT-PCR and Western blot analyses demonstrated that DCA treatment substantially increases uPA, uPAR, and cyclin D1 mRNA (Figure 4, A and B), and protein expression in both cell lines studied versus controls. Low concentration of DCA (5 μ M) significantly increased uPA, uPAR, and cyclin D1 mRNA expression in both cell types studied. Quantitative analysis of uPA, uPAR, and cyclin D1 protein expression demonstrated that 5 μ M DCA causes substantial increase in expressions of these proteins as early as 3 h after incubation and persists up to 6 h.

β -Catenin siRNA Treatment Significantly Reduces DCA-stimulated uPAR and Cyclin D1 Expression

Because DCA induced uPAR and cyclin D1 mRNA and protein expression and these genes are identified as transcriptional targets of β -catenin/TCF, next we sought to determine whether DCA targets uPAR and cyclin D1 via β -catenin. Treatment of colon cancer cells (SW480) with siRNA targeted for β -catenin significantly inhibited β -catenin protein expression (vs. nonspecific RNA-treated controls). Furthermore, DCA (5 μ M, 3 h) treatment of colon cancer cells pretreated with β -catenin siRNA significantly suppressed DCA-induced uPAR and cyclin D1 protein expression (Figure 5) versus nonspecific RNA treated controls, indicating that DCA increases colon cancer cell growth and invasiveness by targeting uPAR and cyclin D1. Transfection efficiency as determined by fluorescein isothiocyanate-labeled oligonucleotide uptake was $89 \pm 4\%$. Treatment of SW480 cells either with Oligofectamine (mock transfection) ($7.2 \pm 1.67\%$ cytotoxicity), siRNA ($7.21 \pm 2.31\%$), or nonspecific RNA ($6.91 \pm 2.11\%$) did not show any significant cytotoxic effect compared with untreated cells (control) ($6.49 \pm 2.3\%$).

Role of uPAR in DCA-induced Colon Cancer Cell Proliferation

Because DCA treatment caused a significant increase in uPA and uPAR expression in colon cancer cells, and uPA and uPAR have been implicated to play crucial roles in tumorigenesis, next we examined the role of uPAR in DCA-mediated increase in colon cancer cell proliferation. Treatment of colon cancer cells (SW480 and LoVo) with a low concentration of DCA (5 μ M; 24 h) significantly increased cell proliferation as reflected by increased uptake of [3 H]thymidine into cellular DNA (Figure 6). Higher concentration of DCA (100 μ M) substantially reduced cell proliferation (Figure 6). Treatment of cells with 100 μ M DCA caused significant cytotoxicity as reflected by a marked increase in LDH released into the cell culture medium ($32.1 \pm 2.57\%$ cytotoxicity; $p = 0.0002$) compared with untreated controls ($6.49 \pm 2.3\%$). Pretreatment of colon cancer cells with uPAR neutralizing antibody caused a modest decrease in DCA-induced (5 μ M) [3 H]thymidine uptake (Figure 6), indicating its relatively lesser role in cell proliferation.

Low Concentration of DCA Stimulates Colon Cancer Cell Invasiveness

Cancer cell invasiveness was assessed using in vitro cell invasion and colony formation assays (Sheng *et al.*, 2001). Low concentration of DCA (5 μ M, 24 h) caused a moderate increase in LoVo cell invasiveness; however, it caused a significant increase in SW480 cells invading through the Matrigel and the porous polycarbonate filters (Figure 7A). Blocking of uPAR with a neutralizing antibody significantly reduced DCA-induced cancer cell invasiveness (Figure 7A). We further characterized cell invasiveness by examining their ability to form colonies in Matrigel. Treatment of SW480 and LoVo cells with a low concentration of DCA (5 μ M, 15 d) markedly increased the number and size of colonies formed in Matrigel (Figure 7, B and C). Higher concentrations of DCA (>50 μ M) did not allow any colony formation in Matrigel with both cell lines studied. Whereas neutralization of uPAR with a specific antibody moderately suppressed colony formation at basal levels (Figure 7, B and C), it significantly reduced the number and size of DCA-stimulated colonies in Matrigel (Figure 7, B and C).

DISCUSSION

The mechanisms of tumor-promoting actions of secondary bile acids remain poorly understood. In this study, we demonstrate for the first time that physiologically relevant concentrations (5 or 50 μ M) of DCA activate β -catenin signaling pathway and increases uPA, uPAR, and cyclin D1 expression in colon cancer cells. Inhibition of β -catenin expression by using siRNA targeted for β -catenin significantly suppressed DCA-induced uPAR and cyclin D1 expression. Furthermore, blocking of uPAR activation with a neutralizing antibody significantly suppressed DCA-mediated colon cancer cell colony formation in Matrigel and reduced the number of invasive cells. On the basis of these data, we propose the following sequence of events. Deoxycholic acid-induced tyrosine phosphorylation stabilizes and translocates β -catenin into the nucleus and stimulates uPA, uPAR, and cyclin D1 expression. Tyrosine phosphorylation dissociates β -catenin from E-cadherin and thus induces loss of cell adhesion. uPA/uPAR-mediated proteolytic degradation of extracellular matrix accompanied by cyclin D1/uPA/uPAR-induced cell proliferation enhances colon cancer growth and progression.

Figure 4. DCA induces uPA, uPAR, and cyclin D1 mRNA and protein expression in colon cancer cells. Top, serum-starved SW480 (A) and LoVo (B) cells were treated either with medium only (control) (Ct) or DCA (5 and 50 μ M) for 2 h. Total RNA was isolated and subjected to RT-PCR by using specific primers for uPA, uPAR, cyclin D1, and β -actin. DNA was visualized by ethidium bromide staining. Middle, serum-starved SW480 and LoVo cells were treated either with medium only (control) (Ct) or DCA (5 μ M) for 3–24 h. Equal amounts of protein (0.1 mg) were subjected to 10% SDS-PAGE and Western blot analysis performed using uPA-, uPAR-, and cyclin D1-specific antibodies. Representative blots from two separate experiments performed in triplicate. Bottom, quantitative analysis of relative densities of bands of uPA, uPAR, and cyclin D1 proteins normalized to β -actin levels from three separate experiments (mean values $\pm$ SE).

Loss of cell adhesion and deregulated cell migration facilitates tumor invasion and metastasis. The tumor suppressing action of APC protein is dependent on its interaction with β -catenin, and loss of functional APC protein results in the accumulation of β -catenin. Cytosolic accumulation and subsequent activation of β -catenin signaling are known to facilitate tumor growth and metastasis (Alexander *et al.*, 2002). Reduced binding of E-cadherin to β -catenin has been shown to accompany loss of intercellular adhesions and acquisition of an invasive phenotype (Hiscox and Jiang, 1999). These findings corroborate our data showing that

DCA causes a significant loss of E-cadherin binding with β -catenin that is associated with an increase in cancer cell invasiveness as reflected by increase in the number of migrating cells, and enhanced number and size of colonies formed in the Matrigel. In normal cells, free cytosolic β -catenin is rapidly phosphorylated by a multiprotein complex that includes APC, axin/conductin, and GSK-3 β . The latter phosphorylates serine and threonine residues on β -catenin, a crucial step required to target the protein for ubiquitination and proteasomal degradation (Brabletz *et al.*, 2001). In our present study, we did not find any significant changes in

Figure 5. β -Catenin siRNA treatment significantly reduces DCA-stimulated uPAR protein expression. Colon cancer (SW480) cells were transfected with either siRNA directed against β -catenin or nonspecific RNA by using Oligofectamine. Twenty-four hours after transfection, cells were serum starved for the next 36 h and then treated with or without DCA (5 μ M) for 3 h. Cells were lysed in lysis buffer and β -catenin, uPAR, cyclin D1, and β -actin protein expressions were assessed by Western blot analysis by using specific antibodies. This figure is a representative blot from two separate experiments performed in duplicate.

GSK-3 β phosphorylation in response to DCA treatment (our unpublished data), indicating that DCA regulates β -catenin signaling in colon cancer cells independently of GSK-3 β .

Colon cancers display elevated expression of uPAR in the invasive front (Pyke *et al.*, 1991) as well as reduced membranous and increased nuclear β -catenin expression during transition of colorectal adenoma to carcinoma (Hao *et al.*, 1997), suggesting a possible correlation between increased uPAR and β -catenin activation. Also, binding of uPA to

uPAR has been shown to activate cell surface-based plasminogen and to generate proteolytic cascade important for matrix degradation and promotion of tumor invasion (Andreassen *et al.*, 1997). Our study showing that neutralization of uPAR with a specific antibody greatly suppresses DCA-induced cell invasiveness and colony formation in Matrigel supports the above-mentioned contention. In addition, we found that blocking of uPAR suppresses DCA-induced colon cancer cell proliferation, which indicates that uPAR may play important role in cell growth as well as in invasiveness. Other studies indicate that bile acids (lithocholic acid) may also promote colon cancer growth and invasiveness by using other signaling pathways involving matrix metalloproteinases and RhoA/Rho-kinase pathways (Halvorsen *et al.*, 2000; Debruyne *et al.*, 2002).

Overexpression of β -catenin has been shown to increase uPAR expression, but the mechanism of this action is unclear. It has been suggested that oncoproteins whose promoters bear TCF binding sites (*c-jun* and *fra-1*) may be involved in mediating this action of β -catenin (Mann *et al.*, 1999). Our current data show that inhibition of β -catenin reduces uPAR expression and that blocking uPAR suppresses DCA-induced colony formation and reduces number of invasive cells. This clearly indicates that bile acids increase metastatic potential of both noninvasive (SW480) and invasive (LoVo) colon cancer cells mostly by targeting uPAR via β -catenin.

Cyclin D1, which regulates progression of cells into the proliferative stage of the cell cycle, plays a key role in tumorigenesis. Overexpression of cyclin D1 in colorectal cancers has been reported (Bartkova *et al.*, 1994; Arber *et al.*, 1996). Conversely, inhibition of cyclin D1 by using antisense cyclin D1 cDNA has been shown to suppress tumor growth in nude mice (Arber *et al.*, 1997). Furthermore, of relevance to our study, cyclin D1 gene has been reported as a target of the β -catenin/lymphocyte enhancer factor 1 (Shtutman *et al.*, 1999). Our present data show that DCA significantly increases cyclin D1 expression and that inhibition of β -catenin by using siRNA greatly reduces DCA-induced cyclin D1 expression. This suggests that DCA

Figure 6. Effect of DCA on colon cancer cell proliferation. Serum-starved colon cancer cells were treated with either 1) medium only, 2) varying concentrations of DCA (5–100 μ M) for 24 h, 3) uPAR neutralizing antibody, or 4) uPAR neutralizing antibody (4 h) followed by DCA (5 μ M, 24 h). Two hours before harvesting, the individual wells were pulsed with [³H]thymidine (0.5 μ Ci), washed with phosphate-buffered saline, and collected on glass wool filters by using a multiple sample cell harvester, and the radioactivity was measured. Values are presented as mean $\pm$ SE of three individual experiments performed in quadruplicate.

Figure 7. DCA enhances colon cancer cell invasiveness. (A) Serum-starved SW480 and LoVo cells were pretreated with or without uPAR neutralizing antibody, and 4×10^4 cells were seeded in the upper chamber in the presence or absence of uPAR antibody. Medium alone or medium containing DCA was added to the lower chamber. After 24 h, cells on the upper surface of the filter were removed. Filters were fixed and stained. The cells on the lower surface were counted under a microscope (magnification 100 $\times$). Five fields were counted per each filter, and four wells were used for each treatment. (B) Serum-starved SW480 and LoVo cells (1×10^4) were suspended in 0.5 ml of 1:2 diluted Matrigel. The cell/Matrigel mixture was plated into 24-well plates and incubated at 37°C. The medium containing uPAR neutralizing antibody (5 μ g/ml) with or without DCA (5 μ M) was then added in fresh serum-free medium every 2 d. Four wells were used for each treatment. After 15 d, images were captured using a camera attached to an inverted

uses β -catenin-cyclinD1 pathway to promote colon cancer growth.

Both genetic and environmental factors play an important role in the development of colorectal carcinogenesis. Loss of APC function due to mutations in the APC gene has been shown to affect the regulation of β -catenin-mediated transcription with resulting uninhibited increase in cell proliferation (Polakis, 1997). We found that DCA increases tyrosine phosphorylation and nuclear β -catenin levels in colon cancer cells and enhances cell proliferation and invasiveness. This indicates that β -catenin is an important molecular target of growth-promoting actions of DCA. The increase in tyrosine phosphorylation of β -catenin and expression of uPA, uPAR, and cyclin D1 associated with enhanced colon cancer cell growth and invasiveness demonstrated in this study were achieved with concentrations of DCA that are present in the human colonic lumen, and in the portal circulation (10–20 μ M) (Stadler *et al.*, 1988; Korpela *et al.*, 1988; Moseley, 1999). In the present study, higher concentrations of DCA (100 μ M) significantly reduced tyrosine phosphorylation of β -catenin, colon cancer cell proliferation, and almost completely inhibited colony formation in the Matrigel. This effect of higher concentration of DCA (100 μ M) was due to cytotoxicity as reflected by a significant increase in LDH release. Our data are in agreement with previous reports of increased colon cancer cell proliferation in response to low concentrations of DCA (<50 μ M) and increased apoptosis in response to higher concentrations of DCA (>100 μ M) (Peiffer *et al.*, 1997; Milovic *et al.*, 2002).

Although our present study demonstrates that DCA regulates β -catenin signaling, the mechanism of this action remains unknown. Previous studies using primary hepatocytes and cholangiocarcinoma cell line have demonstrated that DCA activates epidermal growth factor receptor (Qiao *et al.*, 2001; Yoon *et al.*, 2002). Growth factor receptors, including epidermal growth factor receptor and c-met (hepatocyte growth factor receptor), are known to associate and induce tyrosine phosphorylation of β -catenin (Alexander *et al.*, 2002; Pai *et al.*, 2003). Therefore, DCA-mediated activation of β -catenin signaling pathway in colon cancer cells may involve epidermal growth factor receptor and/or c-met.

In summary, our study provides evidence that secondary bile acids may promote colon cancer growth and invasion/metastasis through the activation of β -catenin-cyclin D1 and -uPAR signaling pathway.

ACKNOWLEDGMENTS

This work was supported by the Department of Veterans Affairs Medical Research Service Merit Review and Research Enhancement Awards (to A.S.T.).

microscope. Quantitative analysis of the number of colonies formed in Matrigel after various treatments. Values are presented as mean $\pm$ SE number of colonies from five fields of each of four wells (total 20 fields) after each treatment. (C) Representative image of cancer cell colonies from one of the five fields captured from each well. Compared with nonstimulated control, DCA (5 μ M) treatment significantly increased the number and size of the cancer cell colonies formation in Matrigel. Whereas pretreatment of colon cancer cells with uPAR neutralizing antibody (5 μ g/ml, 4 h) moderately suppressed the number and size of colonies formed at basal levels, it significantly reduced the number and size of colonies formed in response to DCA (5 μ M) treatment. Bar, 50 μ M.

REFERENCES

- Alexander, N., Wong, C., and Pignatelli, M. (2002). β -Catenin – a linchpin in colorectal carcinogenesis? *Am. J. Pathol.* 160, 389–401.
- Andreasen, P.A., Kjoller, L., Christensen, L., and Duffy, M.J. (1997). The urokinase-type plasminogen activator system in cancer metastasis: a review. *Int. J. Cancer* 72, 1–22.
- Andrews, N.C., and Faller, D.V. (1991). A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucleic Acid Res.* 19, 2499.
- Arber, N., Doki, Y., Han, E.K.-H., Sgambato, A., Zhou, P., Kim, N.-H., Delohery, T., Klein, M.G., Holt, P.R., and Weinstein, I.B. (1997). Antisense to cyclin D1 inhibits the growth and tumorigenicity of human colon cancer cells. *Cancer Res.* 57, 1569–1574.
- Arber, N., Hibshoosh, H., Moss, S.F., Sutter, T., Zhang, Y., Begg, M., Wang, S., Weinstein, I.B., and Holt, P.R. (1996). Increased expression of cyclin D1 is an early event in multistage colorectal carcinogenesis. *Gastroenterology* 110, 669–674.
- Armstrong, B., and Doll, R. (1975). Environmental factors and cancer incidence and mortality in different countries, with special reference to dietary practices. *Int. J. Cancer* 5, 617–631.
- Bartkova, J., Lukas, J., Struss, M., and Bartek. (1994). The PRAD-1/cyclin D1 oncogene product accumulates aberrantly in a subset of colorectal carcinomas. *Int. J. Cancer* 58, 568–573.
- Bayerdorffer, E., Mannes, G.A., Ochsenkuhn, T., Dirschedl, P., Wiebecke, B., and Paumgartner, G. (1995). Unconjugated secondary bile acids in the serum of patients with colorectal adenomas. *Gut* 36, 268–273.
- Ben-Ze'v, A., and Geiger, B. (1998). Differential molecular interactions of β -catenin and plakoglobin in adhesion, signaling and cancer. *Curr. Opin. Cell Biol.* 10, 629–639.
- Brabletz, T., Jung, A., Reu, S., Porzner, M., Hlubek, F., Kunz-Schughart, L.A., Knuechel, R., and Kirchner, T. (2001). Variable β -catenin expression in colorectal cancers indicates tumor progression driven by the tumor environment. *Proc. Natl. Acad. Sci. USA* 98, 10356–10361.
- Breuer, N., and Goebell, H. (1985). The role of bile acids in colonic carcinogenesis. *Klin. Wochenschr.* 63, 97–105.
- Cohen, B.I., and Raicht, R.F. (1981). Effects of bile acids on colon carcinogenesis in rats treated with carcinogens. *Cancer Res.* 41, 3759–3760.
- Comoglio, P.M., and Trusolino, L. (2002). Series introduction: invasive growth: from development to metastasis. *J. Clin. Investig.* 109, 857–862.
- Debruyne, P.R., Bruyneel, E.A., Karaguni, I.M., Li, X., Flatau, G., Muller, O., Zimmer, A., Gespach, C., and Mareel, M.M. (2002). Bile acids stimulate invasion and haptotaxis in human colorectal cancer cells through activation of multiple oncogenic signaling pathways. *Oncogene* 21, 6740–6750.
- Ellis, V., Pyke, C., Eriksen, J., Solberg, H., and Dano, K. (1992). The urokinase receptor: involvement in cell surface proteolysis and cancer invasion. *Ann. N.Y. Acad. Sci.* 667, 13–31.
- Fibbi, G., Barletta, E., Dini, G., Del Rosso, A., Pucci, M., Cerletti, M., and Del Rosso, M. (2001). Cell invasion is affected by differential expression of the urokinase plasminogen activator/urokinase plasminogen activator receptor system in muscle satellite cells. *Lab. Investig.* 81, 27–39.
- Ganesh, S., Sier, C.F., Heering, M.M., Griffioen, G., Lamers, C.B., and Verspaget, H.W. (1994). Urokinase receptor and colorectal cancer survival. *Lancet* 344, 401–401.
- Halvorsen, B., Staff, A.C., Ligaarden, S., Prydz, K., and Kolset, S.O. (2000). Lithocholic acid and sulphated lithocholic acid differs in the ability to promote matrix metalloproteinase secretion in the human colon cancer cell line Caco-2. *Biochem. J.* 349, 189–193.
- Hao, X.P., Tomlinson, I.P., Ilyas, M., Palazzo, J.P., and Talbot, I.C. (1997). Reciprocity between membranous and nuclear expression of β -catenin in colorectal tumors. *Virchows Arch.* 431, 167–172.
- Hiscox, S., and Jiang, W.G. (1999). Hepatocyte growth factor/scatter factor disrupts epithelial tumor cell-cell adhesion: involvement of β -catenin. *Anticancer Res.* 19, 509–518.
- Hoffman, A.F. (1989). Enterohepatic circulation of bile acids. In: *Handbook of Physiology, Section on the Gastrointestinal System*, ed. S.G. Schultz, Bethesda, MD: American Physiological Society, 567–596.
- Jacoby, R.F., Marshall, D.J., Kailas, S., Schlack, S., Harms, B., and Love, R. (1995). Genetic instability associated with adenoma to carcinoma progression in hereditary nonpolyposis colon cancer. *Gastroenterology* 109, 73–82.
- Kinzler, K.W., and Vogelstein, B. (1996). Lessons from hereditary colorectal cancer. *Cell* 87, 159–170.
- Korpela, J.T., Adlercreutz, H., and Turunen, M.J. (1988). Fecal free and conjugated bile acids and neutral sterols in vegetarians, omnivores, and patients with colorectal cancer. *Scand. J. Gastroenterol.* 23, 277–283.
- Mann, B., et al. (1999). Target genes of β -catenin-T cell-factor/lymphoid-enhancer-factor signaling in human colorectal carcinomas. *Proc. Natl. Acad. Sci. USA* 96, 1603–1608.
- Milovic, V., Teller, I.C., Faust, D., Caspary, W.F., and Stein, J. (2002). Effects of deoxycholate on human colon cancer cells: apoptosis or proliferation. *Eur. J. Clin. Investig.* 32, 29–34.
- Morin, P.J. (1999). β -catenin signaling and cancer. *Bioessays* 21, 1021–1030.
- Moseley, R.H. (1999). Bile secretion. In: *Textbook of Gastroenterology*, 3rd ed. Chapter 17, ed. T. Yamada, Philadelphia: Lippincott Williams & Wilkins, 380–403.
- Pai, R., Nakamura, T., Moon, W.S., and Tarnawski, A.S. (2003). Prostaglandins promote colon cancer cell invasion: signaling by cross-talk between two growth factor receptors. *FASEB J.* 17, 1640–1647.
- Pai, R., Soreghan, B., Szabo, I.L., Pavelka, M., Baatar, D., and Tarnawski, A.S. (2002). Prostaglandin E_2 transactivates EGF receptor and triggers mitogenic signaling in gastric mucosa and colon cancer cells. A novel mechanism for colon cancer growth and gastrointestinal hypertrophy. *Nat. Med.* 8, 289–293.
- Peifer, M. (1997). β -Catenin as oncogene: the smoking gun. *Science* 275, 1752–1753.
- Peiffer, L.P., Peters, D.J., and McGarrity, T.J. (1997). Differential effects of deoxycholic acid on proliferation of neoplastic and differentiated colonocytes *in vitro*. *Dis. Dig. Sci.* 42, 2234–2240.
- Poch, B., Gansauge, F., Schwarz, A., Seufferlein, T., Schnelldorfer, T., Ramadani, M., Beger, H.G., and Gansauge, S. (2001). Epidermal growth factor induces cyclin D1 in human pancreatic carcinoma: evidence for a cyclin D1-dependent cell cycle progression. *Pancreas* 23, 280–287.
- Polakis, P. (1997). The adenomatous polyposis coli (APC) tumor suppressor. *Biochim. Biophys. Acta* 1332, F127–F147.
- Polakis, P. (2000). Wnt signaling and cancer. *Genes Dev.* 14, 1837–1851.
- Pyke, C., Kristensen, P., Ralfkiaer, E., Grondahl-Hansen, J., Eriksen, J., Blasi, F., and Dano, K. (1991). Urokinase-type plasminogen activator is expressed in stromal cells and its receptor in cancer cells at invasive foci in human colon adenocarcinoma. *Am. J. Pathol.* 138, 1059–1067.
- Qiao, L., et al. (2001). Deoxycholic acid (DCA) causes ligand-independent activation of epidermal growth factor receptor (EGFR) and FAS receptor in primary hepatocytes: inhibition of EGFR/mitogen-activated protein kinase-signaling module enhances DCA-induced apoptosis. *Mol. Biol. Cell* 12, 2629–2645.
- Reddy, B.S. (1992). Dietary fat and colon cancer: animal model studies. *Lipids* 27, 807–813.
- Rogers, A.E., Zeisel, S.H., and Groopman, J. (1993). Diet and carcinogenesis. *Carcinogenesis* 14, 2205–2217.
- Sheng, H., Shao, J., Washington, M.K., and DuBois, R.N. (2001). Prostaglandin E_2 increases growth and motility of colorectal carcinoma cells. *J. Biol. Chem.* 276, 18075–18081.
- Shutman, M., Zhurinsky, J., Simcha, I., Albanese, C., D'Amico, M., Pestell, R., and Ben-Ze'v, A. (1999). The cyclin D1 gene is a target of the β -catenin/Lef-1 pathway. *Proc. Natl. Acad. Sci. USA* 96, 5522–5527.
- Stadler, J., Yeung, K.S., Furrer, R., Marcon, N., HIMAL, H.S., and Bruce, W.R. (1988). Proliferative activity of rectal mucosa and soluble fecal bile acids in patients with normal colons and in patients with colonic polyps or cancer. *Cancer Lett.* 38, 315–320.
- Tabengwa, E.M., Grenett, H.E., Benza, R.L., Abou-Agag, L.H., Tresnak, J.K., Wheeler, C.G., and Booyse, F.M. (2001). Ethanol-induced up-regulation of the urokinase receptor in cultured human endothelial cells. *Alcohol Clin. Exp. Res.* 25, 163–170.
- Yoon, J.H., Higuchi, H., Werneburg, N.W., Kaufmann, S.H., and Gores, G.J. (2002). Bile acids induce cyclooxygenase-2 expression via the epidermal growth factor receptor in a human cholangiocarcinoma cell line. *Gastroenterology* 122, 985–993.
- Yu, W., Kim, J., and Ossowski, L. (1997). Reduction in surface urokinase receptor forces malignant cells into a protracted state of dormancy. *J. Cell Biol.* 137, 767–775.