# CHAPTER IV

# MATERIALS AND METHODS

## Cell culture: Thawing and recovering cell line

The frozen vials of KKU-100 and KKU-213 cell lines were removed from liquid nitrogen freezer and immediately placed into a 37 $^{o}$C water bath. The vials were agitated continuously until they thawed (approximately 1 minute). The vials were wiped with 70% ethanol before opening. Each type of thawed cell suspension was transferred into a sterile centrifuge tube containing 10 ml cold medium with 20% FBS. The cell suspension was centrifuged at 1200 rpm for 10 min. The supernatant was discarded. The washing step was repeated once more. The cell pellet was gently resuspened in 5 ml of medium containing 20% FBS and then transferred to a culture flask. The cell culture was incubated in a humidified, 37 $^{o}$C, 5% $CO_2$ incubator and checked after 24 hours to ensure that cells attached to the plate. The medium was changed after 3 or 4 days, or when the pH indicator in the medium changed color.

## Trypsinization and subculture method

Medium from cell culture was removed with a sterile pasture pipette. The KKU-100 and KKU-213 cell lines were washed once or twice with sterile PBS without $Ca^{2+}$ and $Mg^{2+}$ to remove any residual FBS that may inhibit the action of trypsin. The 37 $^{o}$C 0.25% trypsin/EDTA solution was added to the culture to cover adhering KKU-100 and KKU-213 cell layers. The culture plates were placed in an incubator at 37 $^{o}$C for 5 min. The cell culture plates were tapped on the countertop to dislodge cells and checked with an inverted microscope to ensure that cells were rounded up and detached from the

surface. The complete medium was added to the culture. Cell suspension was drawn into a pasture pipette and rinsed two or three times to dissociate cells and to dislodge any remaining adherent cells. As soon as the cells were detached, medium containing serum was added to inhibit further trypsin activity that might damage the cells. After the suspension cells were counted with a hemacytometer, and their concentration was adjusted, they were added to each plate or flask. Fresh medium of HAM-12 containing 10% fetal bovine serum (FBS) (GIBCO, Grand Island, NY, U.S.A.) was added to each new culture and incubated in a humidified, 37 $^{o}$C, 5% $CO_2$ incubator.

**Determination of TNFR mRNA expression by RT-PCR**

Approximately 1x10$^{6}$ cells of both KKU-100 and KKU-213 were cultured in a 100-mm culture dish until 80% confluence. The samples then were trypsinized using 0.25% trypsin/EDTA for 5 min, before neutralization with medium containing 10% FBS. These dislodged cells were centrifuged at 1,200 rpm for 10 min and then the supernatant was removed. The cell pellets were washed with PBS (pH 7.4) twice before the RNA was extracted using RNeasy kit. The sequences of both primers for TNF-apha receptor, TNFRI and TNFRII are shown below.

**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’

**The PCR conditions for TNFRI are shown below:**

Thermocycler condition for TNFRI

| | |
|---|---|
| 1 cycle of initial denaturation at 94°C | 1 min |
| 30 cycles of | |
| - Denaturation at 94°C | 30sec |
| - Annealing at 65°C | 30sec |
| - Extension at 72°C | 2 min |
| 1 cycle of final extension at 72°C | 5 min |

**TNFRII primer pair**

A 368-basepair product was expected from this primer pair.

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

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

**The PCR conditions for TNFRII are shown below:**

Thermocycler condition for TNFRI

| | |
|---|---|
| 1 cycle of initial denaturation at 94°C | 1 min |
| 30 cycles of | |
| - Denaturation at 94°C | 30sec |
| - Annealing at 67°C | 30sec |
| - Extension at 72°C | 2 min |
| 1 cycle of final extension at 72°C | 5 min |

The template molecules or molecules of DNA were replicated by the DNA polymerase enzyme, thus doubling the number of DNA molecules. Then each of these molecules was replicated in a second "cycle" of replication, resulting in four times the number of the original molecules. Again, each of these molecules was replicated in a third cycle of replication and replicated until 30 cycles. This process generated of millions copies of the original DNA molecule. The DNA products were separated by 1% agarose gel and detected under UV light of a gel documentation system.

**Determination of apoptotic cells by DAPI staining**

Apoptotic nuclei, characterized by DNA condensation, were stained with 4′-6-diamidino-2-phenylindole (DAPI). The blue-fluorescent DAPI nucleic acid stain preferentially stains dsDNA. It appears to associate with AT clusters in the minor groove. The excitation maximum for DAPI bound to dsDNA is 358 nm, and the emission maximum is 461 nm. Binding of DAPI to dsDNA produces approximately 20-fold fluorescence enhancement. This dye can pass through intact cell membranes but apoptosis increase cell membrane permeability and uptake of DAPI, leaving a stronger blue stain. In addition, apoptotic nuclear morphology is round and clear edged. Sometimes apoptotic cells show irregular edges around the nucleus, along with nuclear pyknosis and an increased number of nuclear body fragments. The role of TNF-alpha in apoptosis was determined using Cholangiocarcinoma cell lines. Cholangiocarcinoma (CCA) cell lines KKU-100 and KKU213 were used to determine the conditions of cells that had undergone apoptosis or were resistant to 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 $^{o}$C. After 24 h, the cells on the coverslip were treated with 160 ng/ml TNF–alpha.

Distilled water was used as negative control, while 15mM Wortmanin served as positive control. Cells and controls were incubated for 24 hours. After the end of incubation period, the cells were washed with PBS pH 7.4, fixed in methanol for 5 min at room temperature, and stained with 300 µl of methanol containing 1 ng/ml DAPI. Subsequently, the specimens were incubated in the dark at room temperature for 15 min and then carefully rinsed briefly with PBS to remove unbound dye. The specimens were mounted on slide using 10 µl of 30% 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**

MTT assay is a method to determine cell survival and cytotoxicity. Briefly, MTT [3-(4, 5-dimethyldiazol-2-yl)-2,5 diphenyl tetrazolium bromide)] is converted to formazan crystals by mitochondrial enzymes of the living cell. The formazan crystals are dissolved by DMSO and their absorbency is determined by microreader plate. The cytotoxicity of the cell after treatment is compared to the untreated control.

Approximately $5x10^3$ cells of KKU-100 and KKU-213 were mixed in 100 µl of HAM-12 medium supplemented with 10% FBS, 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, 15 µM Wortmanin, was added. After 24 h, 10 µl of 5 mg/ml MTT solution (USB, Cleveland, OH, U.S.A.) was added to the individual wells, followed by an incubation for 4 hours at $37^oC$ 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 (Sigma, St. Louis, MO, U.S.A.) before the absorbance was read at 540 nm. The

cytotoxicity was represented in percentages and the procedure was repeated at least three times independent.

**Determination of poly (ADP ribose) polymerase (PARP) by Western Blot Assay**

Poly(ADP ribose) polymerase (PARP) is a highly conserved nuclear enzyme present in higher eukaryotes. The enzyme is a $Zn^{2+}$-dependent DNA binding protein that recognizes DNA stand breaks and is implicated to play a role in DNA repair and in the apoptosis response of cells. It's apoptotic role was demonstrated by observing that PARP cleavage is mediated by caspase-3 activity. This process occurs at the onset of apoptosis, and inhibition of PARP cleavage attenuates apoptosis *in vitro*. PARP catalyzes the poly(ADP-ribosyl)ation of a variety of nuclear proteins with NAD as substrate. Because it is activated by binding to DNA end or stand break, PARP is suggested to contribute the cell death by depleting the cell of NAD and ATP (Berger, N. A. 1985). It was subsequently cleaved into 85-kDa fragments as a marker for apoptosis. The cleavage of PARP is a fairly early event in apoptosis, detectable earlier than other apoptotic events such as DNA fragmentation. In this study, PARP was used to determine the sensitivity of cholangiocarcinoma cell lines to apoptosis after TNF-alpha treatment.

- **Sample conditions**

Approximately $1x10^6$cells/well of KKU-100 and KKU-213 cell lines were spread over a 100 mm x 20 mm plastic tissue culture dish, and culture growth continued in the completed culture medium overnight. After that, the cell lines were treated with 160 ng/ml TNF-alpha for 24 hours. In parallel, the positive and negative controls were treated with 15 mM Wortmanin and distilled water, respectively. After treatment, cells in the culture dishes were washed twice with ice-cold PBS pH 7.4 before adding

100 µl of lysis buffer solution, as shown in the Ingredients section of the Appendix. All culture dishes were placed on ice during the experimental steps. Each cell lysate was scraped after added lysis buffer and transferred to microtubes. Nucleic acid substances were removed from the lysate samples by flushing 4-5 times with a small syringe and needle no.23. The processed lysate samples were incubated on ice for 30 min for complete lysis. To remove cell debris, the lysate samples were centrifuged at 1200 rpm 10 min. The supernatant was kept for further protein separation by electrophoresis system and measurement of protein concentration by Bradford method.

- **Protein concentration determination by Bradford assay**

The Bradford method quantitates the binding of Coomassie Brilliant Blue to an unknown protein and compares this binding value to different amounts of a standard protein, bovine serum albumin. The standard curve was created by adding duplicate aliquots of 0.5 mg/ml (1, 2, 4 and 6 ul) into a 96–well plate. The protein samples were diluted before the measurement. The volume in each well was brought to 10 µl with distilled water. Blank condition was prepared by filling 2 wells with only distilled water. Bradford solution (Biorad) was diluted four times with distilled water and 190 ul of the diluted Bradford solution was added to each well. The solution was mixed and left for 5 min at room temperature. The absorbance was measured at 595 nm (A595) using a 1-cm pathlength microcuvette (1ml). A standard curve was created by plotting absorbance versus protein concentration. The protein concentration from the samples was determined from the standard curve.

- **Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)**

The SDS-PAGE procedure was performed for separating protein samples. The apparatuses used for SDS-PAGE (Amersham) were assembled. The separating solution (12% gel, described in the

Ingredients section of the Appendix) was mixed, leaving TEMED (USB) last. Once TEMED was added, the flask was gently swirled to mix the contents, which then were immediately poured into the space between glass panels integrated with spacers. Distilled water was gently overlaid on the top of the separation solution for smoothing and equalizing the gel level. Then the separating gel was allowed to polymerize. After the separating solution (12%gel) completely polymerized, the stacking gel solution was prepared as described in the Ingredients section of the Appendix. Once the stacking solution mixtures were pipetted into the flask, Ammonium Persulfate (AP) and TEMED were consequently added. The flask was immediately and gently swirled to mix its contents. The separating gel overlay solution (distilled water) was poured off before overlaying the stacking solution. A comb was inserted into the upper part of the sandwiched glass plates with taking care not to trap any bubbles beneath the teeth of the comb, and the solution was left to polymerize. Eighty micrograms of proteins from sample conditions were mixed with SDS sample buffer and boiled for 5 min. The hydrophobic molecules from protein samples were dissolved and covered with many negative charges by the SDS sample buffer. After that, denatured protein samples were added to each well of the stacking gel. The denatured proteins migrated towards the positive pole and were separated by size when electrophoresis was performed at 120 Volts and run for 2 hours in Tris-glycine buffer pH 8.3 (25mM Tris base, 250 mM glycine, 0.1% SDS).

- **Protein Immunoblot assay**

After electrophoresis, the protein samples were transferred to nitrocellulose membrane by electroblotting at 120 volts for 2 hours, 4$^{o}$C in cool-blot buffer containing of 25mM Tris base, pH 8.5, 200mM glycine and 20% methanol (freshly prepared). The proteins in the blot retained the same pattern

of separation they had on the gel. The blot was dried at room temperature for 30 min and then non-specific binding was blocked by covering the blot for 1 hour at room temperature with 5% skim milk (non-fat dry milk) in TBS/T buffer as described in the Ingredients section of the Appendix. Mouse anti-PARP (Monoclonal antibody, $IgG_1$) catalog number MAB 3290 Lot number LV1390313 was used as the primary antibody. The primary antibody was diluted 1: 1000 in 3% skim milk/TBS/T. The blot was incubated overnight with primary antibody at $4^{o}C$. Then excess primary antibody was removed by washing the blot three times with TBS/T for 5 min. The blot was subsequently incubated with horseradish peroxidase conjugated anti-rabbit $IgG_1$ in 3% skim milk/TBS/T and incubated for 1 hour at room temperature. The full-length 116 kDa PARP band and the apoptosis-related 85 kDa PARP cleavage fragment band were visualized with enhanced chemiluminesence (ECL, Amersham Pharmacia Biotech).