RESEARCH ARTICLE

Effects of 5-Aza-2'-Deoxycytidine, Bromodeoxyuridine, Interferons and Hydrogen Peroxide on Cellular Senescence in Cholangiocarcinoma Cells

Benchamart Moolmuang¹, Pattama Singhirunnusorn¹, Mathuros Ruchirawat²

Abstract

Cellular senescence, a barrier to tumorigenesis, controls aberrant proliferation of cells. We here aimed to investigate cellular senescence in immortalized cholangiocyte and cholangiocarcinoma cell lines using five different inducing agents: 5-aza-2’-deoxycytidine, bromodeoxyuridine, interferons (IFNβ and IFNγ), and hydrogen peroxide. We analyzed senescence characteristics, colony formation ability, expression of genes involved in cell cycling and interferon signaling pathways, and protein levels. Treatment with all five agents decreased cell proliferation and induced cellular senescence in immortalized cholangiocyte and cholangiocarcinoma cell lines with different degrees of growth-inhibitory effects depending on cell type and origin. Bromodeoxyuridine gave the strongest stimulus to inhibit growth and induce senescence in most cell lines tested. Expression of p21 and interferon related genes was upregulated in most conditions. The fact that bromodeoxyuridine had the strongest effects on growth inhibition and senescence induction implies that senescence in cholangiocarcinoma cells is likely controlled by DNA damage response pathways relating to the p53/p21 signaling. In addition, interferon signaling pathways may partly regulate this mechanism in cholangiocarcinoma cells.

Keywords: Cholangiocarcinoma cells - cellular senescence - 5-aza-2’-deoxycytidine - bromodeoxyuridine - interferons

Introduction

Cholangiocarcinoma (CCA), a lethal cancer of the biliary tract, is the second most common type of primary liver cancer (Parkin et al., 1993). This cancer is associated with poor prognosis and limited treatment options. Although cholangiocarcinoma is a rare cancer, its incidence is typically more prevalent in Asian countries with the highest incidence rate in the Northeast Thailand (Vatanasapt et al., 1990; Parkin et al., 1993; Khan et al., 2008). The major risk factors of this cancer associate with chronic inflammation of the bile duct epithelial cells, and partial obstruction of bile flow caused by various conditions such as primary sclerosing cholangitis, hepatolithiasis and infestation by liver fluke (Opisthorchis viverrini) (Kubo et al., 1995; Sirica, 2005; Sripa et al., 2007).

Similar to many other cancers, cholangiocarcinoma development is a multistep process with accumulations of genetic and epigenetic changes in regulatory genes. The proposed pathways contributing in the development of cholangiocarcinoma include: 1) self-sufficiency and proliferation, 2) apoptosis resistance, 3) escape from senescence, and 4) tumor invasiveness and metastasis (Lazaridis and Gores, 2005). Cellular senescence is considered to be one of the tumor suppressor mechanisms which are programmed to control cells from aberrant proliferation and provide a barrier to cancer progression (Collado et al., 2005; Munoz-Espin and Serrano, 2014). Abrogation of cellular senescence barrier is, therefore, one of the hallmarks of cancer development. Senescent cells can be characterized by 1) the irreversible cell cycle arrest, 2) changes of cell morphology (cell enlargement and flat), 3) unresponsive to mitotic signals yet remain metabolically active, 4) altered gene expression patterns, and 5) the expression of senescence-associated β-galactosidase (SA-β-gal) (Dimri et al., 1995). Conversely, apart from its role in tumor suppression, characteristics of senescent cells as the senescence-associated secretory phenotype influence the secretion of extracellular matrix and cytokines which can promote tumorigenesis (Kuilman et al., 2008; Coppe et al., 2010; Di et al., 2014).

The interferon signaling pathway has been shown to play a role in cellular senescence (Kulaeva et al., 2003; Fridman et al., 2006). The levels of cytokines are upregulated in senescent cells induced by treatment with various agents such as genotoxic drugs, interferons, and also naturally senescing (Xin et al., 2004; Kim et al., 2009; Novakova et al., 2010). Combined IFNγ and TNF secreted from T-helper-1 cell (Braumuller et al., 2013) and...
IFNγ alone (Hubackova et al., 2015) were demonstrated to induce cell senescence in human cancer cells. Study of cellular senescence in cholangiocarcinoma cells is limited and the involvement of interferon signaling pathway in cellular senescence mechanism of cholangiocarcinoma cells is not well understood. Therefore, we aimed to investigate the role of different agents, including demethylating agent (5-aza-2’-deoxycytidine), genotoxic drug (bromodeoxyuridine), interferons (IFNβ and IFNγ), and oxidative stress (hydrogen peroxide), in the induction of cellular senescence and to examine gene expression changes of interferon signaling related genes in cholangiocarcinoma cell lines. We found that cellular senescence can be induced in cholangiocarcinoma cells by all agents tested with different degree of growth inhibition and senescence induction. Genotoxic drug is the strongest senescence induction agent in these cells.

Materials and Methods

Cell culture

Three cholangiocarcinoma cell lines, KKU100, HuCCA1, RMCCA1, were cultured in HAM’s F12 medium (Hyclone, Thermo Scientific, Utah, USA) supplemented with 10% FBS and 1% penicillin/streptomycin and immortalized biliary epithelial cells MMNK1 were cultured in DMEM medium (Hyclone, Thermo Scientific, USA) supplemented with 10% FBS and 1% penicillin/streptomycin. All cells were maintained at 37°C in 5% humidified CO₂.

Treatments of cholangiocarcinoma cells with 5-aza-2’-deoxycytidine, bromodeoxyuridine, interferons and hydrogen peroxide

All four cell lines were treated with 5-aza-2’-deoxycytidine (5-aza-dC) (Sigma-Aldrich, MO, USA), IFNβ, IFNγ (Gibco, Life technology, MD, USA), bromodeoxyuridine (BrdU) (Sigma-Aldrich, MO, USA) and hydrogen peroxide (H₂O₂) (Merck, Hohenbrunn, Germany) for one week. Briefly, cells (4 x 10⁵ cells in 10-cm dish) were seeded and treated with 5-aza-dC, BrdU, IFNβ, IFNγ next day and every other day for one week, and then cells were observed and harvested for further assays. Cells were treated with H₂O₂ for 2 hours, then the culture media were changed and cells were cultured for the next seven days.

Senescence-associated β-galactosidase assay

Treated cells were stained for SA-β-gal activity by senescence detection kit (BioVision, Moutain View, CA, USA). Cells were prepared in 6-well plate and treated with agents as described above. Treated cells were washed twice with phosphate-buffered saline (PBS) and fixed with fixative solution followed by staining overnight with staining solution. The SA-β-gal-positive cells (blue cells) and total cells were counted from five randomly selected areas on each well using 20x objective.

Colony formation assay

After treatment, cells were seeded at low density on 10-cm culture dishes and cultured for another 11 days. The colonies were washed twice with PBS, fixed with 4 ml cold methanol for 5 minutes, air dried, and stained with 0.5% aqueous crystal violet (Sigma-Aldrich, MO, USA) for 30 minutes. The plates were then rinsed with tap water and air dried. Colony numbers were counted.

Gene expression analysis by quantitative real-time polymerase chain reaction

Treated and untreated cells were harvested by trypsinization, counted and washed with PBS. Total RNA was isolated by RNAeasy mini kit (QIAGEN, Hilden, Germany) and quantified by NanoDrop 1000 spectrophotometer. cDNA was prepared from 1 μg of total RNA using SuperScript III First-strand synthesis system (Invitrogen, Carisbad, CA, USA). The expression of genes from table 1 were investigated by quantitative real-time polymerase chain reaction (qRT-PCR) method using Power SYBR Green PCR Master Mix (Applied Biosystem, Austin, TX, USA) and analyzed by Applied Biosystem Step One Plus Real-Time PCR system. The expression of GAPDH was used as an endogenous control. The experiments were performed at least three independent experiments.

Western blot analysis

Cells were washed with cold PBS and collected by scraping after adding cold lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP40, 0.25% Na-deoxycholate, 1 mM EDTA) containing protease cocktail inhibitor (Sigma-Aldrich, MO, USA). Cell lysates were centrifuged at 14,000 rpm for 15 minutes. Equal amounts of protein were electrophoresed on 10% or 12% polyacrylamide gels, and transferred onto immobilon.
PVDF transfer membranes (Milipore Corporation, Bedford, MA, USA). The membrane was blocked in 5% dry milk in Tris-buffered saline tween-20 (TBST). Proteins were detected by incubating with primary antibodies diluted in 5% dry milk in TBST for 2 hours following by 1 hour incubation with secondary antibodies. The following primary and secondary antibodies were used; p21 (at 1:2000, cell signaling), IFI16 (at 1:1000, Santa Cruz), OAS1 (at 1:1000, Santa Cruz), anti-mouse antibody (at 1:2000, cell signaling). Finally, the proteins were detected by autoradiography using ECL plus western blotting detection system (GE Healthcare, UK).

Statistical analysis

The bar graphs are reported as the mean ± standard deviation. The student’s two-tailed t-test for independent samples was used to test the statistic significant of the effects of treated and untreated cells on SA-β-gal activity and colony formation assay. A p-value ≤ 0.05 was considered to be statistically significant.

Figure 2. Colony Formations are Diminished in all four Cell Lines Treated with 5-aza-dC, BrdU, IFNβ, IFNγ, and H2O2 with Different Inhibitory Effects. After treatments with different agents for one week, cells were seeded at low density and cultured for another 11 days before staining. Figure 3a shows colonies of treated and untreated cells stained with crystal violet and figure 3b demonstrates the relative colony number of treated cells compared to untreated cells. The data represent mean ± SD. Student’s t-test was performed to test the effects of treated and untreated cells. ** p<0.01; * p<0.05

Figure 3. Colony formations are diminished in all four cell lines treated with 5-aza-dC, BrdU, IFNβ, IFNγ, and H2O2 with different inhibitory effects. After treatments with different agents for one week, cells were seeded at low density and cultured for another 11 days before staining. Figure 3a shows colonies of treated and untreated cells stained with crystal violet and figure 3b demonstrates the relative colony number of treated cells compared to untreated cells. The data represent mean ± SD. Student’s t-test was performed to test the effects of treated and untreated cells. ** p<0.01; * p<0.05

Figure 4. Protein expressions of cells treated with 5-aza-dC, BrdU, IFNβ, IFNγ, and H2O2. p21, OAS1, IFI16 were detected and tubulin was used as a loading control. p21 levels were highly increased in MMNK1 and KU100 cells, especially 5-aza-dC and BrdU treated cells. Treatments of IFNβ and IFNγ upregulated IFI16 and OSA1 protein levels in all four cell lines.

Figure 2.

Figure 3.

Figure 4.
Table 2. Gene expression analysis by qRT-PCR of cells treated with 5-aza-dC, BrdU, IFNβ, IFNγ, and H₂O₂. All four cell lines were treated with different agents for one week and the expression of genes were detected. The data represent mean fold changes ± SD of gene expressions from three independent experiments. The fold changes that are greater than 1.5 are shown.

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Table Supplementary 1. Sequences of primers for qRT-PCR

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Results

Treatments of cholangiocarcinoma cell lines with 5-aza-dC, BrdU, IFNβ, IFNγ, and H2O2 decreased cell growth and induced cellular senescence.

Treatments of cholangiocarcinoma cell lines (KKU100, HuCCA1, and RMCCA1) and immortalized biliary cells (MMNK1) with different agents including 5-aza-dC, BrdU, IFNβ, IFNγ, and hydrogen peroxide for one week noticeably decreased cell proliferations of all cell lines (Figure 1). The decrease of cell proliferation was less pronounced in KKU100 cells, which are characterized as poorly-differentiated hilar cholangiocarcinoma, compared to other cell lines. Moreover, cellular senescence was observed from cell morphology changes (cell enlargement) and positive staining of SA-β-gal activity (Figure 2). Notably, senescent cells were induced in MMNK1 and HuCCA1 (intrahepatic cholangiocarcinoma cells) more than other two CCA cell lines. Treatment of BrdU had a highest effect of senescence induction compared to other tested agents.

Colony formations were diminished in CCA cell lines treated with tested agents

Treatments of immortalized cholangiocytes and CCA cell lines with different agents decreased the ability of cells to form the colonies as shown in Figure 3. The degree of growth-inhibitory effects seen in these results was correlated with the ability of senescence induction in each cell line. As a result, BrdU and 5-aza-dC, which were able to highly induce senescence in most cell lines, significantly showed the highest effects of growth inhibition in colony formation assay.

Expressions of genes involved in cell cycle and interferon signaling were changed upon treatments of different stimuli in CCA cell lines

The expressions of cell cycle related genes (p16, p21, and CCNA2) and interferon signaling genes (IRF5, IRF9, IFI16, IFNB, IL6, ISG15, JAK1, MX1, OAS1, STAT1, and SOCS3) were examined by qRT-PCR upon treatments. The results of gene expression changes (Table 1) are shown as fold changes compared to untreated cells. The fold changes that are greater than 1.5 and consistent in at least three independent experiments are presented. p21 gene expressions were upregulated in all cell lines treated with all five agents excepted for RMCCA1 cells treated with BrdU. The expressions of interferon stimulated genes including ISG15, MX1, OAS1, and STAT1 were highly upregulated upon treatment of interferons, especially IFNβ. Interferon gamma-inducible protein 16 (IFI16) expression was also induced upon treatment with IFNβ and IFNγ. However, it is likely that the expressions of interferon related genes were not obviously increased in cells treated with BrdU and 5-aza-dC which showed the highest degree of senescence induction compared with interferons and hydrogen peroxide treatments. Interestingly, H2O2 treatment increased the expressions of IFNB gene in all cell lines.

Analysis of protein levels by western blotting

Protein levels of p21, OAS1 and IFI16 were examined to verify the results of mRNA expressions. p21 protein levels were highly increased in KKU100 cells treated with all tested agents and MMNK1 cells treated with 5-aza-dC and BrdU, which were consistent with the mRNA levels (Figure 4). Nevertheless, treatments of H2O2, IFNβ, and IFNγ did not noticeably stimulate the increase of p21 protein in MMNK1, HuCCA1 and RMCCA1 cell lines. IFI16 proteins were greatly increased after IFNβ and IFNγ treatments in all cell lines. Moreover, H2O2 treatment also induced IFI16 protein levels and treatment of 5-aza-dC, BrdU, IFNB, and IFNγ upregulated OAS1 protein levels in MMNK1, KKU100, and RMCCA1.
**Discussion**

Cellular senescence is the process that can act as a barrier of tumorigenesis. Thus, understanding the molecular mechanism of this pathway may provide the better knowledge of cholangiocarcinogenesis and help to improve treatment outcome. Treatments of demethylating agent (5-aza-dC), genotoxic drug (BrdU), interferons (IFNβ and IFNγ), and oxidative stress (hydrogen peroxide) decreased cell proliferation and colony formation in immortalized cholangiocyte cell line (MMNK1) and three CCA cell lines (KKU100, HuCCA1, and RMCCA1). Induction of cellular senescence, observed by staining of SA-β-gal activity and cell morphology changes, corresponded to the growth-inhibitory effect in all treated stimuli.

5-aza-dC is a DNA methyltransferase inhibitor that is widely used to demethylate DNA and restore gene expression (Haaf, 1995). Silencing of genes by DNA methylation at CpG island regions of most tumor suppressor genes is common in many types of cancers (Rountree et al., 2001). Wehbe et al showed that treatment of 5-aza-dC decreased cell proliferation and growth in soft agar in malignant cholangiocyte Mz-ChA-1 cells (Wehbe et al., 2006). In our study, treatment of 5-aza-dC induced cellular senescence and inhibited colony formation of CCA cells and immortalized biliary cells, with the highest effect in HuCCA1 cell line. Therefore, genes silenced by DNA methylation partly regulate senescence mechanism in cholangiocarcinoma cells.

BrdU is a thymidine analog that incorporates into DNA during the S phase and it was commonly used to quantitate proliferative index of various cell types (Struikmans et al., 1997). Several studies revealed the role of BrdU in senescence induction and growth inhibition in several cell types (Michishita et al., 1999; Suzuki et al., 2001). Our results demonstrated that BrdU significantly induced cellular senescence and inhibited colony formation in immortalized cholangiocytes and CCA cell lines and it showed the strongest effects compared to other tested agents. This indicated that cholangiocarcinoma cells can effectively induced to senesce by genotoxic agent.

Interferons are a family of cytokines with antiproliferative and antiviral activities. Treatment of IFNβ induces senescence through p53-dependent DNA damage signaling pathway in normal human fibroblast IMR-90 (Moiseeva et al., 2006) and in cutaneous human papilloma virus-transformed human keratinocytes (Chiantore et al., 2012). It is recently shown that DNA damage can induce endogenous IFN-β which further activates the p53-p21 signaling to promote cell senescence in mammalian cell (Yu et al., 2015). Here, we found that IFNβ and IFNγ treatments induced senescence in immortalized cholangiocyte MMNK1 cells (16.72% and 12.59%, respectively) and CCA cell lines (less than 10%). The antigrowth effects of IFNβ and IFNγ in colony formation assay were less pronounced than those of BrdU and 5-aza-dC treatments.

Oxidative stress is involved in the damage of DNA and other biomolecules resulting in development of cancer (Halliwell, 2007). However, sublethal doses of oxidative stress, hydrogen peroxide, were able to induce senescence in human diploid fibroblasts (Chen and Ames, 1994) and cancer cells (Yoshizaki et al., 2009). In this study, treatments of H2O2 in immortal biliary cells and CCA cell lines decreased cell proliferation and colony formation with less degree than those of other tested agents. The senescence inductions of H2O2 are slightly increased in all cell lines.

The expressions of p21, the cyclin-dependent kinase inhibitor, were upregulated in almost all cell lines treated with tested agents. However, confirmation by western blotting showed that p21 proteins were increased in most conditions but not all. Increased expression of p21 is one of the common characteristics of senescent cell (Vogt et al., 1998). The upregulation of p21 mRNA, but not p16 (data not shown), may indicate that the p53/p21 signaling controls the senescence mechanism rather than the p16 signaling cascade in cholangiocarcinoma cells. Recent studies showed that oxidative stress and activation of p21 are essential for p53-mediated senescence in head and neck cancer cells (Fitzgerald et al., 2015) and mouse biliary epithelial cells (Sasaki et al., 2008). In our study, upregulations of p21 expressions are likely to be regulated by epigenetic mechanism and DNA damage response pathway (upregulation upon treatment with demethylating and genotoxic agents, respectively).

In addition, the expressions of interferon-stimulated genes, including ISG15, MX1, and OAS1, were highly increased upon treatments of IFNβ and IFNγ. These results implied that the interferon signaling pathway in CCA cells is functionally induced. Increases of interferon signaling genes may be associated with growth inhibition and senescence induction. IFN-inducible protein 16 (IFI16), which was increased in gene and protein expression from this study, was shown to play a role in cellular senescence in human fibroblast (Xin et al., 2004) and human endothelial cells (Kim et al., 2009). IFI 16 expression is increased in old fibroblast (WI-38) compared with young cells and overexpression of IFI16 in immortalized cells inhibits cell proliferation and induces cellular senescence (Clarke et al., 2010).

We conclude that cellular senescence can be induced in immortalized cholangiocytes and CCA cell lines by different agents, suggesting that cellular senescence in cholangiocarcinoma cells is controlled by several mechanisms. The antigrowth effect and senescence induction vary among cell lines depending on cell type and origin. Immortalized cholangiocyte (MMNK1) and well-differentiated CCA cells (HuCCA1 and RMCCA1) appeared to have higher inhibitory effects than KKW100 cells which are classified as poorly-differentiated CCA cells in all agents treated. The induction of senescence in immortalized cholangiocytes and CCA cells by different stimuli including demethylating agent, genotoxic drug, interferons and oxidative stress is likely associated with the p53/p21 signaling pathway rather than p16 signaling. Finally, the interferon signaling pathway is partially involved in senescence mechanism of CCA cells.
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