Differential Effects of TNF-alpha on the Viability of Cholangiocarcinoma Cell Lines

ความแตกต่างของผลกระทบต่อการอยู่รอดของมะเร็งท่อน้ำดีจากการกระตุ้นด้วยทูเมอร์เนโครซิสแฟคเตอร์อัลฟา

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Abstract

The objective of this study is to investigate the sensitivity of cholangiocarcinoma (CCA) cells to TNF-alpha-induced apoptosis. Two cell lines established from Thai patients with cholangiocarcinoma, KKU-100 and KKU-M213, expressed TNF-alpha receptors I (TNFRI) and II (TNFRII) as shown by RT-PCR. These cells were subsequently subjected to a high dose of TNF-alpha (160 ng/ml) for 24 hours before the cytotoxicity was assessed by MTT assay. Neither KKU-100 nor KKU-M213 showed any sign of cytotoxicity by TNF-alpha, despite the presence of TNF-alpha receptors. Lack of effects on cell survival by TNF-alpha was further confirmed by DAPI staining which showed absence of condensed and fragmented nuclei, characteristics of apoptotic cells. These data indicated that the cholangiocarcinoma cell lines were resistant to apoptosis induced by TNF-alpha.

Keywords : Cholangiocarcinoma, TNF-alpha, sensitive or resistance

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บทคัดย่อ

งานวิจัยในครั้งนี้มีจุดประสงค์ในการทดสอบผลของทูเมอร์เนโครซิสแฟคเตอร์อัลฟา (tumor necrosis factor-alpha) ต่อความไวต่อการเกิดอะโพโทซิส (apoptosis) ในเซลล์มะเร็งท่อน้ำดี โดยทำการทดสอบในเซลล์มะเร็งท่อน้ำดีเพาะเลี้ยงของคนไทยสองชนิด คือ KKU-100 และ KKU-M213 เมื่อทดสอบโดยวิธี RT-PCR พบว่ามีแสดงออกของตัวรับ (receptor) ทูเมอร์เนโครซิสแฟคเตอร์อัลฟา ทั้ง TNFRI และ TNFRII เซลล์มะเร็งท่อน้ำดีทั้งสองชนิดนี้ถูกนำไปบ่มกับทูเมอร์เนโครซิสแฟคเตอร์อัลฟาที่ความเข้มข้น 160 ng/mL เป็นเวลา 24 ชั่วโมง และทดสอบความเป็นพิษต่อเซลล์โดยใช้วิธี MTT พบว่าทูเมอร์เนโครซิ-สแฟคเตอร์อัลฟาไม่ทำให้เกิดความเป็นพิษต่อเซลล์เพาะเลี้ยงทั้งสองชนิด แม้ว่าจะพบตัวรับต่อทูเมอร์เนโครซิสแฟคเตอร์อัลฟา นอกจากนี้ การทดสอบยืนยันผลของทูเมอร์-เนโครซิสแฟคเตอร์อัลฟาต่อการอยู่รอดโดยวิธีการย้อมนิวเคลียสด้วย DAPI ในเซลล์มะเร็งท่อน้ำดี พบว่า ทูเมอร์เนโครซิสแฟคเตอร์อัลฟาไม่สามารถกระตุ้นให้เกิดลักษณะเซลล์หดตัว และการแตกของนิวเคลียส ซึ่งเป็นลักษณะของอะโพโทซิสในเซลล์มะเร็งท่อน้ำดีเพาะเลี้ยงทั้งสองชนิดเช่นกัน ดังนั้นจึงสรุปได้ว่า เซลล์มะเร็งท่อน้ำดีทั้งสองชนิดมีความต้านทานต่อการเกิดอะโพโทซิสในภาวะที่กระตุ้นด้วยทูเมอร์เนโครซิสแฟคเตอร์อัลฟา

คำสำคัญ : มะเร็งท่อน้ำดี ทูเมอร์เนโครซิสแฟคเตอร์อัลฟา ความไว หรือ ความต้านทาน

Introduction

Cholangiocarcinoma (CCA) is a malignant tumor derived from the bile duct epithelium, with a rising incidence rate worldwide in recent years (Wiedmann and Mssner. 2010 : 834). Infection by *Opisthorchis viverrini* and chronic inflammation have been implicated in the pathogenesis and progression of CCA. High mortality rate was due to limited detection, along with resistance to chemotherapeutic agents and radiotherapy (Wiedmann and Mssner. 2010 : 834). An ideal anticancer treatment capable of selectively killing cancer cells with limited side effects on normal cells and with minimal drug resistance is warranted. Several biologic response modifiers, such as macrophage-derived cytokine TNF-alpha, have shown potential in cancer treatment. TNF-alpha is a pro-inflammatory cytokine that plays an important role in many cancer types associated with chronic inflammation, including cholangiocarcinoma. TNF-alpha transduces its signal through two distinct receptors, TNFRI and TNFRII. TNFRI is associated with most of

TNF-alpha's biological activities, including apoptosis (Micheau and Tschopp. 2003: 181).

TNF-alpha has been used as an antineoplastic agent for the treatment of patients with locally advanced solid tumors such as melanoma, sarcoma, colorectal cancer and hepato-cellular carcinoma. The overall response rate has been shown to range from 47-100% (n=773) (Mocellin et al. 2005 : 43). In addition, Rossi et al. (2010 : 3006) demonstrated a significant difference between tumor response rates following isolated limb perfusion (ILP) with single chemotherapeutic agents (41.5%) and those observed after the addition of TNF (60.3%) in-transit melanoma metastasis of the limb (*P*=0.036) (Rossi et al. 2010 : 3006). TNF-alpha also improved drug uptake to limb-threatening soft tissue sarcomas in the most distal parts of the limb, where the overall response rate of isolated limb perfusion (ILP) with recombinant human TNF-alpha and melphalan was 71% (n=24), including 33% completed response and 38% partial response. The stable disease after TNF-melphalan isolated limb perfusion is 29%. These results suggested that TNF-alpha and melphalan-base isolated limb perfusion may be effectively used for caring for patients with irresectable soft tissue sarcomas of the distal parts of the limb (Deroose et al. 2012 : 565).

In contrast to the anti-neoplastic role, TNF-alpha has been shown to promote cancer development and progression (Mocellin et al. 2005: 36). In liver tumor model, TNF-alpha is upregulated during hepatic stem cell proliferation induced by a choline-deficient and ethionine-supplemented diet. In TNF receptor type 1 knockout mice, hepatic stem cell proliferation is substantially impaired and tumorigenesis is reduced. These data suggest that TNF signaling participates in the proliferation of hepatic stem cells during the preneoplastic phase of liver carcinogenesis, and that TNF receptor type 1 is necessary for tumor formation (Knight et al. 2000 : 1815). The tumor promoting activity of TNF-alpha has also been demonstrated in cholangiocarcinoma cell lines (KMCH, KMBT and WITT) (Jaiswal LaRusso and Burgart. 2000 : 187). In this study, inflammatory cytokines such as TNF-alpha (500 unit/mL), IL-1b (0.5ng/mL) and IFN-gamma (100 units/mL) have been shown to stimulate iNOs and NO production, leading to NO-dependent DNA

damage and inhibition of NO-dependent DNA repair activity. These data indicated that TNF-alpha is involved in DNA-damaging processes and DNA repair inhibition in cholangiocarcinoma (Jaiswal LaRusso and Burgart. 2000 : 187).

The role of TNF-alpha in cholangiocarcinoma is preliminary. Understanding the role of TNF-alpha CCA may not only provide basic knowledge about tumorigenesis of CCA, but also may lead to efficient and successful therapy.

Objective

The objective of this study is to test the cytotoxic effects of TNF-alpha to cholangiocarcinoma cells.

Methods

Cell culture. The cholangiocarcinoma cell lines used in this experiment are KKU-100 and KKU-M213. These cell lines were obtained from Dr. Banchob Sripa, Department of Pathology, Faculty of Medicine, Khon Kaen University. The KKU-100 and KKU-M213 cells were routinely cultured as monolayer in HAM's F-12 medium supplemented with 10% heat-inactivated fetal bovine serum, 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 100 mg/mL streptomycin, 100 U/mL penicillin G and 0.25 µg/mL amphotericin B. Cells were maintained at 37°C with 5% CO_2 in a humidified incubator and sub-cultured every 2 days.

RT-PCR detection of TNF-alpha receptor I and II. TNF-alpha receptor I and II (TNFRI and TNFRII) expression of KKU-100 and KKU-M213 was determined by reverse transcriptase-polymerase chain reaction (RT-PCR) technique. RNA was extracted using RNeasy Mini Kit (Qiagen, Valencia, CA, U.S.A). The total RNA (2 mg) was transcribed to cDNA by using Superscript RNase H Reverse Transcriptase Kit (Invitrogen™). The template DNA was amplified by polymerase chain reaction (PCR) technique. The reactions for TNFRI and TNFRII were started with an initial heat activation step at 95C for 5 minutes, followed by 30 cycles with a set sequence of thermal conditions: 94°C for 30 seconds, 65C (67°C for TNFRII) for 30 seconds and 72°C for 2 minutes. The sequences of both primers for TNF-alpha receptors in the condition mixture are shown below. The DNA products were separated by 1% agarose gel and detected under UV light of a gel documentation system.

TNFRI primer pair: A 267-base pair product was expected from this primer pair

Sequences: 5' (sense) primer: 5'-AAGGCGATCTCGCAGGACGGTCCTTAG-3'

Sequences: 3' (antisense) primer: 5'-AGATCGATCGGCTGGAGCTGCAGAA-3'

TNFRII primer pair: A 368-base pair product was expected from this primer pair

Sequences: 5' (sense) primer: 5'-AGGTCAATGTCACCTGCATCGTGAAC-3'

Sequences: 3' (antisense) primer: 5'-GAAAGAGCCTCAGAGTCCTAGTGGT-3'

Determination of apoptotic cells by DAPI staining. Cholangiocarcinoma cell lines, KKU-100 and KKU-M213, were used to determine the conditions of cells that had undergone apoptosis. A cell suspension of approximately $1x10^5$ cells was seeded on a sterile coverslip that was placed in a 35 mm plate and then incubated at 37°C. After 24 hours, the cells were treated with 160 ng/mL TNF–alpha. Distilled water was used as negative control, while 15 μM Wortmannin served as positive control. Cells were incubated for 24 hours. After the end of incubation period, the cells were washed with phosphate buffer saline (PBS) pH 7.4, fixed in 70%(v/v) methanol for 5 minutes at room temperature, and stained with 300 μL of methanol containing 1 mg/mL DAPI (4',6-diamidino-2-phenylindole). Subsequently, the specimens were incubated in the dark at room temperature for 15 minutes and then carefully rinsed briefly with PBS to remove unbound dye. The specimens were mounted on slide using 10 μL of 30%(v/v) glycerol in PBS, covered with coverslip and viewed under fluorescent microscope. The experiments were repeated at least three times independently.

Determination of cytotoxicity by MTT assay. Approximately $5x10^3$ cells of KKU-100 and KKU-M213 were mixed in 100 μL of HAM's F-12 medium supplemented with 10%v/v fetal bovine serum, and the mixture was added in a 96-well plate in triplicates (Corning, New York, U.S.A.). After 18 hours, 160 ng/mL of TNF-alpha, sterile distilled water and 15 mM Wortmannin were added. After 24 hours, 10 μL of 5 mg/mL MTT [3-(4, 5-dimethyldiazol-2-yl)-2,5 diphenyl tetrazolium bromide)] solution (USB, Cleveland, OH, U.S.A.) was added to the individual wells, followed by an incubation for 4 hours at 37°C in a humidified atmosphere containing 5% CO_2. MTT converted to

insoluble formazan dye in live cells was then dissolved by adding 200 µL DMSO (dimethyl Sulfoxide, Sigma, St. Louis, MO, U.S.A.) before the absorbance was read at 540 nm using MultiscanEX microplate reader. The surviving cells were represented in percentages and the procedure was repeated at least three times independently.

Statistical analysis. Statistical examination was performed by using one-way ANOVA, SPSS at 95% confidence interval for mean.

Results

Tumor necrosis factor receptor (TNFR) expression. KKU-100 and KKU-M213 expressed both TNFRI and TNFRII. The predicted amplification products of TNFRI (267 bp) and TNFRII (368 bp) mRNA were detected in both cell lines (Figure 1).

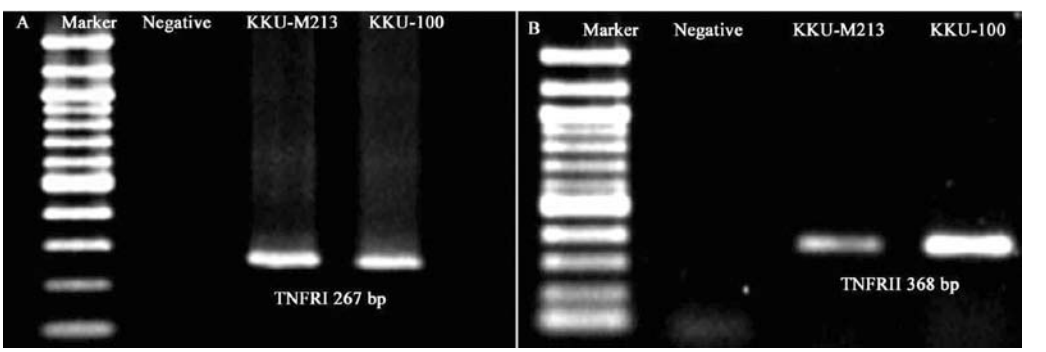

Figure 1 Expression of TNFRI (A) and TNFRII (B) of the KKU-M213 and KKU-100 cell was determined by RT-PCR. The predicted 267 bp and 368 bp products of TNFRI and TNFRII cDNA were detected in both KKU-100 and KKU-M213 cell lines.

Cytotoxic effect of TNF-alpha in cholangiocarcinoma cell lines. KKU-100 (Figure 2) or KKU-M213 (Figure 3) cells were exposed to 160 ng/mL TNF-alpha or 15 mM Wortmannin (positive control) for 24 hours before cell viability was determined by MTT assay. Cells exposed to distilled water were used as negative control. Treatment with TNF-alpha did not significantly affect viability of KKU-100, whereas cells treated with Wortmanin showed a reduction of cell viability to 39% compared to negative control. In contrast to KKU-100, KKU-M213 showed a

significantly increased viability up on exposure to TNF-alpha (121% viability compared to negative control), whereas those treated with Wortmanin showed a significant reduction of cell viability to 66% of negative control. These results demonstrated that TNF-alpha have differential effects on the two CCA cell lines, although it has no cytotoxic effect to either cell line.

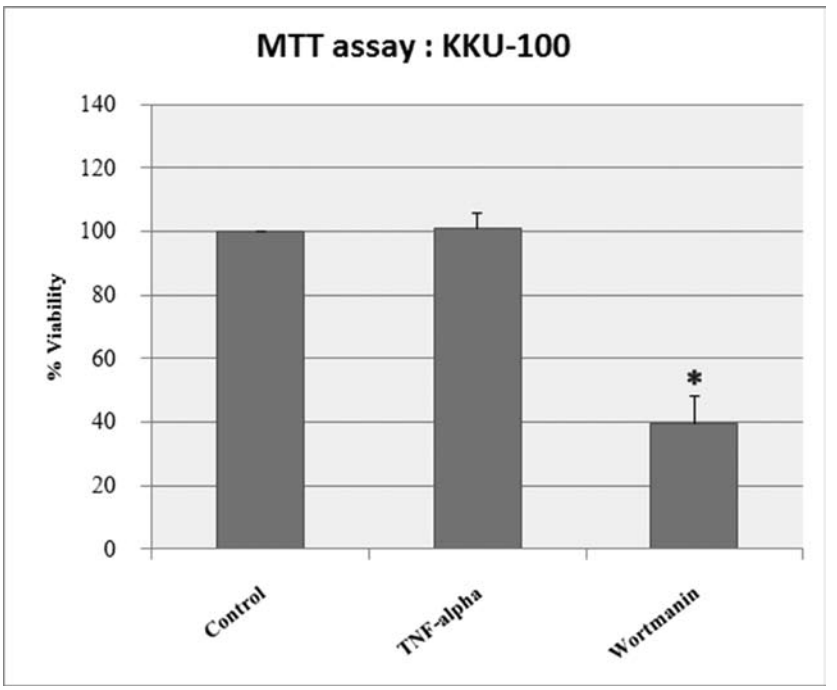

Figure 2 The effect of TNF-alpha on KKU-100 cell viability. KKU-100 cells were exposed to deionized water (negative control), 160 ng/mL TNF-alpha or 15 μM Wortmannin for 24 h before being subjected to MTT assay. Cell viability was not significantly affected by TNF-alpha treatment, whereas that of Wortmanin-treated cells showed a significant reduction ($P<0.05$).

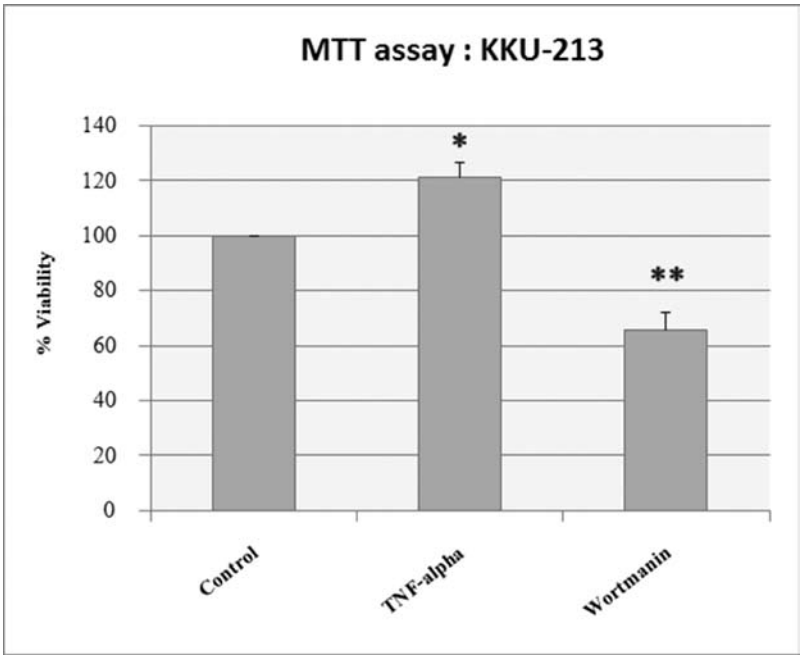

Figure 3 The effect of TNF-alpha on KKU-M213 cell viability. KKU-M213 cells were exposed to deionized water (negative control), 160 ng/ml TNF-alpha or 15 μM Wortmannin for 24 h before being subjected to MTT assay. Cell viability was significantly increased by TNF-alpha treatment (*P<0.05), whereas that of Wortmanin-treated cells showed a significant reduction (**P<0.05).

Apoptotic effect of TNF-alpha in cholangiocarcinoma cell lines. Cholangiocarcinoma cell lines, KKU-100 and KKU-M213, were stained with DAPI (4′-6-diamidino-2-phenolindole). Both cell lines were incubated in the absence (negative control) (Figure 4 A and 5 A) or presence (Figure 4 B and 5 B) of 160 ng/mL TNF-alpha for 24 hours. Positive control were CCA cells treated with 15 μM Wortmannin for 24 hours (Figure 4 C and 5 C). The hallmark of apoptosis such as nuclear condensation and fragmentation was detected under fluorescence microscope, where as the cell morphology was observed under light microscope. The nuclei of control and TNF-alpha-treated cells showed round and intact nuclei by DAPI staining, where as the cell morphology showed brick-like cells (KKU-100) and fusiform-like cells (KKU-M213) with spread and tight adhesion. In

contrast, CCA cells treated with Wortmanin showed condensed and fragmented nuclei, characteristics of apoptotic cells, by DAPI staining. Besides, cell morphology showed shriveled appearance, patchy condensation, pyknosis and an increased number of nuclear body fragments. Hence, both CCA cell lines were resistant to apoptosis induced by TNF-alpha.

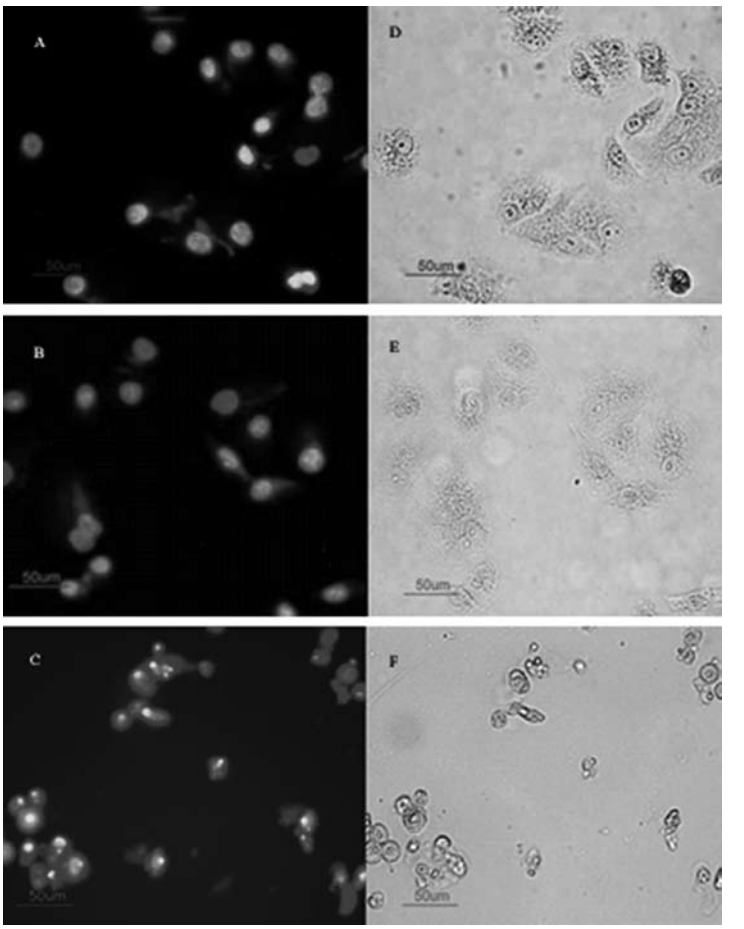

Figure 4 Fluorescence microscope images of KKU-100 cell line stained with DAPI. Cells were treated with (A) negative control (deionized water), (B) 160 ng/mL TNF-alpha and (C) 15 μM Wortmannin, for 24 hours before being subjected to DAPI staining.

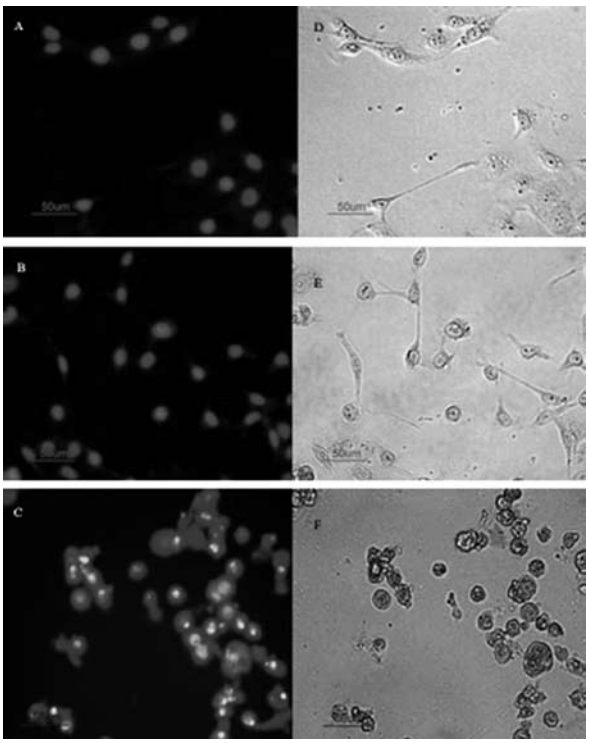

Figure 5 Fluorescence microscope images of KKU-M213 cell line stained with DAPI. Cells were treated with (A) negative control (deionized water), (B) 160 ng/mL TNF-alpha and (C) 15 μM Wortmannin, for 24 hours before being subjected to DAPI staining.

Discussion

In this study, the effect of TNF-alpha on cell viability of two CCA cell lines with different eitology were investigated. KKU-100 was classified as poorly-differentiated, where as KKU-M213 was classified as well-differentiated. Our data indicated that exposure to high dosage of TNF-alpha (160 ng/ml) for 24 h did not have a significant effect on the viability of KKU-100, where as it significantly enhanced the viability of KKU-M213 (P<0.05). Enhancement of cell viability/ proliferation was previously reported by Li et al. (2501 : 1138 ,2014), showing that TNF-alpha (20 ng/mL) significantly induces proliferation in rheumatoid arthritis (RA)-fibroblast-like synoviocytes (FLS) after having been administered for 72 hours

(Li et al. 2014 : 1138). PI3K/Akt signaling pathway has been shown to mediate of TNF-alpha activation in cholangiocarcinoma cells (Mocellin et al. 2005 : 36 ; Tanimura. 2005 : 211; Leelawat et al. 2009 : 7). Treatment of CCKS1 cell line with a low dosage of TNF-alpha (10 ng/mL) stimulated the MAPK- and PI3K/Akt signaling (Tanimura et al. 2005 : 211). On the contrary, combination of oxaliplatin and a PI3K inhibitor (10 μM LY294002) arrested cell proliferation and induced apoptosis in KKU-100 and RMCCA-1 cell lines (Leelawat et al. 2009 : 7). Moreover, Prakobwong et al. (2011 : 1379) showed that curcumin suppressed cell proliferation of CCA cell lines by inhibiting NF-kB and STAT-3 signaling pathway, PI3K/Akt pathway, and anti-apoptotic proteins.

Accordingly, a PI3K inhibitor (15 μM Wortmannin), was used as a positive control to test the potential of TNF-alpha to suppress cell viability and to induce apoptotic cell death in this study. Indeed, our data showed that Wortmannin efficiently reduced cell viability and induced apoptosis in both CCA cell lines. Insensitivity to TNF-alpha exhibited by our CCA cell lines contrasted that observed by Utaisincharoen et al. (1999 ; 45) who showed that TNF-alpha combined with actinomycin D (protein synthesis inhibitor) induced apoptosis in cholangiocarcinoma cell lines HuCCA-1 and HuCCA-1Nu. Cleavage of poly (ADP-ribose) polymerase (PARP) induced by TNF-alpha treatment was suppressed by a monoclonal antibody to TNFRI, indicating that TNFRI, not TNFRII, was involved in induction of apoptosis. (Utaisincharoen et al. 1999 : 45). However, the effect of TNF-alpha alone on the CCA cells has not been reported. In addition, previous report has shown that exposure of ameloblastoma (AM-1 cell line) to a high dose of TNF-alpha (100 ng/mL) for 24 hours significantly induced apoptosis (Sandra et al. 2006 : 41). Moreover, *in vitro* studies demonstrated that apoptosis of melanoma cell lines (Mel-RM, Mel007, Mel-JD) induced by atmospheric gas plasma (AGP) was mediated by TNF-receptor pathways through induction of caspase 3/7 activity. Blocking of the TNF-receptor I (TNFRI) with a TNFRI-neutralizing antibody inhibited AGP-induced apoptosis signal regulating kinase1 (ASK1) (Ishaq et al, 2014 : 1527).

Conclusion

The two CCA cell lines in our study expressed TNFRI and TNFRII receptors, and TNF-alpha has different effect on the viability of these cell lines. The viability of KKU-100 did not change significantly, where as that of KKU-M213 significantly increased, by TNF-alpha. DAPI staining & fluorescence microscopy revealed intact nuclei and lack of nuclear fragmentation, indicating absence of apoptosis in both cell lines. Nevertheless, these cell lines showed a drastic reduction of cell viability and induction of apoptosis up on treatment with Wortmanin, a PI3K inhibitor, indicating that PI3K/AKT signaling, but not TNF-alpha signaling, regulates the viability of these CCA cells. However, further studies may be required to determine how the viability is controlled in the CCA cells, and how they resisted TNF-alpha.

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