Acidified bile acids increase hTERT expression via c-myc activation in human gastric cancer cells

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Abstract. Human telomerase reverse transcriptase (hTERT) is upregulated in most cancer cell types as well in immortalized cells. The underlying mechanism for such upregulation, however, remains largely unknown. We report here that bile acids under acidified media increase hTERT expression via c-myc activation in primary human gastric cancer cell lines. Human gastric cancer MKN28, MGC803 and SGC7901 cells were treated with 100 μM deoxycholic acid (DCA) or chenodeoxycholic acid (CDCA) with or without acidified media in the presence or absence of the c-myc inhibitor 10058-F4 for 24 h. hTERT and c-myc protein levels were determined by western blot analysis. hTERT and c-myc mRNA levels were determined by RT-PCR. The promoter activities of hTERT and c-myc transcription were determined using promoter reporter luciferase assays for both. Telomerase enzyme activity was analyzed by stretch PCR. hTERT mRNA and protein levels were significantly increased by bile acids in acidified media and were accompanied with enhanced telomerase activity. No changes were found at a pH of 7.0 or with acidified media alone. Similarly, the mRNA and protein levels of c-myc were also increased by bile acids in acidified media but not at a pH of 7.0 or with acidified media alone. Importantly, pharmacologic inhibition of c-myc using 10058-F4 prevented hTERT induction by DCA or CDCA in gastric cancer cells under acidic conditions. Bile acids (DCA and CDCA) under acidic conditions increased hTERT expression in human gastric cancer cells by activation of c-myc transcription. This suggests that acidified bile acids may promote tumorigenesis and affect cell ageing via telomerase activation.

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Introduction

The human telomerase complex consists of an RNA component (hTR), which acts as a template for the telomeric repeat synthesis and human telomerase reverse transcriptase (hTERT) (1). Telomerase activity is closely correlated with cell proliferation and is required for the unlimited proliferation potential of cancer cells (2). Active telomerase is found in ~80% of cancer cells and rapidly proliferating tissues during early development (3). Increased hTERT expression leads to enhanced telomerase activity, which in turn, affects cell ageing, cell cycle progression and tumorigenesis (4). As such, targeting hTERT represents a promising strategy for the development of anticancer drugs.

Reflux of duodenal contents has been shown to be involved in the pathogenesis of Barrett's esophagus (5,6). Moreover, bile acid was found to promote intestinal metaplasia and gastric carcinogenesis (7). For example, DCA and CDCA, the main constituents of the refluxate (pH 4.0-7.0) (8), have been reported to promote esophageal carcinogenesis likely by activating the tuberous sclerosis complex 1/mammalian target of rapamycin (TSC1/mTOR) or nuclear factor κB (NF-κB) pathways (9,10). Bile acids under acidified media were recently reported to upregulate the expression of the proto-oncogene c-myc in Barrett's metaplasia and esophageal adenocarcinoma (11). c-myc is a gene transcription factor and upregulated in many types of cancer. Among the well-documented direct targets of c-myc is hTERT (12). Expression of c-myc in human epithelial cells and fibroblasts induces the transcription of endogenous hTERT; thereby activating telomerase (4). Hence, we hypothesized that acidified bile acids may upregulate hTERT gene transcription by activating c-myc in gastric cancer cells.

In the present study, we investigated the effects of acidified bile acids (pH 5.5) on hTERT gene expression and telomerase activity in gastric adenocarcinoma cells and explored the underlying molecular mechanisms. We found that acidified bile acids enhanced the expression and activity of hTERT by increasing c-myc levels in gastric adenocarcinoma cells.

Materials and methods

Reagents. DCA, CDCA and small-molecular-weight c-myc inhibitor (10058-F4; [Z,E]-5-[4-ethylbenzylidine]-2-thioxothiazolidin-4-one) were obtained from Sigma (St. Louis, MO, USA) and dissolved in dimethylsulfoxide (DMSO). Rabbit monoclonal antibodies specific for hTERT and c-myc were obtained from Sigma. TurboFect™ Transfection reagent was acquired from Thermo Fisher. Dual-Glo® Luciferase Assay system was from Promega. RNeasy total RNA extraction kit was from Qiagen (Hilden, Germany). Takara *Taq* DNA polymerase was from Takara Bio (Dalian, China).

Cell culture and treatment. Human gastric cancer cell lines MKN28, MGC803 and SGC7901 were maintained in RPMI-1640 medium supplemented with 10% calf fetal serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin. The three cell lines were treated as follows: i) acidified growth media at pH 5.5 only; ii) acidified growth media at pH 5.5 containing 100 μM DCA; iii) growth media at pH 7.0 containing 100 μM DCA; iv) acidified growth media at pH 5.5 containing 100 μM CDCA; and v) growth media at pH 7.0 containing 100 μM CDCA. Cells were stimulated continuously for 12 or 24 h and then incubated in neutral growth media (growth media at pH 7.0) for 3 h. For inhibitor assays, cells were pretreated with inhibitors for 24 h before exposure to acidified growth media containing bile acids. All experiments were performed in triplicate.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR). Cells were harvested after treatment and total RNA was extracted with an RNA extraction kit from Qiagen. Total RNA was first transcribed into cDNA in 25 μl of reagent mix, 1 μl of which was amplified by PCR in a 25 μl reaction using Takara *Taq* DNA polymerase with 0.5 μl primers (20 μM). Primers for hTERT were: (forward) 5'-ATC AGACAGCACTTGAAGAG-3' and (reverse) 5'-GTAGTC CATGTTCACAATCG-3'; for c-myc, (forward) 5'-TCAAGA GGTGCCACGTCTCC-3' and (reverse) 5'-TCTTGGCAGCA GGATAGTCCTT-3'. The PCR reaction mixture was denatured at 94˚C for 3 min followed by 30 cycles at 94˚C for 30 sec, 51˚C (TERT) and 61˚C (c-myc) for 30 sec, and 72˚C for 60 sec. Primers for β-actin, (forward) 5'-CTTAGCACCCCTGGC CAAG-3', (reverse) 5'-GATGTTCTGGAGAGCCCCG-3' were used to amplify β-actin as an internal control. Primers were synthesized by Sangon Biotech (Shanghai, China). PCR products were analyzed by Image Lab version 4.1 software.

Western blotting. Cells were lysed on ice with NP-40 buffer containing 40 mM Tris-HCI, pH 6.9, 2 mM ethylenediaminetetraacetic acid, 100 mM sodium fluoride, 150 mM NaCl, 10 mM sodium pyrophosphate, 1% Nonidet P40 (NP-40), 2 mM orthovanadate, 1% Triton X-100, 0.3 mM phenylmethanesulfonyl fluoride, and 1 Mini Tablet Protein Inhibitor (Roche, South San Francisco, CA, USA). Cell lysates were centrifuged at 12,000 x g for 10 min at 4˚C, and the supernatants were stored at -80˚C. Protein quantification was performed with the BCA protein assay (Pierce, Rockford, IL, USA). Equal amounts of protein from different groups were denatured in SDS sample buffer and separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes. β-actin, hTERT and c-myc antibodies (1:2,000 dilution) were used to detect the corresponding proteins. Blots were developed using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific Pierce, Rockford, IL, USA).

Transient transfection and luciferase reporter assays. Plasmids were prepared using the Omega Plasmid Midi kit from Omega Bio-Tek Inc. (Norcross, GA, USA). The 295-bp promoter (13) region of the human TERT gene (GenBank accession no. AN097365) was amplified by PCR from human genomic DNA, using the following primer pairs: TERT (forward) 5'-CTAGCTAGCCACAGACGCCCAGGACCGC GCTTC-3' and (reverse) 5'-CCCAAGCTTCCACGTGCGCC CACGTGCGCCCAC-3'. The 295-bp fragment was inserted upstream of the luciferase reporter pGL4.20 vector (Promega) and verified by DNA sequencing to obtain plasmid pGL4.20-hTERTp.

The 225-bp promoter (14,15) of the human v-myc avian myelocytomatosis viral oncogene c-myc gene (GenBank accession no. NG007161) was generated by PCR from human genomic DNA, using the following primer pairs: (forward) 5'-ATAGATCTCTCTTACTCTGTTTACATCCTAGAGC-3' and (reverse) 5'-CTAAGCTTCCGGGAGGGGCGCTTAT GGGGAGGG-3'. The 225-bp amplified fragment was cloned into pGL4.20 to obtain the plasmid pGL4.20-mycp (15).

The cells were seeded at a concentration of 5,000 cells/well in 96-well plates the day before transfection. The cells were transfected with plasmids pGL4.20-hTERTp or pGL4.20-mycp together with pGL4.74 containing the TK promoter using TurboFect™ Transfection reagent following the manufacturer's protocol. Twenty-four hours later, the cells were incubated in different media with or without bile acids or inhibitors for an additional 24 h, followed by measurement of the luciferase activity.

Telomerase activity assay. Telomerase activity was assayed by the stretch PCR method (16-18) using the TeloChaser (Toyobo Co., Ltd., Osaka, Japan) according to the manufacturer's instructions. In brief, cells were pelleted and washed in ice-cold PBS buffer. After washing, the pellets were homogenized in a 200 μl volume of ice-cold lysis buffer and incubated on ice for 30 min. The lysate was centrifuged at 15,000 rpm for 20 min at 4˚C. The supernatant was frozen and stored at -80˚C. Nine micrograms of protein were used for the PCR assay. For the negative control, each extract was pre-incubated with 1 μg of RNase A for 30 min at 37˚C. After incubation at 37˚C for 60 min, the DNA products were isolated, heated at 90˚C for 3 min and subjected to 30 cycles of PCR including denaturation at 94˚C for 45 sec, annealing at 50˚C for 30 sec, and extension at 72˚C for 60 sec. The PCR products were electrophoresed on a 10% polyacrylamide gel and stained with ethidium bromide. Images were captured using the FLA-3000G image analyzer (Fuji Film Corp., Tokyo, Japan).

Statistical analyses. Results are shown as means ± standard error. Differences were evaluated with unpaired two-tailed Student's t-tests with unequal variance for multiple comparisons using the SPSS software, version 16.0. The difference between

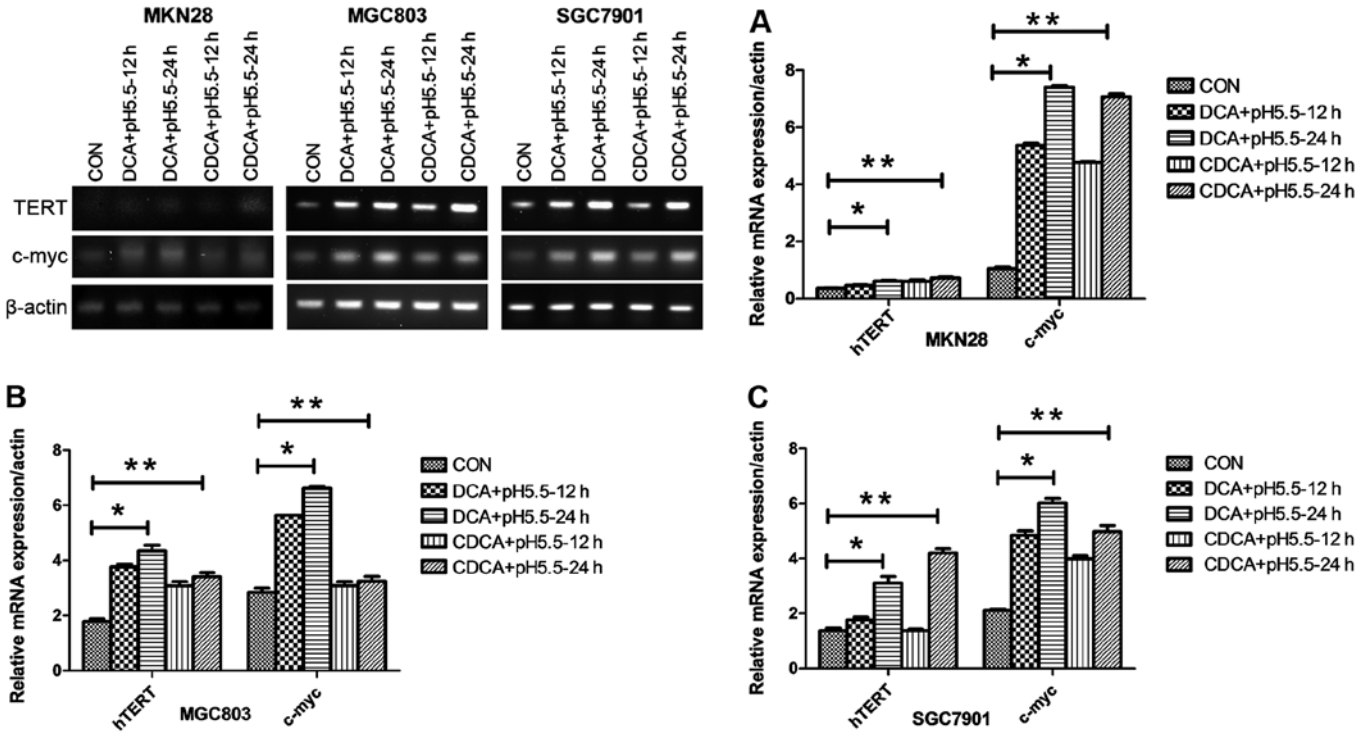

Figure 1. DCA or CDCA in acidified growth media promotes hTERT and c-myc expression in gastric cancer cells. MKN28, MGC803 and SGC7901 cells were treated with 100 μM DCA or CDCA in pH 5.5 for 12 and 24 h. The mRNA levels of hTERT and c-myc were determined by RT-PCR with β-actin as internal control. Integrated optical density was measured to evaluate the hTERT and c-myc mRNA expression relative to the expression of β-actin in the (A) MKN28, (B) MGC803 and (C) SGC7901 cells. *P<0.05 compared with the 12-h treatment group; **P<0.05 compared with the 24-h treatment group; n=3 per group.

two values was considered statistically significant at P<0.05. Experiments were repeated independently at least three times.

Results

Bile acids in acidified growth media upregulate hTERT expression at the transcriptional level in gastric cancer cells. To determine the effect of bile acids in acidified growth media on hTERT expression in gastric cancer cells, we used the bile acids DCA and CDCA as they are common constituents of the refluxate and are widely studied in cell culture models. A concentration of 100 μM was used as it has been shown to be a physiological concentration in our cell culture model (11). In addition, as the pH of reflux constituents ranges between 4.0 and 7.0 (8), we used a median value pH value of 5.5. We treated human gastric cancer MKN28, MGC803 and SGC7901 cells with 100 μM DCA or CDCA with acidified growth media at pH 5.5 for 12 or 24 h and determined the mRNA and protein levels of hTERT by RT-PCR and western blotting, respectively. In the three cell lines, the addition of 100 μM of either DCA or CDCA to acidified growth media at pH 5.5 for either 12 or 24 h significantly increased hTERT mRNA expression (Fig. 1). In sharp contrast, the mRNA levels of hTERT were not affected by DCA or CDCA at pH 7.0, or acidified media alone over 24 h (Fig. 2A). Consistent with the induction of hTERT mRNA, western blotting results showed that either DCA or CDCA at pH 5.5 but not at pH 7.0 significantly increased the protein levels of hTERT in all the 3 cell lines (Fig. 3). These results demonstrated that DCA and CDCA under acidic conditions upregulated the expression of hTERT in gastric cancer cells.

To determine whether bile acids increase hTERT promoter activity, we employed an hTERT promoter luciferase construct pGL4.20-hTERTp, which contains a 295-bp human hTERT promoter core region fused to a luciferase reporter gene. After transient transfection of human gastric cancer cells with pGL4.20-hTERTp for 24 h, the cells were treated with different conditions and luciferase activities were determined. As shown in Fig. 4A, 100 μM DCA or CDCA with media of pH 5.5 induced the hTERT promoter driven luciferase activities in these cells, with a maximum 3-fold increase with CDCA in the SGC7901 cells. These results indicated that acidified bile acids in acidic conditions but not neutral pH induced hTERT upregulation at the transcriptional level.

Bile acids in acidified growth media increase the telomerase activity in gastric cancer cells. We assessed the telomerase activity of gastric cancer cells stimulated with bile acids in acidic conditions. Specifically, three gastric cancer cell lines were treated with 100 μM DCA or CDCA with acidified media for 24 h. Telomerase activity was then measured by stretch PCR and expressed as a ladder of 6-bp bands or multiples of 6-bp intervals. The percentage of telomerase activity was calculated using the band intensity. Telomerase activity was significantly increased following treatment with bile acids in acidified growth media when compared with the untreated cells (Fig. 5).

Bile acids in acidified growth media induce c-myc expression at the transcriptional level in gastric cancer cells. Previous reports have suggested that c-myc is important in mediating

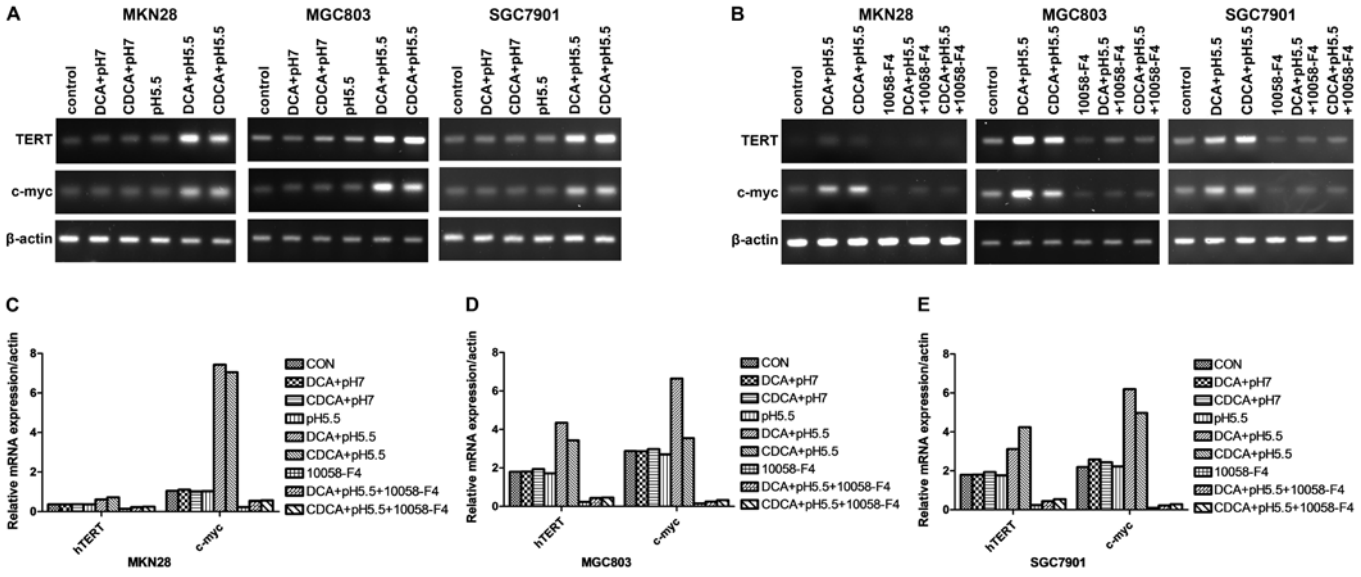

Figure 2. Inhibition of c-myc prevents the induction of hTERT by DCA or CDCA in acidic conditions in gastric cancer cells. (A) MKN28, MGC803 and SGC7901 cells were treated with 100 μM DCA or CDCA in pH 5.5 or pH 7.0 media, or pH 5.5 medium alone for 24 h. The mRNA levels of hTERT and c-myc were determined by RT-PCR with β-actin as the internal control. (B) MKN28 MGC803 and SGC7901 cells were pretreated with 10058-F4 for 24 h followed by additional incubation with 100 μM DCA or CDCA with or without acidified media for 24 h. The mRNA levels of c-myc and hTERT were determined by RT-PCR. Integrated optical density was measured to evaluate the hTERT and c-myc mRNA expression relative to the expression of β-actin in the (C) MKN28, (D) MGC803 and (E) SGC7901 cells.

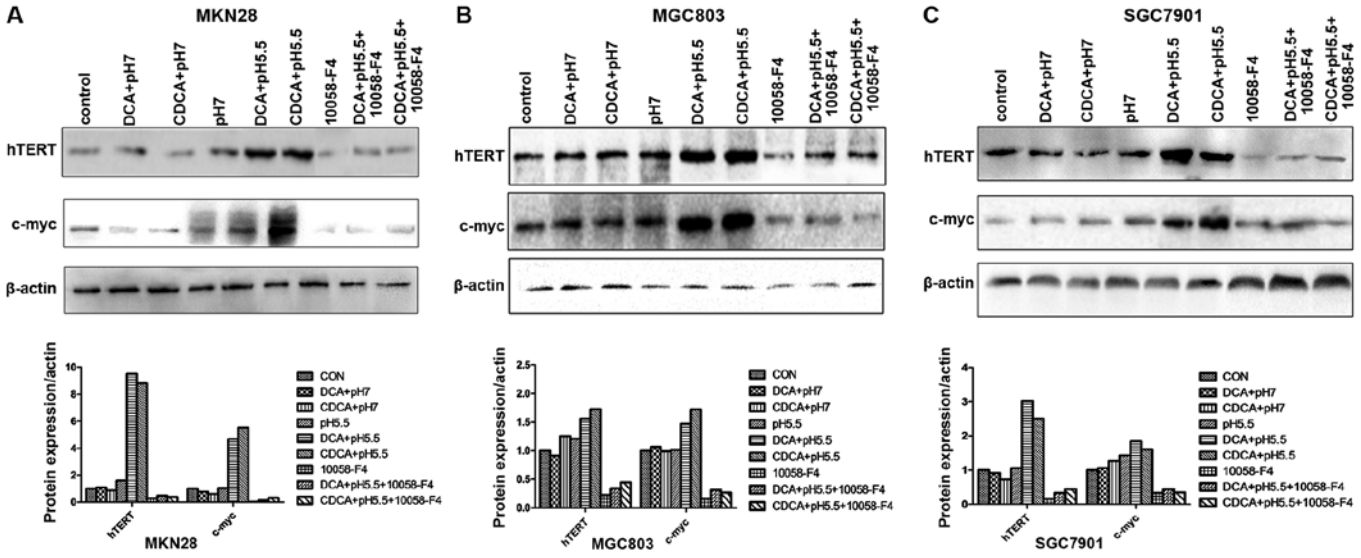

Figure 3. DCA or CDCA in acidified media induces hTERT protein expression through c-myc activation. MKN28 MGC803 and SGC7901 cells were pretreated with 10058-F4 for 24 h, followed by additional incubation with 100 μM DCA or CDCA, with or without acidified media for 24 h, or incubated in pH 7.0 medium in the presence or absence of DCA or CDCA. The protein levels of c-myc and hTERT were determined by western blotting. Integrated optical density was measured to evaluate hTERT and c-myc protein levels relative to the expression of β-actin in the (A) MKN28, (B) MGC803 and (C) SGC7901 cells.

bile acid signaling (11). To test whether bile acids in acidified growth media induce the expression of hTERT via c-myc, we evaluated c-myc protein levels and gene transcription in 3 human gastric cancer cell lines. Indeed, in all the three cell lines, DCA or CDCA with acidified media increased c-myc mRNA expression in a time-dependent fashion with a maximum increase of ~7-fold (Fig. 1). Moreover, consistent with changes in hTERT mRNA levels, DCA or CDCA at pH 7.0, or acidified media alone did not increase c-myc gene transcription. In contrast, DCA or CDCA under acidified media conditions elicited clear effects (Fig. 2A). To determine whether bile acids increase c-myc promoter activity, we employed a c-myc promoter luciferase construct pGL4.20-mycp containing a 225-bp human c-myc promoter core region. We found that DCA and CDCA with acidified media induced c-myc promoter driven luciferase activities in these cells, with a maximum increase of 7.5-fold using 100 μM DCA in MKN28 cells. In contrast, 100 μM DCA or CDCA at pH 7.0, or acidified media alone failed to induce c-myc promoter driven luciferase activity at 24 h (Fig. 4B). These results indicated

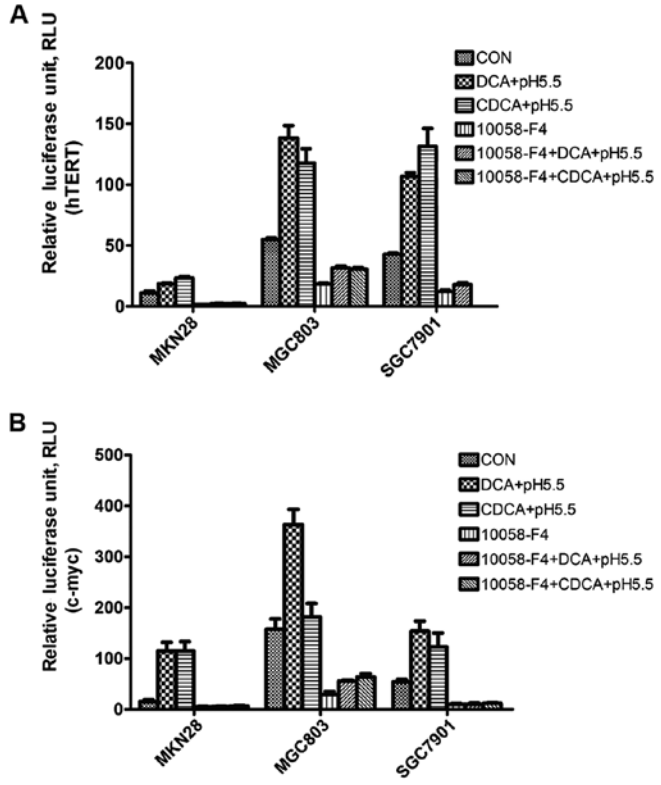

Figure 4. DCA and CDCA under acidified media induce hTERT and c-myc promoter activity. MKN28 MGC803 and SGC7901 cells were transfected with hTERT and c-myc promoter luciferase constructs pGL4.20-hTERTp or pGL4.20-mycp for 24 h, followed by treatment with or without 100 μM DCA or CDCA in the presence or absence of c-myc inhibitor 10058-F4 for 24 h. Luciferase activity for (A) hTERT and (B) c-myc was measured and normalized to an internal transfection control (pGL4.74). Means ± SD of triplicate assays.

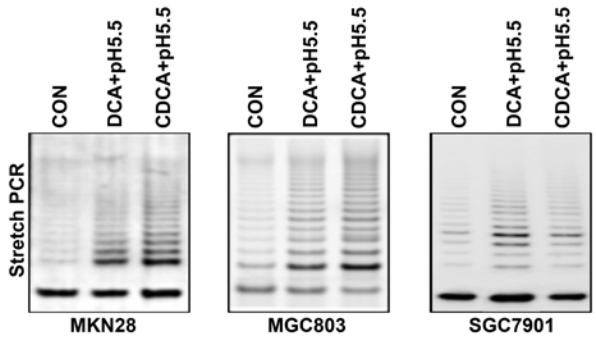

Figure 5. Bile acids in acidified growth media increase the telomerase activity in gastric cancer cells. MKN28, MGC803 and SGC7901 cells were incubated with 100 μM DCA or CDCA with acidified media for 24 h. The telomerase activity was measured by stretch PCR. The percent increase in telomerase was calculated from the band intensity. Similar results were obtained in three separate sets of experiments.

that acidic conditions were indeed required for bile acid to induce c-myc expression at the transcriptional level.

Pharmacologic inhibition of c-myc prevents the induction of hTERT by DCA or CDCA under acidic conditions in gastric cancer cells. As hTERT is a direct target of c-myc, our results suggested that induction of c-myc is one of the mechanisms by which bile acids under acidified growth media increase hTERT expression in gastric cancer cells. To test this hypothesis, we pretreated these cells with 10058-F4, a specific inhibitor of c-myc, followed by incubation with bile acids in acidified growth media. The protein levels and gene transcription of c-myc and hTERT were determined by immunoblotting and RT-PCR, respectively. In all three cell lines, 10058-F4 reduced the mRNA levels of c-myc and hTERT compared to the control. Intriguingly, pretreatment with 100 μM 10058-F4 for 24 h (19,20) significantly abolished the induction of the mRNA levels of c-myc and hTERT by either DCA or CDCA in media at pH 5.5 (Fig. 2B). Consistent with the effect on c-myc and hTERT mRNA levels, western blot analysis demonstrated that either DCA or CDCA at pH 5.5 significantly prevented the induction of the protein levels of c-myc and hTERT in all 3 cell lines (Fig. 3). These results implied that DCA and CDCA under acidic conditions upregulated the expression of hTERT by activating c-myc in gastric cancer cells.

To extend our observations further, we asked whether 10058-F4 blocked hTERT promoter activity. We transiently transfected cells with plasmid pGL4.20-hTERTp or pGL4.20-mycp, treated with 100 μM DCA or CDCA under acidified growth media for 24 h with or without 10058-F4, and then determined the luciferase activities. In the 3 human gastric cancer cell lines, 10058-F4 inhibited hTERT and c-myc promoter-driven luciferase activities and apparently prevented the induction of hTERT (Fig. 4A) and c-myc (Fig. 4B) promoter-driven luciferase activities by DCA or CDCA under acidified growth media. Our studies indicated that pharmacologic inhibition of c-myc prevented the induction of hTERT by both DCA and CDCA in acidic conditions in gastric adenocarcinoma cells, suggesting that activation of c-myc contributes to the induction of hTERT by bile acids under acidified growth media in gastric cancer cells.

Discussion

In the present study, we found that bile acids in acidified media significantly increased the expression of both c-myc and hTERT in gastric cancer cells. This increase was abolished by the c-myc specific inhibitor 10058-F4. Our results demonstrated that bile acids under acidified conditions induced hTERT overexpression in human gastric cancer cells by activation of c-myc transcription and suggest that acidified bile acids may promote tumorigenesis via activation of telomerase.

Reflux of juices from the duodenum has been closely associated with the development of gastric adenocarcinoma (21-23), but the effects of duodenal gastric reflux (DGR) on the proliferation of gastric cancer have not yet been well studied. The unconjugated bile acids DCA and CDCA have been shown to be the main components in DGR (24), and have been widely studied in cell culture models. Moreover, bile acids under acidified media have been reported to induce the c-myc gene expression in esophageal cells, but the molecular events responsible for hTERT gene expression and telomerase activity have not been studied in gastric adenocarcinoma cells (11). In the present study, we found that bile acids increased hTERT expression at the transcriptional level in gastric cancer

cells only under acidic conditions. Furthermore, this effect depended on c-myc expression.

The telomerase RNA subunit is essential for telomerase activity, and is expressed in both normal cells and cells that continuously divide beyond replicative senescence (25). Data from both *in vitro* and *in vivo* studies clearly demonstrate that TERT is the determinant of telomerase activity (26). The transcriptional activity of the TERT gene promoter is significantly higher in telomerase-positive cells than in telomerase-negative cells (2). In addition, studies with dual-luciferase gene assays indicated that hTERT gene expression is controlled mainly at the transcriptional rather than the post-transcriptional level. As telomere length is thought to be the limiting factor in determining the lifespan of a cell (27), stimulation of telomerase activity may be important for the ability of hTERT to promote cell proliferation. We found that exposure of three gastric adenocarcinoma cell lines to acidified bile acids induced the expression of hTERT, an effect that was accompanied by increased telomerase activity. Our data suggest that acidified bile acids may promote carcinogenesis of gastric cancer by stimulating the expression of hTERT.

c-myc is an important transcription factor that mediates expression of multiple genes in important biological processes, including cell proliferation, growth arrest and apoptosis (28). c-myc was recently reported to enhance the activity of the hTERT promoter, an effect that was completely abrogated by deletion of a single Myc/Max binding site within the core promoter (12). However, Wu *et al* reported that the 5' region of human TERT contains 29 potential Myc/Max binding sites, including 18 canonical (CACGTG) and 11 specific non-canonical (CA(C/T)GCG) sites (29). Nonetheless, it was found that the upregulation of TERT expression by c-myc was a direct effect and was independent of new protein synthesis (29). Other transcription factors including AP-1, c-Jun and Jun have been shown to regulate hTERT expression in other contexts (30). Activity of these transcription factors may also be required for hTERT expression in gastric adenocarcinoma cells. In agreement with the previous finding that c-myc regulates the expression of hTERT (4), our data revealed that 10058-F4, an inhibitor of c-myc, reduced the endogenous as well as acidified bile acid-mediated upregulation of hTERT. In addition, c-myc expression and transcription activity coincided with hTERT induction, while inhibition of c-myc markedly suppressed the upregulation of hTERT by acidified bile acids, indicating that c-myc is indeed involved in hTERT transcription induced by bile acids under acidified media.

In conclusion, the present study revealed that acidified bile acids induced c-myc and hTERT expression in gastric adenocarcinoma cells. Our findings suggest that bile acid-induced upregulation of c-myc may be a mechanism through which acidified bile acids regulate telomerase activity in gastric cancer.

Acknowledgements

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