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

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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 μM) 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).

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 tumorogenesis as well as 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 deoxicholic 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 expression 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 experiments were performed within 10 passages after obtaining the cell lines. Serum-starved cells were incubated with DCA (5–100 μM; 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; 2–24 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 μg/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 μg) were resolved on 7.5% SDS-PAGE and immunoblotted following the procedure described 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 μg/ml leupeptin, 5 μg/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× 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)-3β and GSK-3β antibodies from Cell Signaling Technologies (Beverly, MA). 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 RNAqueous 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′-AAATCTGTGTCGTGTAAGC-3′ and antisense 5′-CTGGCTCCCTGAGAAT-3′; uPAR sense 5′-TGCAAGTGTAGAAGCCACCCGGG-3′ and antisense 5′-AATAGGTTGACAGCCCCGCAAGAT-3′; and cyclin D1 sense 5′-CTGGAGCCCGCTGAAAAGACG-3′ and antisense 5′-CTGGAGACAGACCGCTGAGG-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 cells (SW480) 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 24 h and were transfected with or without DCA (5 μM) for 5 h. Cells were lysed in lysis buffer, and Western blot analysis was performed using β-catenin, uPAR, and cyclin D1 antibodies following the protocol described above. For the proliferation 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 lactate dehydrogenase (LDH) release into the cell culture medium using a CytoSens LDH-toxicity assay kit (Bioloworld, Dublin, OH).

**Cell Proliferation Assay**

The effect of DCA on cell proliferation was studied by determining the incorporation of [3H]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 μM) for 24 h, 3) uPAR neutralizing antibody (5 μg/ml), or 4) uPAR neutralizing antibody (5 μg/ml) followed by DCA (5 μM) treatment for 4 h. Two hours before termination of the experiment, 0.5 μCi of [3H]thymidine was added to each well. After incubation, the cells were washed three times with phosphate-buffered saline, collected on glass wool filters by using a multiple sample cell harvester, and the radioactivity was measured.

**Colony Formation Assay**

Colony formation assay was performed after the protocol described previously (Sheng et al., 2001). In brief, colon cancer (SW480 and LoVo) cells (1 × 10^5) were suspended in 0.5 ml of 1:2 diluted Matrigel (Collaborative Research, Bedford, MA). The cell/Matrigel mixture was plated into 24-well polystyrene plates and incubated at 37°C. The uPAR neutralizing antibody (5 μg/ml) with or without DCA (5 and 50 μM) was then added in fresh medium. 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 grown in six-well plates to attain 70–75% confluence. Cells were serum starved for 24 h. Transwell chambers, equipped with 8-μm Matrigel-coated filters (24-well format) (BD Biosciences, Franklin Lakes, NJ) were rehydrated, and 4 × 10^5 cells in 400 μl of serum-free medium in presence or absence of uPAR neutralizing antibody (5 μg/ml, 4 h) were seeded in the upper chamber. Serum-free medium or medium-containing DCA (5 μM) 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
lower surface were counted under a microscope (magnification 100×). 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 ± 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 μM) significantly increase tyrosine phosphorylation of β-catenin at 30 min (Figure 1). In contrast, higher concentration of DCA (100 μM) 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 levels versus control (Figure 2). To determine whether this effect was due to reduced GSK-3β phosphorylation (negative regulator of β-catenin), we assessed GSK-3β phosphorylation levels in response to DCA (5 and 50 μM, 30 min). DCA did not cause any significant change in GSK-3β 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-3β 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 μM, 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...
whether DCA induces uPA, uPAR, and cyclin D1 mRNA and protein expression in colon cancer cells. RT-PCR and Western blot analysis demonstrated that DCA treatment substantially increases uPAR, uPAR, and cyclin D1 mRNA expression (Figure 4, A and B), and protein expression in both cell lines studied versus controls. Low concentration of DCA (5 μM) significantly increased uPAR, uPAR, and cyclin D1 mRNA expression in both cell types studied. Quantitative analysis of uPA, uPAR, and cyclin D1 protein expression demonstrated that 5 μM DCA causes substantial increase in expression 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 μM, 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 ± 4%. Treatment of SW480 cells either with Oligofectamine (mock transfection) (7.2 ± 1.67% cytotoxicity), siRNA (7.21 ± 2.31%), or nonspecific RNA (6.91 ± 2.11%) did not show any significant cytotoxic effect compared with untreated cells (control) (6.49 ± 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 μM, 24 h) significantly increased cell proliferation as reflected by increased uptake of [3H]thymidine into cellular DNA (Figure 6). Higher concentration of DCA (100 μM) substantially reduced cell proliferation (Figure 6). Treatment of cells with 100 μM DCA caused significant cytotoxicity as reflected by a marked increase in LDH released into the cell culture medium (32.1 ± 2.57% cytotoxicity; p = 0.0002) compared with untreated controls (6.49 ± 2.3%). Pretreatment of colon cancer cells with uPAR neutralizing antibody caused a modest decrease in DCA-induced cell proliferation (5 μM) [3H]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 μM, 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 μM, 15 d) markedly increased the number and size of colonies formed in Matrigel (Figure 7, B and C). Higher concentrations of DCA (>50 μM) 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 μM) 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.
Loss of cell adhesion and deregulated cell migration facilitates tumor invasion and metastasis. The tumor suppressive 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-3β. 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 4](image-url). 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) (C) or DCA (5 and 50 μM) 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) (C) or DCA (5 μM) 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 ± SE).
GSK-3β phosphorylation in response to DCA treatment (our unpublished data), indicating that DCA regulates β-catenin signaling in colon cancer cells independently of GSK-3β.

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 oncogenes 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 N 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 7. DCA enhances colon cancer cell invasiveness. (A) Serum-starved SW480 and LoVo cells were pretreated with or without uPAR neutralizing antibody, and $4 \times 10^5$ 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 100x). Five fields were counted per each filter, and four wells were used for each treatment. (B) Serum-starved SW480 and LoVo cells ($1 \times 10^5$) 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 $\mu$g/ml) with or without DCA (5 $\mu$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 microscope. Quantitative analysis of the number of colonies formed in Matrigel after various treatments. Values are presented as mean ± 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 $\mu$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 $\mu$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 $\mu$M) treatment. Bar, 50 $\mu$M.

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