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Interleukin-6 decreases senescence and increases telomerase activity in malignant human cholangiocytes

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Abstract

Background/aims: Cellular senescence results in irreversible growth arrest. In malignant cells, senescence is prevented by maintenance of chromosomal length by telomerase activity. Telomerase activity is increased in malignant, but not in normal cholangiocytes. Interleukin-6 (IL-6) is an autocrine promoter of cholangiocarcinoma growth. Our aims were to assess the relationship between IL-6 activated p38 mitogen-activated protein kinase (MAPK) pathways and senescence in malignant cholangiocytes.
Methods: Cell senescence and telomerase activity was assessed in Mz-ChA-1 malignant human cholangiocytes. The effect of inhibitors of p38 MAPK and telomerase activity on cell proliferation was assessed, and the interaction between these inhibitors was quantitated by median effects analysis.
Results: Mz-ChA-1 cells rapidly underwent senescence during repeated passaging. IL-6 increased telomerase activity and decreased cellular senescence during repeated passaging. However, basal telomerase activity was increased by inhibition of p38 MAPK. Inhibition of telomerase activity decreased IL-6 induced proliferation and had a synergistic effect with p38 MAPK inhibitors. Thus, IL-6 increases telomerase activity independent of p38 MAPK signaling and maintenance of telomerase activity promotes cholangiocarcinoma growth.
Conclusion: Enhanced telomerase activity in response to IL-6 stimulation can prevent cellular senescence and thereby contribute to cholangiocarcinoma growth. Inhibition of telomerase activity may therefore be therapeutically useful in biliary tract malignancies.

Keywords: Cholangiocarcinoma; p38 MAPK; Proliferation; Biliary tract; Cytokines

Introduction

Cellular senescence is a condition of irreversible growth arrest and serves as an important tumor suppressive mechanism by limiting cell growth (Reddel, 2000; Ishikawa, 2000). Replicative senescence occurs when normally dividing cells enter into a non-replicative state after undergoing a finite amount of cell division (Harley, 1991). Senescence is related to progressive shortening with each cell division of simple, tandemly repeated DNA sequences located at the ends of chromosomes termed telomeres (Variri and Benchimol, 1998; Bodnar et al., 1998; Karlseder et al., 2002). When the telomeric ends are either critically shortened or their function is disrupted, cells undergo senescence, which is characterized by cessation of cell division and replication. Telomerase is a multi-subunit ribonucleoprotein reverse transcriptase enzyme complex that serves to elongate the telomeric ends and compensate for the progressive shortening associated with DNA replication during each cycle of cell division (Greider and Blackburn, 1985; Blackburn, 1992). Thus, telomere shortening and senescence can be inhibited by maintenance of telomerase activity. Although it is repressed in most somatic tissues and non-malignant cells, telomerase is expressed in rapidly proliferating cells such as malignant cells, germ-line cells and stem cells (Broccoli et al., 1995; Meyerson, 2000; Kim et al., 1994). Telomerase expression in rapidly proliferating tumor cells maintains chromosomal length and prevents senescence (Variri and Benchimol, 1998; Bodnar et al., 1998; Greider, 1998).

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Tumors arising from biliary tract epithelia, or cholangiocarcinomas, are difficult to treat and are associated with a poor prognosis (Gores, 2003; Sirica, 2005). The dramatic increase in the global incidence and mortality from cholangiocarcinoma along with their unresponsiveness to conventional chemotherapeutic agents underscores the need for novel and improved treatments for this tumor (Patel, 2001, 2002). Telomerase activity is increased in malignant cholangiocytes, and is present in dysplastic cholangiocytes, but not in normal cholangiocytes (Iki et al., 1998; Itoi et al., 2000; Ozaki et al., 1999; Morales et al., 1998). Escape from senescence may be an important contributor to malignant transformation or tumor progression in biliary tract epithelia. However, the molecular mechanisms of telomerase regulation in human cholangiocarcinoma are unknown.

The inflammatory cytokine interleukin-6 (IL-6) is mitogenic for biliary tract epithelia and promotes growth of human cholangiocarcinoma (Matsumoto et al., 1994; Yokomuro et al., 2000; Park et al., 1999a,b; Okada et al., 1994). We and others have shown that IL-6 can act as an autocrine factor and promote proliferation of malignant cholangiocytes. Proliferation in response to IL-6 involves activation of the p38 mitogen-activated protein kinase (MAPK) signaling pathways in malignant but not in non-malignant cholangiocytes (Tadlock and Patel, 2001; Park et al., 1999b). p38 MAPK signaling mediates a transformed cell phenotype in malignant cholangiocytes manifest by decreased serum dependency, anchorage independent growth and enhanced xenograft growth in nude mice. Despite the close association between telomerase expression and cellular proliferative capacity, the relationship between mitogenic signaling and regulation of telomerase activity in malignant cholangiocytes is unknown. Recent studies suggest that premature senescence represents a cellular mechanism of chemoresistance suggesting that strategies to prevent senescence may improve therapeutic responses (Schmitt et al., 2002; Te Poele et al., 2002). Thus, the overall aim of our study was to investigate the effect of the biliary epithelial mitogen IL-6 on telomerase activity and to examine the relationship between maintenance of telomerase activity and IL-6 induced cell proliferation in malignant human cholangiocytes. We addressed the following questions in our study: Does stimulation by IL-6 alter cellular senescence or telomerase activity? Is regulation of telomerase activity dependent on p38 MAPK signaling? If so, is p38 MAPK signaling involved in modulation of telomerase activity by IL-6? Does inhibition of telomerase activity modulate mitogenic effect of IL- 6?

Materials and methods

Cells

Mz-ChA-1 cells, derived from metastatic gall bladder cancer, were provided by Dr. J.G. Fitz (University of Texas Southwestern, Dallas, TX) and cultured in CMRL 1066 media with 10% fetal bovine serum, 1% L-glutamine and 1% penicillin (100 IU/ml)/streptomycin (100 mg/ml) mix. Cells were used at 50–60% confluency, washed twice with phosphate-buffered saline (PBS) and incubated in serum-free media for 24 h prior to study. KMCH, malignant intrahepatic cholangiocytes, and H69 cells, non-malignant intrahepatic cholangiocytes, were obtained and cultured as previously reported (Park et al., 1999b). For MAPK or telomerase inhibitor studies, cells were pre-incubated with the inhibitors for 1 h prior to the experiments.

Generation of stably transfected cell lines

Mz-ChA-1 cells were stably transfected with an expression plasmid containing full-length IL-6 under the control of a CMV promoter and designated as Mz-IL-6. Control cells were transfected with empty vector. Plasmids were purified using the Qiagen Plasmid Midi Kit (Qiagen Inc., Valencia, CA) and linearized by restriction enzyme digestion prior to transfection using Trans-IT (Panvera, Madison, WI). A mixed population of stable transfectants was obtained by selection in media containing G418 for 3 weeks. Stable transfection was confirmed using an IL-6 bioassay to verify IL-6 over-expression. Basal expression of IL-6 was increased two- to three-fold in Mz-IL-6 cells relative to controls. For studies of p38 MAPK signaling pathways, Mz-p38DN cells were derived by stable transfection of Mz-ChA-1 cells with an inactive upstream activator of p38 MAPK signaling MKK3(A). These cells demonstrate decreased constitutive expression of p38 MAPK and were generated as we have recently described for KMCH cells (Yamagiwa et al., 2003).

Senescence assay

Cellular senescence was detected by staining for senescence associated acidic β-galactosidase (SA-β-gal) (Dimri et al., 1995; Bodnar et al., 1998). Cells were fixed with 2% formaldehyde and 0.2% glutaraldehyde in PBS and incubated for 24 h at 37 °C with β-gal staining solution containing 1 mg/ml X-gal, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM $MgCl_2$, 40 mM citric acid with Na_2HPO_4 (pH 6.0). The proportion of senescent cells at each time point was assessed by counting the percentage of SA-β-gal positive cells in at least 2×10^3 total cells using light microscopy.

Cell proliferation

Cells were seeded into 96-well plates (10,000 cells/well) and incubated in a final volume of 200 μl medium. The cell proliferation index was assessed as we have previously described using a commercially available colorimetric assay (CellTiter 96AQueous, Promega Corp., Madison, WI) (Park et al., 1999b).

Telomerase assay

Telomerase activity in cell extracts was measured by the PCR-based telomere repeat amplification protocol (TRAP) using the TRAPeze kit (Intergen, Gathiesburg, MD). Briefly, the cells were grown in 6-well plates, washed in PBS, and

homogenized for 30 min on ice in buffer containing 10 mM Tris–HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.5% 3-[3-cholamidopropyldimethyyammonio-]-1-propanesulfonate (CHAPS). Protein concentration in the cell extract was determined using the Bradford assay. 50–100 ng of proteins from each cell extract were added directly to the TRAP reaction mixture containing dNTPs, template substrate primer, reverse primer mix and *Taq* polymerase. The extended telomerase products were then amplified by two-step PCR (94 °C for 30 s, 59 °C for 30 s and 72 °C for 60 s for 36 cycles, followed by incubation at 55 °C for 25 min for extension). The telomerase activity was quantitated by measuring the ratio of a 36-bp internal standard to the extended telomerase products as described by the manufacturer using a fluorometer (Cytofluor 4000, Perseptive Biosystems, Foster City, CA). Telomerase activity was expressed as total product generated (TPG) units per microgram protein, with 1 unit equivalent to the number of telomerase substrate primers (in 1×10^3 amol) extended with at least three telomeric repeats in 30 min at 30 °C. To confirm telomerase activity, polyacrylamide gel electrophoresis (PAGE) was performed on the reaction products on a 10% non-denaturing gel followed by image analysis using a CCD based imaging system (ChemiImager 4000, Alpha Innotech, San Leandro, CA).

PCR analysis

Total cellular RNA was extracted from cells using the Ultraspec RNA isolation reagent (Biotecx Laboratories, Inc., Houston, TX). RNA was treated (10 min at 20 °C) with amplification grade DNAse I (Invitrogen, San Diego, CA) followed by heat inactivation (5 min at 75 °C). For TERT, real-time PCR analysis was performed in a final volume of 20 μl containing 2 μl of cDNA sample, 3 mM $MgCl_2$, 0.5 μM each of the TERT primers or 0.3 μM each of the GADPH primers, 1 μl of LC-Fast Start Reaction Mix SYBR Green I and 1 μl of LC-Fast Start DNA Master SYBR Green I/Enzyme mix. Samples were incubated for 2 min at 95 °C, followed by 40 cycles (94 °C for 1 min, 62 °C for 1 min and 72 °C for 1 min). PCR product accumulation was monitored using a Mx3000PTM Real-Time PCR System (Stratagene, Cedar Creek, TX). The mean cycle threshold value (*Ct*) from quadruplicate samples was used to calculate relative gene expression as described by the manufacturer. PCR products were normalized to levels of GAPDH. The primers used were as follows: human TERT 5′-CGTGGTTTCTGTGTGGTGTC-3′(sense) and 5′- TGGAACCCAGAAAGATGGTC-3′(antisense); GAPDH 5′-TGCCAGTGAGCTTCC-3′ (sense) and 5′-CACCATGGAGAAGGC-3′ (antisense). For each reaction, a no template control and a no reverse transcriptase control were included. Post-amplification dissociation curves were performed to verify the presence of a single amplification product in the absence of DNA contamination. Analysis of expression of other components of the telomerase complex including telomeric repeat binding proteins (TRF)-1, 2 and telomerase associated protein 1 (TP-1) was performed using the CytoXpressTM Multiplex kit (BioSource International Inc., Camarillo, CA) as per the manufacturer's recommendations, and gene expression was normalized against S18.

Immunoblot analysis

Cells were washed with PBS, then suspended in lysis buffer containing 50 mM Tris base, 2 mM EDTA, 100 mM NaCl, 1% Triton X-100, 2 μg/ml pepstatin A, 2 μg/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride and 1 miniprotease inhibitor cocktail tablet/25 ml, and sonicated for 20 s on ice. Protein concentration was determined by the Bradford assay. Extracts were then separated on a 4% to 12% gradient polyacrylamide gel (Invitrogen, San Diego, CA) and transferred to nitrocellulose membrane (Bio-Rad laboratories, Hercules, CA). The membrane was pre-blocked in 5% non-fat dry milk, 20 mM Tris base, 137 mM NaCl, 0.1% Tween 20 (TBST) for at least 4 h at 4 °C, washed with TBST, and incubated overnight with the primary antibodies diluted with TBST at 1:200 dilutions for TERT and 1:1000 dilution for Actin. The membranes were washed three times for 10 min with TBST, incubated with the secondary antibody, a rabbit anti-goat horseradish peroxidase (Zymed, San Francisco, CA), at a dilution of 1:1000 for 2 h at 4 °C, and washed three times for 10 min with TBST. Protein expression was detected using the enhanced chemiluminescence kit (ECL Plus, Amersham Pharmacia biotech, Buckinghamshire, UK) and quantitated using a CCD-camera based image analyzer (ChemiImager 4000, Alpha Innotech, San Leandro, CA).

Analysis of drug interactions

Quantitation of the interactions between telomerase inhibitors and p38 MAPK inhibitor was performed by a median effect analysis as described by Chou and Talalay (1984) and using a commercial software package (Calcusyn, Biosoft). The median effect equation used is: $f_a/f_u=(D/D_m)^m$ where D is the dose, D_m is the dose required for 50% inhibition of cell growth, f_a is the fraction affected by D (e.g. 0.75 if cell growth is inhibited by 75%), f_u is the unaffected fraction and m is the coefficient of sigmoidicity of the dose–effect curve. The dose effect curve was plotted using a logarithmic conversion of this equation to: $\log(f_a/f_u)=m\ \log(D)-m\ \log(D_m)$ for the median effect plot: $x=\log(D)$ versus $y=\log(f_a/f_u)$, which determines m (the slope) and D_m (anti-log of x intercept) values. A combination index (CI) was determined where $CI=(D)_1/(Dx)_1+(D)_2/(Dx)_2$, where $(Dx)_1$ is the dose of agent 1 (telomerase inhibitor) required to produce the same X percentage effect in combination with $(D)_2$. Similarly, $(Dx)_2$ is the dose of agent 2 (p38 MAPK inhibitor) required to produce X percentage effect alone and $(D)_2$ is the dose required to produce the same effect in combination with $(D)_1$. A CI of 1 indicates an additive effect, whereas a $CI>1$ indicates an antagonistic effect and a $CI<1$ indicates a synergistic effect.

Statistical analysis

Data are expressed as the mean ± standard error (S.E.) from at least three separate experiments performed in triplicate,

unless otherwise noted. The differences between groups were analyzed using a double-sided Student's *t*-test. Statistical significance was considered as $p<0.05$. Statistical analyses were performed with the GB-STAT statistical software program (Dynamic Microsystems Inc., Silver Spring, MD).

Materials

All cell culture reagents were from Gibco BRL (Rockville, MD) except for fetal bovine serum which was obtained from Sigma (St. Louis, MO). IL-6 was obtained from R&D systems, Inc. (Minneapolis, MN). The kinase inhibitors SB203580, PD098059 and LY290042 were purchased from Calbiochem-Novabiochem Co. (San Diego, CA). SYBR Green I and all PCR reagents were from Roche (Indianapolis, IN). 3,3′-Diethyloxadicarbocyanine iodide (DODCI) was obtained from Sigma (St. Louis, MO). TERT and Actin antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Results

Cellular senescence in Mz-ChA-1 malignant human cholangiocytes

In order to assess the potential impact of short-term telomerase regulation on cellular immortality and tumor growth, we ascertained the rate of cellular senescence during serial culturing of Mz-ChA-1 malignant human cholangiocytes. Cell senescence was quantitated at each cell passage by counting the percentage of cells expressing the senescence marker SA-β-gal. The number of SA-β-gal positive cells increased with serial cell passaging from 1.9±0.4% to 4.7±0.5% after 20 population doublings (Fig. 1). In contrast, the number of SA-β-gal positive cells was not significantly altered in either non-malignant H69 human cholangiocyte cells (0.4±0.3% to 0.7±0.5%) or in malignant KMCH cells

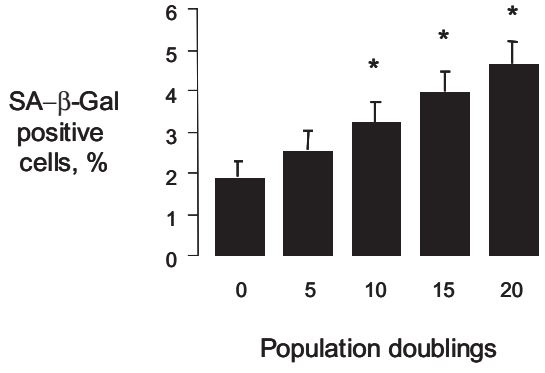

Fig. 1. Mz-ChA-1 malignant cholangiocytes undergo progressive senescence during cell culture. Mz-ChA-1 cells were serially passaged and cell number assessed. The number of senescent cells was assessed at various time points after staining cells for the senescence marker acidic β-galactosidase (SA-β-gal) and examining at least five high-power fields by light microscopy. With each progressive cell passage, there was an increase in the number of SA-β-gal positive cells. Data represents the mean and standard deviation of three separate determinations. *$p<0.05$ compared to initial value.

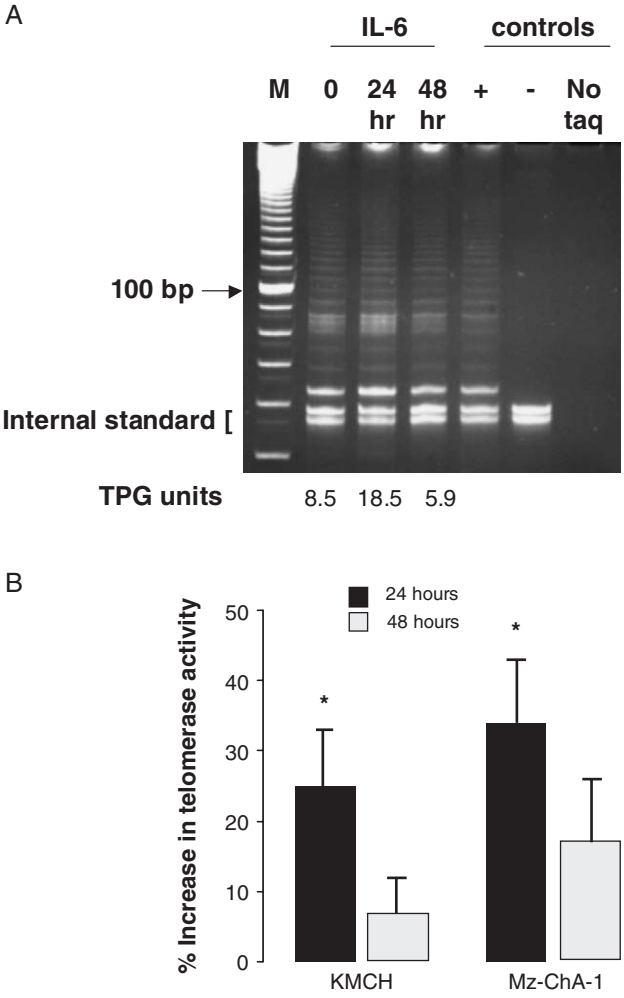

Fig. 2. IL-6 increases telomerase activity in Mz-ChA-1 cells. Mz-ChA-1 cells were serum starved for 24 h prior to stimulation with IL-6 (10 ng/ml). At the indicated time points, cells were lysed to obtain protein extracts. Telomerase activity was measured as described in the Methods section. (A) A polyacrylamide gel image of the PCR products. M represents a 100-bp marker. Controls samples are from a cell line known to express telomerase (positive control +), internal control primers only (negative control −) or from a telomerase expressing cell line but with the omission of *taq* polymerase (no *taq* control). IL-6 transiently increases telomerase activity. Results are from one of three representative experiments. (B) Telomerase activity was quantitated in cell lysates obtained from Mz-ChA-1 or KMCH malignant human cholangiocytes after stimulation with IL-6 (10 ng/ml) for 24 or 48 h. Results represent the mean±standard error of the increase in telomerase activity compared to unstimulated cells from three separate determinations. *$p<0.05$ compared to unstimulated controls.

(0.1±0.1% to 0.5±0.2%) after 20 population doublings. In rapidly proliferating cells, replicative senescence is associated with shortening of telomeres. Since cell replication stops and senescence occurs when the telomeric lengths are shortened to a critical length, the rapid increase in senescent cells suggests that Mz-ChA-1 malignant cholangiocytes have short telomere lengths close to the Hayflick limit (Hayflick and Moorhead, 1961). Thus, maintenance of telomerase activity is essential for the continued immortality. Because of their susceptibility to short-term alterations in telomerase activity, Mz-ChA-1 cells are suitable for the study of factors regulating telomerase

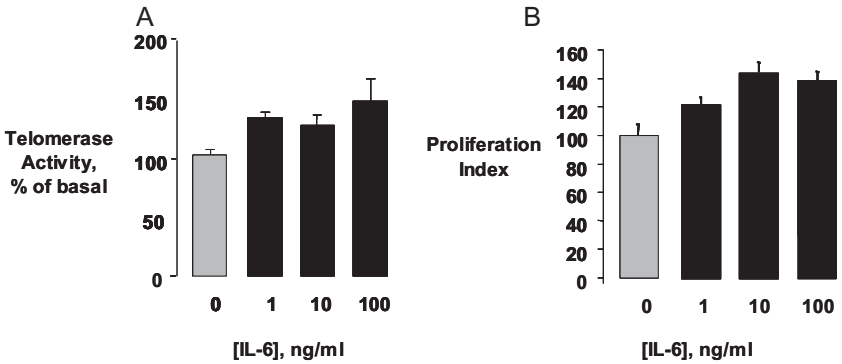

Fig. 3. IL-6 increases telomerase activity and proliferation in a concentration-dependent manner. Mz-ChA-1 cells at ~50% confluency were incubated in serum-free media for 24 h prior to stimulation with IL-6 (0–100 ng/ml). (A) Telomerase activity was measured using a modified TRAP assay employing fluorescence energy transfer primers in cell lysates obtained after 24 h. IL-6 increased telomerase activity in a concentration dependent manner. (B) Cell proliferation was assessed using a viable cell assay after 24 h. IL-6 increased cell proliferation in a concentration dependent manner. Data represent the mean±standard error from three separate experiments. A significant change was observed at all concentrations for both telomerase activity and proliferation, $p<0.05$ compared to unstimulated basal values.

activity and to assess the relationship between telomerase activity and growth in human cholangiocarcinoma.

IL-6 increases telomerase activity in malignant cholangiocytes

The inflammatory cytokine IL-6 is mitogenic for biliary epithelia and promotes the growth of human cholangiocarcinoma. The effect of IL-6 on telomerase activity in Mz-ChA-1 malignant cholangiocytes was assessed using a modified TRAP assay and the results were correlated with polyacrylamide gel electrophoresis of the PCR products (Uehara et al., 1999). An increase in telomerase activity occurred in cells stimulated with IL-6 (10 ng/ml) for 24 h, with activity returning to basal levels by 48 h (Fig. 2A). Telomerase activity was significantly increased to 146±18% of controls following incubation with 100 ng/ml IL-6 for 24 h (*t*-test, $p=0.01$). However, telomerase activity at 48 h was decreased to levels that were not significantly different from basal values (*t*-test, $p=0.34$). Thus, stimulation with IL-6 transiently enhances telomerase activity. Similar effects of IL-6 on telomerase activity were also observed in another IL-6-responsive human malignant cholangiocyte cell line KMCH-1 (Fig. 2B). Notably, activation of telomerase by IL-6 was observed at concentrations of IL-6 that induce a mitogenic response (Fig. 3). In order to assess the effect of persistent IL-6 stimulation on telomerase activity, Mz-ChA-1 cells were stably transfected to over-express full-length IL-6 (Mz-IL-6) or empty vector (controls). Telomerase activity in Mz-IL-6 cells was 145±15% of controls (*t*-test, $p<0.05$). Thus, under conditions of enforced IL-6 expression, there is an increase in basal telomerase activity.

IL-6 decreases senescence in Mz-ChA-1 malignant human cholangiocytes

Our next studies addressed the relevance of the effects of IL-6 on telomerase activity to tumor cell senescence. We assessed the number of population doublings and proportion of early passage Mz-ChA-1 cells undergoing senescence during serial passaging in the presence or absence of IL-6. Although the number of population doublings at the end of each passage was not significantly altered, there was a reduction of SA-β-gal positive cells in cells continuously incubated with IL-6 (10 ng/ml) compared to controls (Fig. 4). These changes in the

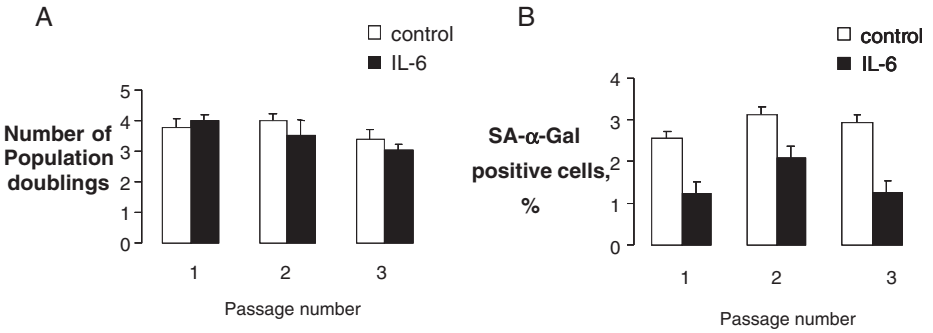

Fig. 4. IL-6 decreases cellular senescence during serial passaging of Mz-ChA-1 cells. Mz-ChA-1 cells were continuously incubated in the absence (control) or presence of IL-6 (10 ng/ml). (A) The amount of population doubling was calculated from measurements of total cell number at the beginning and end of each passage. IL-6 did not alter the number of population doublings per passage. Results represent the mean±standard error of three separate determinations. The differences between the groups were not statistically significant. (B) The number of cells expressing the senescence-associated β-galactosidase (SA-β-gal) was identified in confluent cells during each passage. At least 10 high-power fields were examined. IL-6 decreased the number of senescent cells during early serial passaging. Results represent the mean and standard error of three separate determinations. The differences between the two groups were significant at each passage number ($p<0.05$).

number of senescent cells were significant even at very early passages. Thus, IL-6 mediated increases in telomerase activity are associated with a reduction in cellular senescence. Telomerase activity in the Mz-ChA-1 cells continuously incubated with 10 ng/ml IL-6 was increased to 118+8% of initial values (*t*-test, $p=0.07$) after three passages. IL-6 is mitogenic for biliary epithelia and an autocrine factor implicated in cholangiocarcinoma growth. The demonstration that IL-6 also decreases senescence and increases telomerase activity thus raise the possibility that the regulation of cell proliferation and telomerase activity may be mediated by common intracellular signaling pathways.

Telomerase activity is repressed by constitutive p38 MAPK signaling

We next sought to examine the relationships between signaling mechanisms involved in regulation of cell proliferation and telomerase activity in malignant cholangiocytes. Our previous studies have shown activation of p38 MAPK signaling pathways in malignant cholangiocytes but not in non-malignant cholangiocytes by IL-6. Inhibition of p38 MAPK signaling decreases transformed cell growth in vitro and reduces cholangiocarcinoma xenograft growth in vivo. These observations indicate that signaling via p38 MAPK contributes to tumor cell growth and maintenance of the tumor cell phenotype. First we confirmed the involvement of p38 MAPK signaling in the mitogenic response to IL-6. Pre-incubation for 1 h with the p38 MAPK inhibitor SB203580 (25 μM) decreased IL-6 (10 ng/ml) induced cell proliferation in Mz-ChA-1 cells by 78±11% after 24 h. We have reported similar effects of p38 MAPK inhibition in other human cholangiocarcinoma cell lines (Park et al., 1999b). We next determined the effects of inhibition of p38 MAPK activation on telomerase activity. Mz-ChA-1 cells were incubated with the p38 MAPK inhibitor SB203580 (25 μM) and telomerase activity assessed after 24 h. Surprisingly, SB203580 increased telomerase activity to 144±12% of diluent controls in unstimulated cells. Although IL-6 also increased telomerase activity, SB203580 did not significantly alter telomerase activity in IL-6 stimulated cells. These results were confirmed by studies in Mz-p38DN cells, which are stably transfected with a mutated upstream kinase, MKK3 that acts as a dominant negative inhibitor of p38 MAPK activation. In these cells, telomerase activity was increased to 152±19% of control cells under basal conditions. Taken together, these results indicate that constitutive cellular MAPK activity represses telomerase activity.

The human telomerase reverse transcriptase (TERT) is the catalytic component of telomerase (Nakamura et al., 1997; Meyerson et al., 1997; Bodnar et al., 1998; Nakayama et al., 1998). Regulation of telomerase activity can occur by transcriptional regulation of TERT expression during physiological or pathological conditions such as hypoxia (Minamino et al., 2001). TERT is repressed in most human somatic cells, but is over-expressed in most tumor cells (Nakayama et al., 1998; Minamino et al., 2001; Dhaene et al., 2000). TERT mRNA expression was significantly increased by pre-incubation with either SB203580 (to 132±8% of diluent controls) but not by the p44/p42 MAPK inhibitor PD098059 (to 104±11% of diluent controls). Furthermore, telomerase activity was increased by 61.3±23.7% of controls after 24 h in cells incubated with the protein synthesis inhibitor cycloheximide (100 μg/ml) in unstimulated cells. In combination, these studies demonstrate that constitutive p38 MAPK signaling represses telomerase activity in malignant cholangiocytes by repressing expression of TERT.

Telomerase activation by IL-6 occurs via a p38 MAPK independent pathway

Although IL-6 stimulation results in p38 MAPK activation in malignant cholangiocytes and constitutive p38 MAPK activity represses telomerase activity under basal conditions, IL-6 induced telomerase activity was not inhibited by inhibition of p38 MAPK. Furthermore, incubation with IL-6 (10 ng/ml) for 24 or 48 h did not alter TERT mRNA expression assessed by quantitative real-time PCR. We next assessed the effect of IL-6 on the expression of non-catalytic components of the telomerase complex by multiplex PCR. Incubation with 10 ng/ml IL-6 for 24 h increased telomeric repeat binding protein 2 (TRF-2) mRNA expression by 38±8% and increased telomerase associated protein 1 (TP- 1) mRNA expression by 30±6%. In contrast, the telomerase repressor protein TRF-1 mRNA was not detected in Mz-ChA-1 cells. The mechanism by which IL-6 regulates telomerase activity may thus involve alterations in expression of non-catalytic components of the telomerase complex, or in the expression of stimulatory molecules that interfere with the complex and increase in catalytic activity without directly altering TERT expression. Thus, IL-6 stimulation modulates telomerase activity independent of p38 MAPK mediated regulation of TERT expression. These data thus suggest the presence of a dominant IL-6 stimulated pathway regulating telomerase activity despite concomitant activation of p38 MAPK signaling. Recent studies have implicated phosphatidylinositol 3-kinase (PI3-kinase) mediated signaling in cytokine modulation of telomerase activity in multiple myeloma cells (Akiyama et al., 2002). Although IL-6 can activate PI3-kinase signaling, the selective PI3-kinase inhibitors LY290042 or Wortmannin did not alter either basal or IL-6 stimulated telomerase activation in Mz-ChA-1 cells.

Inhibition of telomerase activity reduces mitogenic effect of IL-6

Since telomerase activity appeared to be regulated independently from p38 MAPK, and hence proliferative signaling, we studied potential relationships between telomerase activity and cell proliferation. First, we assessed the effect of the telomerase inhibitor, 3,3′-diethyloxadicarbocyanine iodide (DODCI), on proliferation of malignant cholangiocytes (Fu et al., 1999). We began by confirming the effect of DODCI on telomerase activity in Mz-ChA-1 cells. Incubation with 5 μM DODCI significantly decreased telomerase activity to 19±4% of

controls (t-test, $p<0.01$) without significant cytotoxicity. Pre-incubation with 5 μM DODCI decreased proliferation following stimulation with IL-6 although it did not have any direct effect in unstimulated cells (Fig. 5). These observations suggested that simultaneous targeting of both telomerase and the p38 MAPK signaling pathway may be therapeutically useful. Thus, we examined the interactions between DODCI and p38 MAPK inhibitors on inhibition of growth of malignant cholangiocytes. The nature of the interaction was quantitated by using the median effects analysis to derive a combination index. The results of the median effects analysis are depicted in Fig. 6. Combinations of DODCI and SB202190 were synergistic with a CI at an effective dose for 50% inhibition (ED50) of 0.19±0.11 for SB202190. Similar results were observed with the p38 MAPK inhibitor SB203580, with a CI of 0.62±0.22 at ED50. Thus, maintenance of telomerase activity facilitates the mitogenic effect of IL-6 on malignant cholangiocytes, and simultaneously targeting both telomerase activation and p38 MAPK signaling has a synergistic effect.

Discussion

Escape from senescence is an important element of malignant transformation. We show that Mz-ChA-1 malignant human cholangiocytes undergo senescence during repeated passaging and are susceptible to short-term alterations in telomerase activity. In addition to enhanced telomerase activity, malignant tumors are characterized by aberrant growth signaling mechanisms that permit escape from physiological controls on cell growth. The major findings of our studies are that IL-6, a biliary epithelial mitogen, increases telomerase activity in Mz-ChA-1 cells independent of mitogenic responses involving the activation of p38 MAPK. Thus, autocrine or paracrine IL-6 stimulation may contribute to cholangiocarcinoma growth by decreasing senescence as well as promoting cellular immortality despite rapid proliferation.

IL-6 has been implicated as an autocrine promoter of cholangiocarcinoma growth. Thus, the regulation of telomerase activity by this cytokine may thus represent a unique mechanism resulting in tumor growth and progression. Our results support the presence of a dominant IL-6 stimulated pathway that increases telomerase activity despite concomitant activation of p38 MAPK. Although the specific signaling mechanisms by which telomerase activity is regulated by IL-6 have not been identified by the present studies, this pathway is distinct from and can overcome the repressive effects of constitutive p38 MAPK activity. The synergistic effect of telomerase and p38 MAPK inhibitors in decreasing growth suggests that maintenance of telomerase activity during IL-6 stimulation may facilitate the mitogenic effect of IL-6 on malignant cholangiocytes. Furthermore, the maintenance of telomerase activity may contribute to resistance to apoptosis (Holt et al., 1999). By decreasing senescence in susceptible cells, cytokine modulation of telomerase activity may serve as a mechanism promoting tumor growth independent of effects on cellular proliferation.

Modulation of telomerase activity can be mediated by transcriptional or post-translational mechanisms (Ducrest et al.,

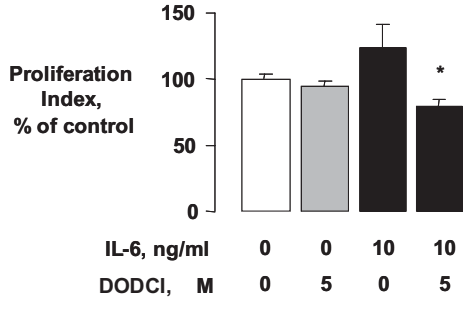

Fig. 5. The telomerase inhibitor DODCI decreases IL-6 induced proliferation. Mz-ChA-1 cells were serum-starved for 24 h, and pre-incubated with DODCI (5 μM) or diluent control for 1 h prior to treatment with IL-6 (0 or 10 ng/ml). Cell proliferation was assessed after 24 h. DODCI did not alter basal proliferation, but decreased IL-6 induced cell proliferation. Results represent mean±standard error of three separate experiments. *$p<0.05$ compared to proliferative index in the absence of DODCI.

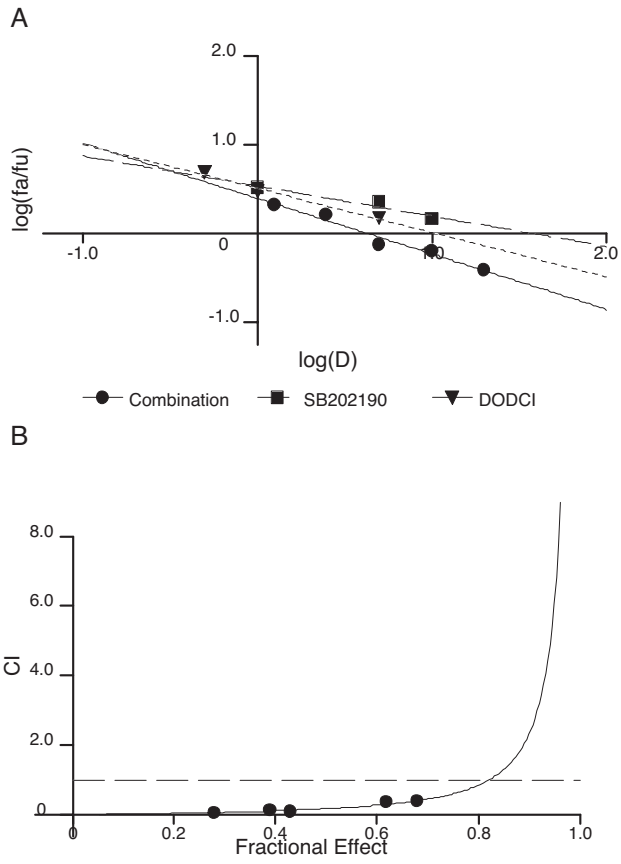

Fig. 6. Synergistic interaction between the p38 MAPK inhibitor SB202190 and the telomerase inhibitor DODCI. (A) A median effects plot of log (f_a/f_u) plotted against the log of the concentration (D) for SB202190, DODCI or the combination of DODCI and SB202190 in equimolar concentrations, as outlined in the Methods section. (B) Combination index versus fractional effect (f_a) plots derived from the median effects analysis. The curve is a computer stimulated fraction affected (f_a)–CI plot based on parameters (m and D_m) values for the combination of DODCI and SB202190 in equimolar concentrations. Actual combination data plots are plotted as closed circles. CI<1, =1 and >1 indicates synergism, additive effects and antagonism, respectively.

2002). Although transcriptional regulation of the human telomerase gene TERT is an important mediator of telomerase activity, IL-6 regulation of telomerase activity does not involve alterations in the expression of TERT. Similar observations have recently been reported in myeloma cells using higher concentrations of IL-6 (50 ng/ml) (Akiyama et al., 2002). Interestingly, in this study, phosphorylation of TERT was demonstrated to occur via an Akt mediated pathway and correlated with alterations in telomerase activity. Because we were unable to identify a direct involvement of PI3-kinase signaling, other mechanisms are likely to be present in malignant cholangiocytes. Telomerase activity can be regulated by modulation of expression of proteins involved in the assembly of the telomerase enzyme complex. Thus, the effects of IL-6 on telomerase activity could be related to effects on other protein subunits of the telomerase complex. Indeed, modest increases were seen in TRF-2 and TP-1 mRNA expression. TRF-2 is involved in the maintenance of single-strand telomeric DNA and can delay senescence by protecting telomeric ends despite increasing the rate of telomere shortening (Karlseder et al., 2002). TP-1 is involved in assembly of the telomerase complex. Although TP-1 mRNA is increased in human cholangiocarcinoma cells (Ozaki et al., 1999), the significance of these changes to telomerase activity is unclear since alterations in TP-1 levels do not correlate with telomerase activity in the absence of changes in TERT (Ancelin et al., 2002). An alternative mechanism may involve altered expression by IL-6 of regulatory factors that influence the reverse transcriptase activity of TERT. Cellular factors such as p53 and the retinoblastoma protein have also been implicated in the regulation of telomerase in various cancers and further study is required to ascertain their role in the telomerase activation due to IL-6 in cholangiocytes (Kang et al., 1999; Li et al., 1997, 1998). Telomerase activity may also be regulated by post-translational modifications involving phosphorylation by intracellular kinases.

Conclusion

Despite some progress in understanding the molecular basis of cholangiocarcinoma, the tumor remains uniformly fatal and effective treatment options do not exist. The demonstration that IL-6 affects telomerase activity and delays senescence establishes a novel effect for this cytokine in promoting long-term survival in malignant cholangiocytes. Because of the central role of IL-6 as an autocrine growth factor for cholangiocarcinoma, these observations may have therapeutic implications. By promoting replicative senescence as well as by decreasing proliferation, telomerase inhibitors may be potential useful for biliary tract malignancies. The independence of telomerase regulation from known IL-6 mediated mitogen signaling pathways provides a rationale for combination approaches targeting both mechanisms. Since p38 MAPK signaling maintains a transformed phenotype in human cholangiocarcinoma, combining telomerase inhibitors with specific p38 MAPK inhibitors would be a logical strategy for the treatment of human cholangiocarcinoma.

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