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Proinflammatory cytokine-induced cellular senescence of biliary epithelial cells is mediated via oxidative stress and activation of ATM pathway: A culture study

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Abstract

Cellular senescence is reportedly involved in cholangiopathy in primary biliary cirrhosis and oxidative stress is proposed as a pathogenetic factor in biliary epithelial cells (BECs). This study investigated the involvement of proinflammatory cytokines (IFN-β, IFN-γ and TNF-α) and ataxia telangiectasia-mutated (ATM)/p53/ $p21^{WAF1/Cip1}$ pathway with respect to oxidative stress in cellular senescence of BECs. H_2O_2 treatment (oxidative stress) induced phosphorylation (activation) of ATM and p53 and also $p21^{WAF1/Cip1}$ expression in BECs. Treatment with inflammatory cytokines generated reactive oxygen species (ROS) in cultured BECs followed by activation of the ATM/p53/$p21^{WAF1/Cip1}$ pathway and the induction of cellular senescence. Pre-treatment with ATM inhibitor (2-aminopurine) and antioxidant (N-acetylcysteine) significantly blocked the cellular senescence of BECs induced by oxidative stress or inflammatory cytokines. In conclusion, proinflamamtory cytokines induce ROS generation and activate the ATM/p53/$p21^{WAF1/Cip1}$ pathway, followed by biliary epithelial senescence. This senescent process may be involved in the development of destructive cholangiopathy in humans.

Keywords: *Biliary epithelial cells, cytokine, cellular senescence, ataxia telangiectasia-mutated (ATM), oxidative stress*

Introduction

Cellular senescence is defined as a condition in which a cell no longer has the ability to proliferate. Senescent cells are irreversibly arrested at the G1 phase of the cell cycle, but remain metabolically active. Senescent cells display several characteristics, including cytologic changes [1,2], shortened telomeres, increased expression of $p16^{INK4}$ and $p21^{WAF1/Cip1}$ and increased activity of senescence-associated β-galactosidase (SA-β-gal) [3]. Oxidative stress is known as a factor causing cellular senescence [4,5].

Primary biliary cirrhosis (PBC) is an organ-specific autoimmune disease and presents with chronic, progressive cholestasis and liver failure [6–8]. PBC is characterized histologically as chronic non-suppurative destructive cholangitis (CNSDC) involving small bile ducts, eventually followed by extensive loss [7–9]. Recently, we demonstrated a cellular senescence of biliary epithelial cells (BECs) in damaged small bile ducts in PBC; these cells showed several senescent features such as the expression of SA-β-gal and the increased expression of $p16^{INK4}$ and $p21^{WAF1/Cip1}$, in addition to cellular features of cellular senescence [10]. Similar features of BECs are also reported in the bile ducts with chronic hepatic allograft rejection showing extensive bile duct loss [11]. These findings suggested that the cellular senescence may be involved in the pathogenesis of duct loss in immune-mediated cholangiopathy in PBC and chronic hepatic allograft rejection [10].

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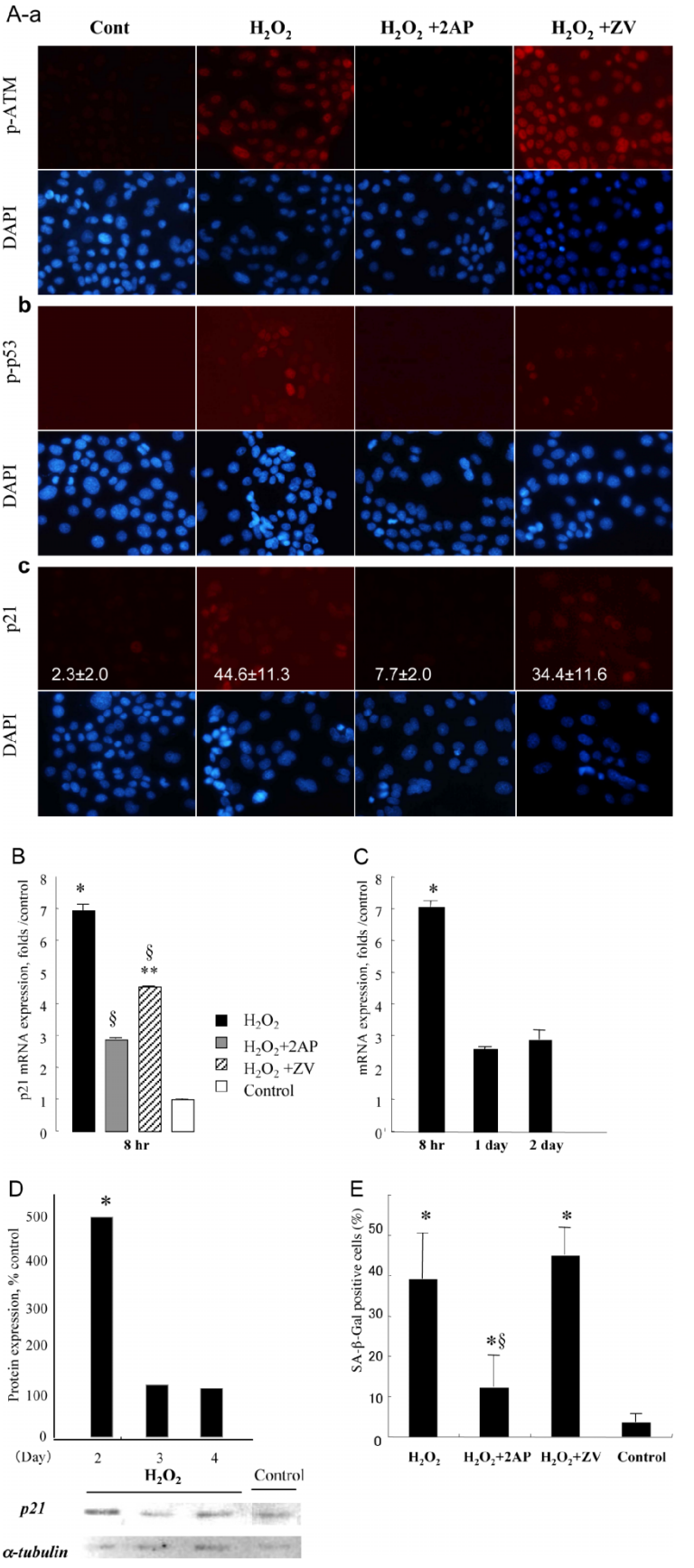

Figure 1 (*Continued*)

We have recently reported that a polycomb group repressor, Bmi1, may be related to the increased expression of $p16^{INK4}$ by oxidative stress in cultured BECs [12], however the signalling pathway regarding the induction of $p21^{WAF1/Cip1}$ by oxidative stress in the affected small bile ducts of PBC has not been fully clarified.

Recent reports suggested that the protein kinase ataxia -telangiectasia mutated (ATM)/p53/$p21^{WAF1/Cip1}$ pathway is involved in cellular senescence induced by oxidative stress [13,14] and is related to the cell fate decision of cells either to apoptosis or senescence [15]. Furthermore, inflammatory cytokines, such as interferon-β, induce cellular senescence via the ATM-signalling pathway [16]; however, it remains unexplored whether inflammatory cytokines and the ATM-signalling pathway are responsible for the induction of cellular senescence in BECs and also in damaged bile ducts in PBC.

We took advantage of mBECs, since non-neoplastic human BECs are not always conveniently available and the features of senescent mBECs are reportedly similar to those of senescent human BECs [11]. Immortalized human BECs [17] and cholangiocarcinoma cell lines [18] appear to be inappropriate for the present study, since SV40-T-antigen transformation and the genetic and epigenetic alterations in carcinoma cells directly affect the pathway of cellular senescence. In this study using cultured mouse BECs, we investigated whether proinflammatory cytokines are involved in the induction of oxidative-stress induced cellular senescence in BECs and whether the ATM/p53/$p21^{WAF1/Cip1}$ pathway is involved in the induction of cellular senescence in the cultured BECs due to cytokines and oxidative stress.

Materials and methods

Cell culture and treatments of mouse intrahepatic BECs

Mouse intrahepatic BECs (mBECs) were purified and cultured as described previously [19]. BECs during 10–15 passages were used, since the conditions of cells, such as viability and growth rate, were stable during 10–15 passages in a preliminary study. BECs were cultured for up to 8 days in experiments. The confluency of BECs was kept below 80% during each experiment. Recombinant cytokines (IFN-β, 200–2000 U/ml (Biosource, Camarillo, CA); IFN-γ, 100–1000 U/ml (R&D Systems, Minneapolis, MN); TNF-α, 1–10 μg/ml; (R&D Systems)) were used for the treatment of BECs in several experiments. mBECs were treated with H_2O_2 (100 μM) for 2 h, washed throughly to remove H_2O_2 and cultured in fresh medium. For treatment with 2-aminopurine (2-AP) as the ATM inhibitor [15], BECs were incubated with 2-AP (10 mM) (Sigma, St. Louis,

Figure 1. Oxidative stress induces the phosphorylation of ATM (ser1981) and p53 (ser15), $p21^{WAF1/Cip}$ expression and cellular senescence in cultured biliary epithelial cells. (Aa) Phosphorylated ATM (ser1981) labelled by red fluorescence was evidently detected in the nuclei of BECs after H_2O_2 treatment. In the experiment with H_2O_2 (100 μM, 2 h) + pre-treatment with 2-aminopurin (AP) (10 mM), ATM inhibitor, the phosphorylaiton of ATM was markedly decreased, while H_2O_2 + pre-treatment with Z-VAD (20 μM), pan-caspase inhibitor, failed to show an inhibitory effect on phosphorylation. Eight hours after H_2O_2 treatment. Immunofluorescence for phosphorylated ATM (ser1981). (Ab) Phosphorylated p53 (ser15) labelled by red fluorescence was expressed in the nuclei of BECs treated with H_2O_2 (100 μM, 2 h). Expression of phosphorylated p53 in the nuclei was clearly blocked by H_2O_2 + pre-treatment with 2-AP (10 mM), but such a block was incomplete by Z-VAD (20 μM). One day after H_2O_2 treatment. Immunofluorescence for phosphorylated p53 (ser15). (Ac) Expression of $p21^{WAF1/Cip1}$ labelled by red fluorescence in the nuclei of BECs was significantly increased after H_2O_2 treatment (100 μM, 2 h) (labelling index (LI), 44.6 ± 11.3%; control, 2.3 ± 2.0%) ($p < 0.01$). This increased expression of $p21^{WAF1/Cip1}$ was significantly inhibited by H_2O_2 + pre-treatment with 2-AP (10 mM), (LI, 7.7 ± 2.0%) ($p < 0.01$), but not by pre-treatment with Z-VAD (20 μM) (LI, 34.4 ± 11.6%). The LI of $p21^{WAF1/Cip1}$ (percentage of positive cells in at least 1000 cells counted in each group) is expressed as the mean ± standard deviation. Two days after H_2O_2 treatment. Immunofluorescence for $p21^{WAF1/Cip1}$. Lower row is a nuclear counterstain with DAPI. H_2O_2+AP, pre-treatment with 2-AP (10 mM); H_2O_2+ZV, pre-treatment with Z-VAD (20 μM). (B) $p21^{WAF1/Cip1}$ mRNA expression was significantly high (7-fold the control) in BECs 8 h after H_2O_2 treatment (100 μM, 2 h). This increased expression of $p21^{WAF1/Cip1}$ mRNA was significantly blocked by pre-treatment with 2-AP and Z-VAD. Expression of mRNA was quantified with real-time PCR. The expression of $p21^{WAF1/Cip1}$ is shown as a ratio using GAPDH as a housekeeping gene. Data are expressed as the mean ± standard deviation. $N = 3$ for each group. * $p < 0.01$ compared to H_2O_2+AP, H_2O_2+ZV and control; § $p < 0.01$ compared to control; **, $p < 0.01$ vs H_2O_2+AP. H_2O_2+AP, pre-treatment with 2-AP (10 mM); H_2O_2+ZV, pre-treatment with Z-VAD (20 μM). (C) Expression of $p21^{WAF1/Cip1}$ mRNA in BECs which was high (7-fold the control) 8 h after H_2O_2 treatment (100 μM, 2 h), decreased on day 1 and 2 after H_2O_2 treatment (100 μM, 2 h). Expression of mRNA was quantified with real-time PCR. The expression of $p21^{WAF1/Cip1}$ mRNA is shown as a ratio using GAPDH as a housekeeping gene. Data are expressed as the mean ± SD. $N = 3$ for each group. * $p < 0.01$ compared with days 1 and 2, respectively. (D) Immunoblot analysis for $p21^{WAF1/Cip1}$ protein expression in BECs treated with H_2O_2 (100 μM, 2 h). $p21^{WAF1/Cip1}$ protein expression was high on day 2 after H_2O_2 treatment and then decreased to the control level thereafter. Protein samples prepared in three independent experiments were used and the analysis was performed twice. Intensity of each band in immunoblot analysis was quantified by densitometry, shown as a ratio using α-tubulin as an internal control. * $p < 0.01$, compared to control. (E) Percentage of BECs positive for senescence associated-β-galactosidase activity (SA-β-gal) was significantly higher in BECs treated with H_2O_2 (100 μM, 2 h) (percentage of SA-β-gal positive cells, 39.04 ± 11.25%), when compared with the control (3.58 ± 1.94%) ($p < 0.01$). Pre-treatment with 2-aminopurine (AP) significantly decreased SA-β-gal-positive cells (12.24 ± 7.78%), when compared with BECs with H_2O_2 treatment alone. Pre-treatment with Z-VAD (20 μM) failed to decrease SA-β-gal-positive cells (44.88 ± 6.80%), when compared with BECs with H_2O_2 treatment alone. Cellular senescence was assessed by expression of SA-β-gal on day 6 after H_2O_2 treatment + pre-treatment with 2-AP or Z-VAD. Three independent experiments were performed. Data are expressed as the mean ± standard deviation. * $p < 0.01$ compared to the control. § $p < 0.01$ compared to H_2O_2 treatment.

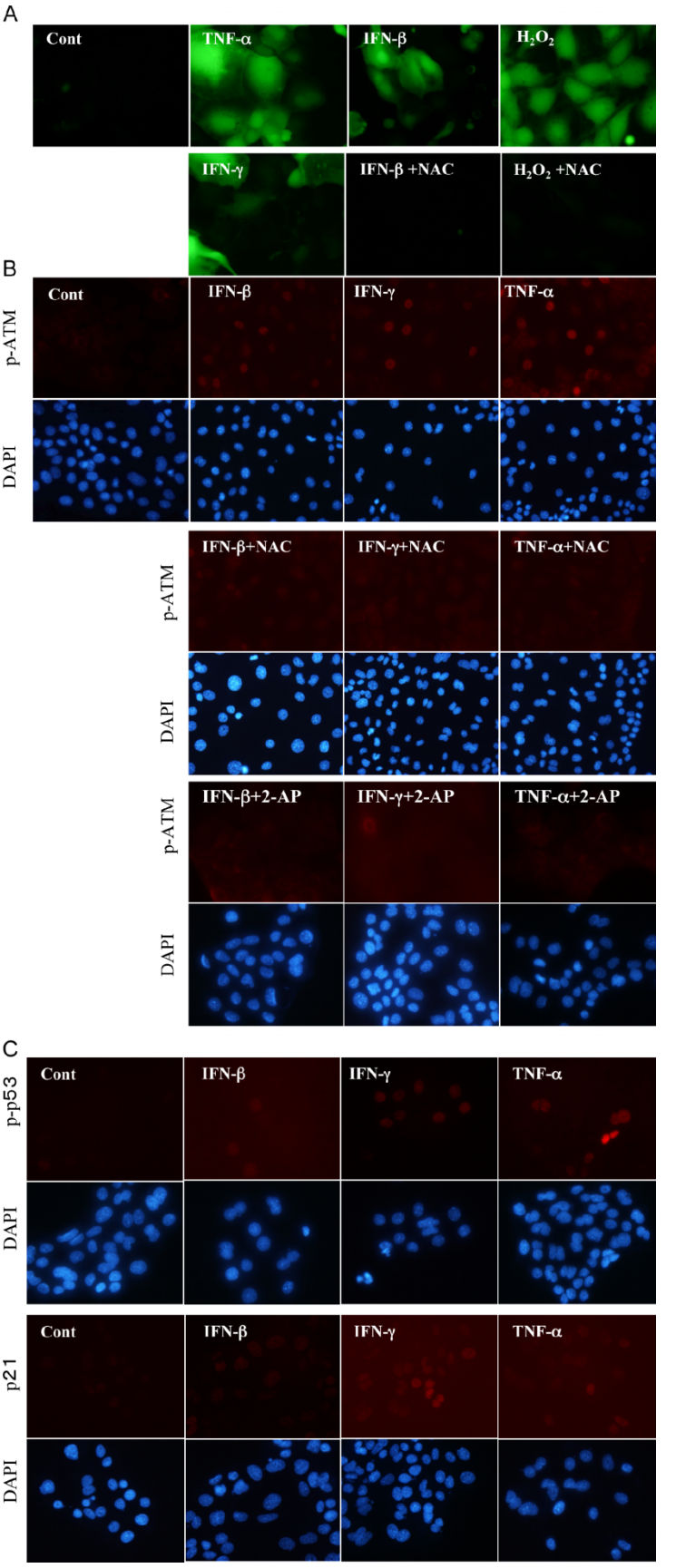

Figure 2 (*Continued*)

MO) 30 min before H_2O_2 treatment and incubated during experiments. Pan-caspase inhibitor Z-VAD-FMK (Z-VAD, Calbiochem, San Diego, CA) was used to examine the participation of the apoptotic process in the comparison with cellular senescence. Z-VAD (20 μM) was added to cell cultures for 30 min before H_2O_2 treatment and incubated during experiments. N-acetylcystein (NAC, Sigma) (5 mM) was also used in pre-treatment as an antioxidant.

Detection of reactive oxygen species (ROS)

To measure ROS, BECs were incubated with dichlorodihydrofluorescein diacetate (H_2DCFDA) (Molecular Probes, Eugene, OR). Fluorescence was monitored by immunofluorescence microscopy.

Immunofluorescence for cultured cells

The mBECs were grown in a Lab-Tek chamber and immunocytochemical detection was performed as described previously [12] using primary antibodies for phosphorylated-ATM at serine 1981 (p-ATM) (clone 10H11.E12, Upstate, Lake Placid, NY), phosphorylated p-53 at serine 15 (p-p53 (Ser15)) (rabbit polyclonal, Cell Signaling, Beverly, MA) and $p21^{WAF1/Cip1}$ (clone sx118, Dako, Glostrup, Denmark). The labelling index of $p21^{WAF1/Cip1}$ was the percentage of positive cells in at least 1000 cells counted in each group.

RNA extraction and real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the cells and real-time quantitative RT-PCR analysis for $p21^{WAF1/Cip}$ mRNAs was performed as described previously [12]. Sequence-specific primers and probes for mouse $p21^{WAF1/Cip}$ and GAPDH as the internal control were purchased from PE Applied Biosystems (Warrington, UK).

Immunoblotting

The cell lysate samples (15 μl) were solubilized, resolved by SDS-PAGE and transferred to a nitrocellulose membrane as described previously [20]. After transfer, the membranes were processed for immunoblotting as described previously [20]. The primary antibody for $p21^{WAF1/Cip1}$ was described above. Mouse monoclonal anti-α-tubulin (clone TU-01, Zymed, South San Francisco, CA) was used as an internal control and the protein expression of $p21^{WAF1/Cip1}$ was quantified relative to α-tubulin by using NIH image.

Assays for cellular senescence

Cellular senescence was assessed by SA-β-gal activity. SA-β-gal activity was detected by using the senescence detection kit (BioVision, Mountain View, CA) according to the manufacturer's protocol [3]. The proportion of senescent cells in each condition was assessed on day 6 by counting the percentage of SA-β-gal-positive cells in at least 1000 total cultured BECs counted using light microscopy in each experiment.

Cell growth and BrdU incorporation

Cell proliferation activity was assessed on day 4 after treatment by using a 5-bromo-2′-deoxy-uridine (BrdU) Labelling and Detection Ki1 I (Roche, Nonenwald, Germany), according to the manufacturer's protocol. The nuclei were simultaneously stained with DAPI. At least 1×10^3 total cells were checked and counted to assess the BrdU-labelling index with a conventional fluorescence microscope (Olympus).

Statistics

Statistical analysis of the difference between the *in vitro* assays was performed with Student's *t*-test. When the *p*-value was less than 0.05, the difference was considered significant.

Results

Oxidative stress immediately increased $p21^{WAF1/Cip}$ expression via phosphorylation (activation) of ATM and p53 and induced cellular senescence in cultured BECs

Immunofluorescence study showed that the treatment with H_2O_2 (100 μM) clearly phosphorylated ATM at serine 1981 in cultured BECs and

Figure 2. Cytokines induce the generation of reactive oxygen species (ROS) and activation of the ATM pathway in cultured biliary epithelial cells (BECs). (A) Treatment with IFN-β (2000 U/ml), IFN-γ (1000 U/ml) or TNF-α (10 μg/ml) induced ROS generation labelled by green fluorescence in the cytoplasm of BECs. H_2O_2 treatment also yielded a positive signal of ROS in BECs. Pre-treatment with N-acetylcystein (NAC) (5 mM), an antioxidant, blocked ROS generation in these cells. One day after treatment with each cytokine or H_2O_2. Fluorescence for ROS sensor H_2DCFDA. (B) Treatment with IFN-β (2000 U/ml), IFN-γ (1000 U/ml) or TNF-α (10 μg/ml) induced the nuclear expression of phosphorylated ATM (ser1981) labelled by red fluorescence in BECs. Pre-treatment with NAC (5 mM, antioxidant) and 2-aminopurine (2AP, ATM inhibitor, 10 mM) blocked the phosphorylation (activation) of ATM. One day after treatment with each cytokine or H_2O_2. Immunofluorescence for phosphorylated ATM (ser1981). (C) Phosphorylated p53 (ser15) labelled by red fluorescence was expressed in the nuclei of BECs treated with IFN-β (2000 U/ml), IFN-γ (1000 U/ml) or TNF-α (10 μg/ml). One day after treatment. Expression of $p21^{WAF1/Cip1}$ labelled by red fluorescence also increased after treatment with IFN-β (2000 U/ml), IFN-γ (1000 U/ml) or TNF-α (10 μg/ml). Two days after treatment. Immunofluorescence for phosphorylated p53 (ser15) and $p21^{WAF1/Cip1}$. Lower row is a nuclear counterstain with DAPI.

pre-treatment with the ATM inhibitor, 2-AP, blocked this phosphorylation of ATM, but not by pan-caspase inhibitor (Z-VAD) (Figure 1A). P53 was phosphorylated at serine 15 by H_2O_2 treatment (Figure 1A) and the phosphorylation of p53 was blocked by the pre-treatment with 2-AP (Figure 1A).

The $p21^{WAF1/Cip1}$ protein and mRNA expression was significantly increased in cultured BECs after H_2O_2 treatment ($p < 0.05$) and the increased expression was significantly inhibited by pre-treatment with 2-AP ($p < 0.05$) (Figures 1A and B). Pre-treatment with Z-VAD failed to show such a clear block. Real-time PCR showed that $p21^{WAF1/Cip1}$ mRNA expression was immediately increased to 7.00 ± 0.19-fold 8 h after H_2O_2 treatment and gradually decreased thereafter (Figure 1C). Immunoblot analysis showed that $p21^{WAF1/Cip1}$ protein expression followed $p21^{WAF1/Cip1}$ mRNA expression after 1–2 days, as expected; that is $p21^{WAF1/Cip1}$ protein expression was transiently high on day 2 after H_2O_2 treatment and fell to the control level thereafter (Figure 1D). These findings suggest that ATM signalling is involved in the induction of $p21^{WAF1/Cip1}$ mRNA and protein expression in BECs induced by H_2O_2 treatment.

Senescent cells labelled by SA-β-gal were significantly increased in BECs with H_2O_2 treatment ($39.04 \pm 11.25\%$), when compared with control BECs ($3.58 \pm 1.94\%$) ($p < 0.01$) (Figure 1E). Pre-treatment with 2-AP ($12.24 \pm 7.78\%$) significantly prevented the cellular senescence induced by H_2O_2 treatment ($p < 0.01$). There was no difference in the pre-treatment group with Z-VAD ($44.88 \pm 6.80\%$) for the blockage of apoptosis and the group with H_2O_2 treatment alone ($p > 0.05$).

Cytokines induced the generation of ROS, activation of the ATM pathway and cellular senescence in cultured BECs

To examine the generation of ROS induced by cytokines, we stained the cells with H_2DCFDA, a compound that fluoresces in the presence of high levels of ROS. We found that cytokines (IFN-β, IFN-γ and TNF-α) generated reactive oxygen species (ROS) in cultured BECs (Figure 2A). To prove the role of ROS in the signalling pathway activated by these cytokines, we pre-treated the cells with antioxidant, NAC. As expected, pre-treatment with NAC blocked the generation of ROS. When examined by immunocytochemistry, these cytokines activated ATM, the phosphorylation of p53 at serine 15 and the induction of $p21^{WAF1/Cip1}$ expression (Figure 2A). Pre-treatment with NAC or 2-AP (ATM inhibitor) blocked the phosphorylation of ATM (Figure 2B). These findings suggest that cytokines induced ROS and this was followed by phosphorylation of the ATM pathway in BECs.

Cellular senescence of mBECs treated with cytokines (IFN-β, IFN-γ and TNF-α) was assessed by the detection of SA-β-gal. As shown in Figure 3A, the SA-β-gal labelling index was significantly higher in BECs with cytokine treatment (IFN-β, $40.50 \pm 6.77\%$; IFN-γ, $35.60 \pm 11.34\%$; and TNF-α, $33.21 \pm 8.73\%$) and H_2O_2 ($38.00 \pm 12.35\%$), when compared with control BECs ($6.67 \pm 3.67\%$) ($p < 0.01$). The BrdU labelling index on day 4 after the treatment was significantly lower in BECs treated with cytokines (IFN-β,

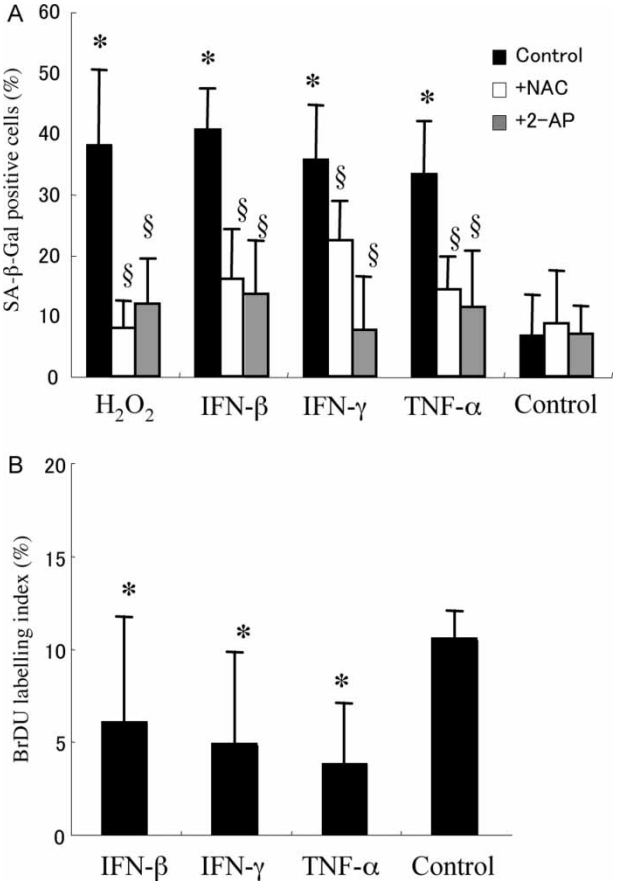

Figure 3. Antioxidant and ATM inhibitor blocked cytokine-induced cellular senescence in cultured biliary epithelial cells (BECs). (A) Percentage of cells positive for senescence associated-β-galactosidase activity (SA-β-gal) was significantly higher in BECs treated with IFN-β, IFN-γ, TNF-α or H_2O_2, when compared with the control (Cont), respectively. Pre-treatment with antioxidant N-acetylcystein (NAC, 5 mM) and ATM inhibitor, 2-aminopurin (2-AP, 10 mM) significantly decreased the percentage of SA-β-gal-positive cells, when compared with BECs with either cytokines or H_2O_2 treatment alone. Data are expressed as the mean $\pm$ standard deviation. * $p < 0.01$ compared to the control. § $p < 0.01$ between the presence and absence of pretreatment with NAC. Cellular senescence was assessed by SA-β-gal on day 6 after treatment with IFN-β (2000 U/ml), IFN-γ (1000 U/ml), TNF-α (10 μg/ml) or H_2O_2 (100 μM, 2 h) with and without pre-treatment with NAC (5 mM) or 2-AP (10 mM). (B) Cell proliferation was assessed by 5-bromo-2′-deoxyuridine (BrdU) assay. The labelling index of BrdU was significantly lower in BECs treated with cytokines (IFN-β, $5.97 \pm 5.71\%$; IFN-γ, $4.81 \pm 4.97\%$; and TNF-α, $3.75 \pm 3.30\%$), when compared with control mBECs ($10.49 \pm 1.50\%$) ($p < 0.05$). Data are expressed as the mean $\pm$ SD. * $p < 0.01$ and ** $p < 0.05$ compared to the control. Day 4 after treatment.

$5.97 \pm 5.71\%$; IFN-γ, $4.81 \pm 4.97\%$; and TNF-α, $3.75 \pm 3.30\%$), when compared with control mBECs ($10.49 \pm 1.50\%$) ($p < 0.05$) (Figure 3B). These findings suggest that treatment with cytokines may decrease cell proliferation of mBECs by the induction of cellular senescence. Pre-treatment with NAC or 2-AP (ATM inhibitor) significantly blocked cellular senescence in BECs induced by IFN-β, IFN-γ and TNF-α and H_2O_2 ($p < 0.01$) (Figure 3A).

Discussion

Oxidative stress is suggested to be involved in the pathogenesis of the cellular senescence of BECs of CNSDC in PBC [10,21,22], however, the generator of oxidative stress and the signalling pathway involved have not been fully clarified. It is well known that a number of proinflammatory cytokines such as TNF-α and IFN-γ were increased in the inflammatory lesions around the damaged bile ducts in PBC [21,23,24]. Accumulating data suggest that ROS is a critical chemical mediator of cell signalling induced by cytokines such as IFN-β [16] and TNF-α [25]. Furthermore, the ATM/p53/p21$^{WAF1/Cip1}$ pathway is suggested to be involved in cellular senescence related to oxidative stress [13–15,26]. Taking this into consideration, we examined whether the proinflammatory cytokines and the ATM-signalling pathway are involved in the induction of oxidative stress-induced cellular senescence in cultured BECs.

First, it was found in this study that the ATM-signalling pathway was involved in oxidative stress-induced cellular senescence in BECs; that is, oxidative stress induced phosphorylation of ATM at Ser-1981, p53 at Ser-15 and immediately increased the expression of p21$^{WAF1/Cip1}$ and also cellular senescence in cultured BECs. The ATM inhibitor significantly prevented oxidative stress-induced cellular senescence, clearly suggesting the involvement of the ATM pathway. Reportedly, p21$^{WAF1/Cip1}$ also plays a role in the induction of cellular senescence, while p16^{INK4a} is important for the maintenance of cellular senescence [27–29]. Mitogenic signalling and the p16^{INK4a}-Rb pathway cooperate to enforce irreversible cellular senescence [30]. Recent reports showed that the ability of the cell to relieve senescent cell cycle arrest of reverse senescence depends on the expression levels of p16 ^{INK4a} [15,31,32]. Zhang et al. [15] also demonstrated that the inhibition of ATM can reverse senescence that expresses no or a low level of p16^{INK4a}. We have recently reported that decreased bmi1 expression in damaged small bile ducts in PBC may be responsible for the subsequent increased expression of p16^{INK4a} and the development of cellular senescence [12]. Taken together, it is likely that oxidative stress causes the immediate increase of p21$^{WAF1/Cip1}$ via ATM signalling for the induction of cellular senescence and then bmi1-dependent increase of p16 for the establishment of cellular senescence.

Secondly, this study revealed that inflammatory cytokines, IFN-β, IFN-γ and TNF-α, generate ROS, activate the ATM-signalling pathway and the subsequent induction of cellular senescence in BECs. The decreased cell proliferation rate after treatment with cytokines was compatible with the induction of cellular senescence by cytokines. The antioxidant (NAC) and the ATM inhibitor (2-AP) prevented cytokine-induced cellular senescence, suggesting the involvement of ROS and the ATM pathway in cytokine-induced cellular senescence. TNF-α and IFN-γ were increased in inflammatory lesions around the damaged bile ducts in PBC [21,23,24]. It was reported that the administration of IFN-β induced cellular senescence in fibroblasts [16]; therefore, it is conceivable that inflammatory cytokines play a role in the generation of endogenous ROS and therefore the induction of cellular senescence in BECs.

In conclusion, activation of the ATM/p53 pathway by proinflammatory cytokines and oxidative stress is involved in the increased expression of p21$^{WAF1/Cip1}$ in PBC and then in the induction of cellular senescence in BECs. This may be at least partly responsible for the progressive bile duct loss in PBC.

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