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Synergistic Cytotoxicity and Apoptosis Induced in Human Cholangiocarcinoma Cell Lines by a Combined Treatment with Tumor Necrosis Factor-Alpha (TNF-α) and Triptolide

Tasanee Panichakul[1], Thiwaree Wanun[1], Vichai Reutrakul[2] and Stitaya Sirisinha[1,3]

SUMMARY Cholangiocarcinoma is known to be relatively resistant to chemotherapy. One alternative approach is to use a combination of an immunomodulating agent with an anticancer drug. Here we studied the synergistic actions of TNF-α and triptolide (a diterpene epoxide prepared from *Tripterygium wilfordii*), previously shown to have antitumor activity against hamster cholangiocarcinoma (CCA) cells. Three human CCA cell lines (HuCCA-1, HubCCA-1, KKU-100 cell lines) were subjected to a combined treatment of TNF-α (0.1-10 ng/ml) and triptolide (5-50 ng/ml) for 24 hours in microculture plates. The combination of TNF-α and triptolide had a significantly increased cytotoxic activity over that of triptolide alone ($p < 0.05$). Under the same conditions, TNF-α by itself was not cytotoxic to these cell lines. Similarly, the combined treatment could also accelerate apoptotic cell death in all three human cholangiocarcinoma cell lines. The combined treatment of TNF-α at 10 ng/ml and triptolide at 50 ng/ml for 6-10 hours achieved a percentage of apoptotic cells shown by DAPI staining of 18-65%, compared to only 6-20% apoptotic cells for triptolide alone. Analyzing the possible mechanisms of the combined treatment, we found by Western blot that at 6 hours, there was a poly (ADP-ribose) polymerase (PARP) cleavage which was not detectable by the treatment of either TNF-α or triptolide alone. The cleavage of PARP was inhibited when the cells were pretreated with the enzyme inhibitor AC-DEVD-CMK, suggesting that apoptosis induced by the combination of TNF-α and triptolide involved activation of caspase 3. These results indicate that apoptosis of human cholangiocarcinoma cell lines as induced by a combination of TNF-α and triptolide is mediated through caspase 3 activation.

Cholangiocarcinoma is a malignant tumor of the bile duct's epithelial cells (cholangiocytes). It is found predominantly in several Asian countries where the liver fluke is endemic, e.g. the northeastern part of Thailand.[1] Most cases are probably sporadic, but chronic inflammatory and other conditions confer an increased risk of developing cholangiocarcinoma. These include chronic liver fluke infestations, exposure to various chemicals, certain congenital diseases of the biliary tree and primary sclerosing cholangitis.[2,3] The cancer is invariably fatal, as currently there is neither an appropriate laboratory method available for early diagnosis nor effective chemotherapy; thus, the disease has a poor prognosis. The major problems in the treatment of this cancer are the resistance to conventional anticancer chemotherapeutic agents.[4] Hence, a new therapeutic approach is warranted.

One alternative approach is to use a combination of an immunomodulating agent with an anticancer drug. TNF-α is a member of the tumor necrosis factor ligand family, and it induces apoptosis in some cancer cell lines through a TNF receptor (e.g. TNF-R1). However, by itself TNF-α rarely triggers apoptosis in some tumor cell types unless the protein synthesis is blocked.[5] A previous study showed that TNF-α combined with actinomycin D could

From the [1]Laboratory of Immunology, Chulabhorn Research Institute, [2]Department of Chemistry, and [3]Department of Microbiology, Faculty of Science, Mahidol University, Bangkok 10210, Thailand.
Correspondence: Stitaya Sirisinha

induce apoptosis in cholangiocarcinoma cells but TNF-α alone could not.[6] Triptolide is a diterpenoid triepoxide extracted from the Chinese herbal remedy *Tripterygium wilfordii* Hooh F with potent anticancer activity against several human cancer cell lines such as leukemia, breast cancer and cholangiocarcinoma.[7,8] With some tumors, both TNF-α and triptolide are required to induce apoptosis.[5,8] It seems that TNF-α and triptolide may share common intracellular signaling pathways leading to cell death. Indeed, we have studied the synergistic action of TNF-α and triptolide in the cytotoxic activity and apoptosis induction.

MATERIALS AND METHODS

Cell cultures

HuCCA-1 and KKU-100 cell lines were established from tumor tissues of bile ducts obtained from Thai patients as previously reported.[9,10] HubCCA-1 cells were kindly provided by A. Wongkajornsilp (Department of Pharmacology, Faculty of Medicine Siriraj Hospital, Mahidol University). They were established from tumor cells in intrahepatic bile duct of a patient with pathologically proven cholangiocarcinoma. The three cell lines were cultured in Ham's F12 culture medium (Hyclone Laboratories, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone Laboratories), 100 U/ml penicillin and 100 μg/ml streptomycin and incubated at 37°C with 5% CO_2.

Reagents

Triptolide powder (99% pure, extracted from *T. wilfordii* by ethanol extraction and purified as previously described by Shamon *et al.*[7]) was solubilized in 0.01% dimethylsulfoxide in culture medium at a concentration of 100 μg/ml. Then the solution was sterilized with a 0.2 μm Millipore® filter. Lyophilized TNF-α (10 μg, R & D Systems, Minneapolis, MN, USA) was reconstituted in 1 ml of 0.1% sterile bovine serum albumin in phosphate-buffered saline (PBS, pH 7.2). The stock solution was kept at -70°C.

Cytotoxic assay

The assay was performed as described previously.[8] Briefly, 100 μl per well with 3 x 10^5 cell/ml were cultured in a 96-well tissue culture plate for 24 hours at 37°C. TNF-α, triptolide or the combination of both were added to the cultured cells and incubated further for an additional 24 hours. The medium was removed and the monolayers were washed. The cells were fixed with 95% ethanol and stained with 0.5% crystal violet. The stained cells were lysed with 100 mM HCl in absolute methanol and the optical density (OD) was determined by a microtiter plate reader (Multiskan Ascent, Labsystem), set to read at a wavelength of 540 nm. Cytotoxic activity from a quadruplicate assay was calculated as follows:

Percent cytotoxicity = (1- OD_{540nm} of treated cells/ OD_{540nm} of control) x 100

DNA staining with DAPI (4', 6-diamidine-2'-phenylindole dihydrochloride)

Two millions tumor cells cultured in a 25 cm^2 tissue culture flask were treated with 10 ng/ml TNF-α, 50 ng/ml triptolide or their combinations. After different time intervals, the treated cells were harvested by trypsinization, thoroughly washed with PBS and stained at room temperature for 15 minutes with 2 μg/ml DAPI (Roche Diagnostics Gmbh, Roche Molecular Biochemicals, Mannheim, Germany) as described.[8] The stained cells were examined under a fluorescence microscope fitted with a 340/380 nm excitation filter. The percentage of condensed and fragmented apoptotic cells were calculated from a total of 1,000 cells counted.

Western blot analysis

After treatment of the HuCCA-1 and HubCCA-1 cells with 10 ng/ml TNF-α, 50 ng/ml triptolide or their combination, the treated cells were trypsinized and washed with sterile cold PBS before centrifugation at 200 *g* for 5 minutes at 4°C. The pellets were suspended in 0.1 ml of reducing buffer (62.5 nM Tris pH 6.8, 6 M urea, 10% glycerol, 2% SDS, 0.03% bromphenol blue and 5% 2-mercaptoethanol) followed by sonication on ice for 20 seconds. The samples were subjected to a 10% SDS-PAGE[11] and the electrophoresed components were transferred onto a nitrocellulose membrane.[12] The nitrocellulose membrane was washed with PBS containing 1% Tween 20 (Sigma Chemicals Co., St Louis, MO, USA) and blocked for 1 hour with 5% non-fat powdered milk (GIBCO, Laboratories, Grand Island, NY, USA) before staining with rabbit polyclonal antibodies specific to poly (ADP-ribose) polymerase (PARP) (Santa Cruz, Biotechnology, Inc.) at 4°C overnight. The membrane was then incubated with horseradish peroxidase-conjugated swine anti-rabbit immunoglobulins (Dako A/S, Glostrup, Denmark) for 1 hour at room temperature and the reactions were visualized by enhanced chemiluminescence reaction (Super Signal, Pierce, Illinois, USA).

Statistical analysis

All determinations were made in triplicates, and the results

were expressed as the mean ± SD. Statistical significance ($p < 0.05$) was determined by Student's t test.

RESULTS AND DISCUSSION

We investigated the cytotoxic effect of a combination of TNF-α and triptolide against three human cholangiocarcinoma cell lines (HuCCA-1, HubCCA-1 and KKU-100) *in vitro*. A combination of TNF-α (0.1-10 ng/ml) and triptolide (5–50 ng/ml), in a 24-hour cytotoxic test showed a significantly increased cytotoxic activity ($p < 0.05$) over that of triptolide or TNF-α alone (Fig. 1). The combination of TNF-α (10 ng/ml) and triptolide (50 ng/ml) could induce cytotoxicity up to 71-83%. The triptolide alone at 50 ng/ml could induce only 23-48% cytotoxicity, while the TNF-α alone at a concentration of 10 ng/ml showed only 2-6% cytotoxicity against all three cell lines. Moreover, this combined treatment could induce cytotoxic activity to occur earlier than when either of the triptolide or TNF-α was used alone. For instance, even as early as 10 hours, the combined treatment could induce cytotoxicity up to 50% while the triptolide alone induced at most 12% and the TNF-α alone showed only negligible activity against these three cell lines (Fig. 2). It should be mentioned that this synergistic cytotoxicity did not require their presence together at the same time, as a similar degree of cytotoxicity could be observed when either one was added to the cells prior to the other (data not shown).

In order to analyze a possible molecular mechanism associated with cell cytotoxicity of this combined protocol, the following experiments were conducted. HuCCA-1 and HubCCA-1 cells were subjected to the combined TNF-α and triptolide treatment for

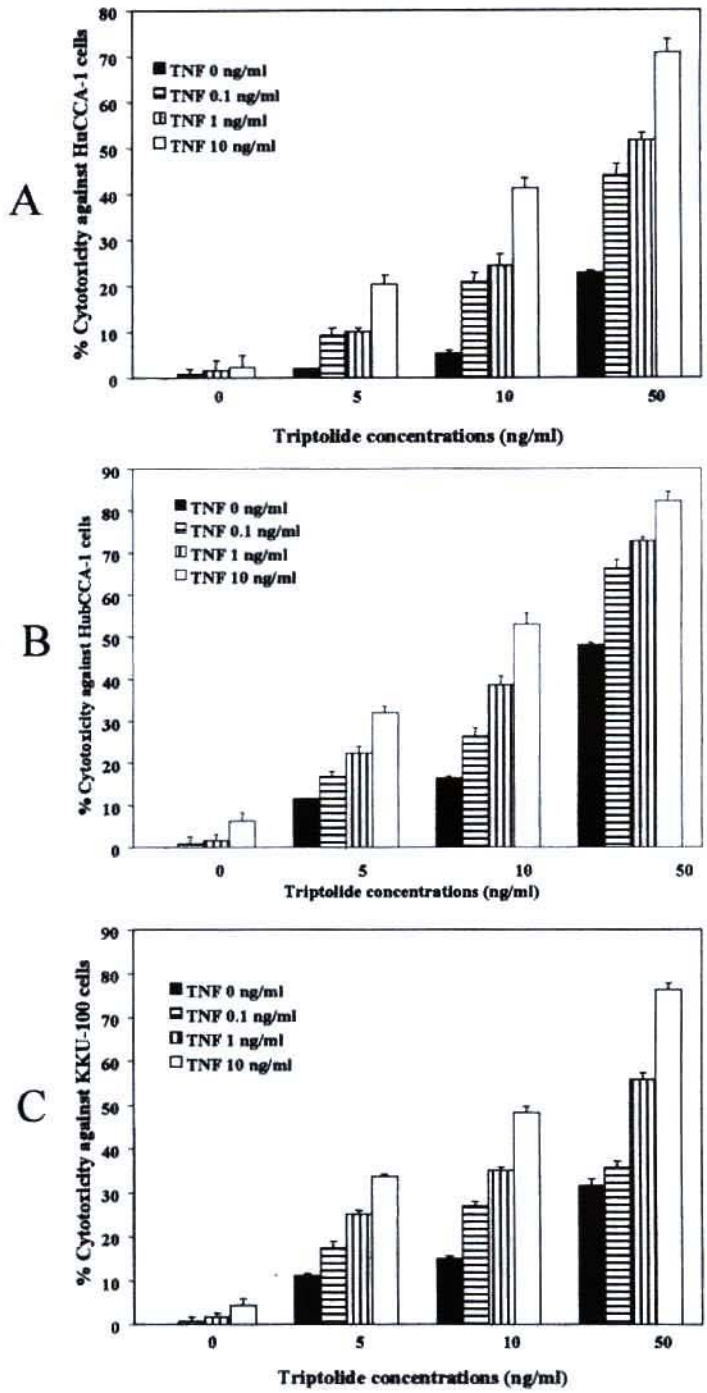

Fig. 1 The synergistic cytotoxic effect of TNF-α and triptolide against human CCA cell lines. HuCCA-1 (A), HubCCA-1 (B) and KKU-100 (C) cell monolayers were subjected to the combined treatments of TNF-α (0.1-10 ng/ml) and triptolide (5-50 ng/ml) for 24 hours in a cytotoxicity test. Data are expressed as mean ± SD determined from three independent experiments. Enhanced cytotoxic activity of the combined treatment was statistically significant at $p < 0.05$.

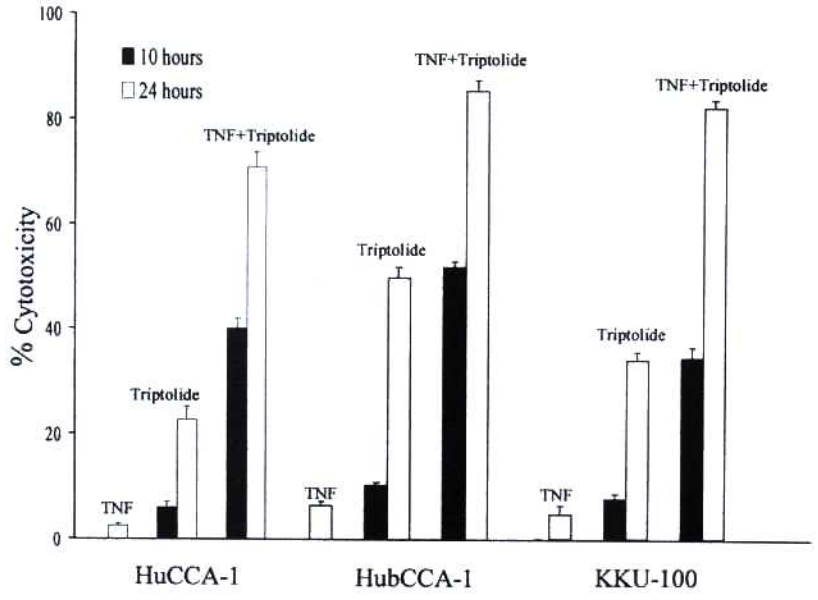

Fig. 2 The cytotoxic activity of TNF-α, triptolide and their combined treatment against human CCA cell lines. HuCCA-1, HubCCA-1 and KKU-100 cells were treated with TNF-α 10 ng/ml, triptolide 50 ng/ml and their combination for 10 (black bar) and 24 (open bar) hours in cytotoxicity tests. Data are expressed as mean ± SD determined from three independent experiments.

Table 1 Percentage of apoptotic cells of cholangiocarcinoma cells at different time intervals after treatment with a combination of TNF-α (10 ng/ml) and triptolide (50 ng/ml) comparing with either triptolide or TNF-α alone

Treatment	Time (hours)									
	2	4	6	8	10	2	4	6	8	10
	HuCCA-1 cells					HubCCA-1 cells				
TNF-α and triptolide	2	4	18	34	45	2	6	25	50	65
Triptolide alone	1	2	6	10	15	1	2	12	15	20
TNF-α alone	0	0	1	1	2	0	0	1	2	2
Medium	0	0	1	2	2	0	0	1	2	2

DAPI staining
All values carried out in triplicates were based on counting a total of 1,000 cells

various time intervals (2, 4, 6, 8, 10 hours) and at each time point the degree of apoptosis was determined by staining DNA with DAPI. The treated HuCCA-1 and HubCCA-1 cells showed distinct nuclear condensation and fragmentation after 6 hours of treatment (Fig. 3). The kinetic study summarized in Table 1 showed a gradual increase in the number of apoptotic cells from 2 to 10 hours when the experiment was terminated. In concordance with results of the cytotoxicity test, a combined treatment markedly enhanced the degree of apoptosis in both cell lines compared to either one alone. When the DNA from treated cells were subjected to electrophoresis, DNA fragmentation could be readily observed, judging from the appearance of the DNA ladder (data not shown).

One of the mechanisms of the apoptosis-inducing compounds is known to be activation of the caspases, which in turn cleaves and inactivates the DNA repairing enzyme, poly (ADP-ribose) polymerase (PARP). In this study, the kinetic of the PARP cleavage induced by a combined treatment was analyzed by tracking the increase of the appearance of the cleaved PARP product. This was achieved by immunoblotting of the treated cells using polyclonal antibodies specific for PARP. The results pre-

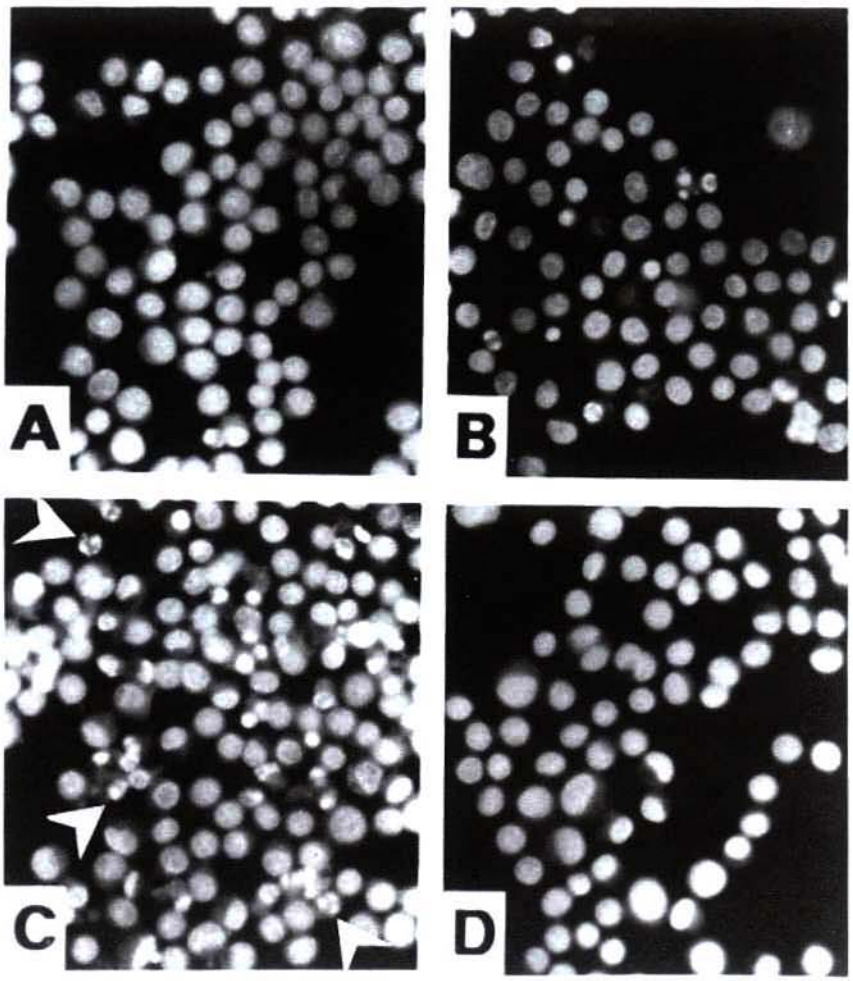

Fig. 3 Fluorescence photomicrographs of TNF-α and triptolide treated HubCCA-1 cells. Tumor cells were treated with 10 ng/ml TNF-α (A), 50 ng/ml triptolide (B), or both (C) for 10 hours. The treated cells were stained with DAPI as described in Materials and Methods. There was a marked increase in the number of apoptotic cells with characteristic chromatin condensation, nuclear fragmentation and cell shrinkage (arrow heads) as depicted in (C) compared with those of TNF-α alone (A) or untreated cells culture (D) which showed no apoptotic cells. A trace number of apoptotic cells can be noted with the concentration of triptolide used (B). Magnification, 400x.

sented in Fig. 4 showed that an 85-kD fragment of the cleaved PARP product could be readily detected after 6 hours of treatment. No degradation of PARP was noted when HuCCA-1 cells were exposed to either TNF-α or triptolide alone. However, a trace of PARP degradation could also be detected at 8 and 10 hours of triptolide treatment of the HubCCA-1 but not the HuCCA-1 cells. It appeared therefore that HubCCA-1 cells were more susceptible to the action of triptolide. This conclusion is consistent with the cytotoxicity data presented earlier in Fig. 1.

The activation of caspase enzymes in the cleavage of PARP was analyzed using a caspase inhibitor (AC-DEVD-CMK). The cells were pretreated with 10, 25, 50 μM of AC-DEVD-CMK for 2 hours prior to the combined treatment of TNF-α and triptolide. AC-DEVD-CMK at concentration of 50 μM could significantly ($P < 0.05$) inhibit the cytotoxic activity of the combined treatment, i.e. 73% for HuCCA-1 cells and 47% for HubCCA-1 cells (Fig. 5). The effect of AC-DEVD-CMK on the PARP cleav-

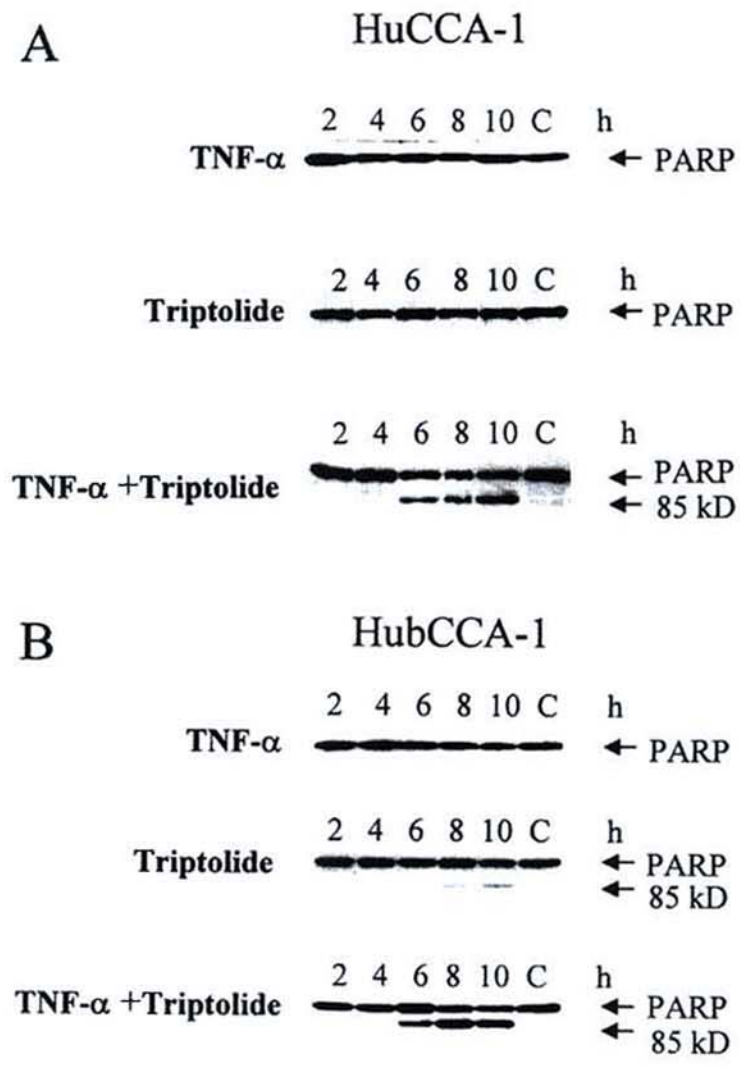

Fig. 4 Western blot profiles showing a cleavage of Poly (ADP-ribose) polymerase (PARP) in human cholangiocarcinoma cell lines. HuCCA-1 (A) and HubCCA-1 (B) cells treated with 10 ng/ml TNF-α, 50 ng/ml triptolide or both were analyzed by immunoblot with antibody to PARP and visualized by enhanced chemiluminescence reaction. Fragmentation of PARP from 116 kDa to 85 kDa can be visualized in the combined treatment group.

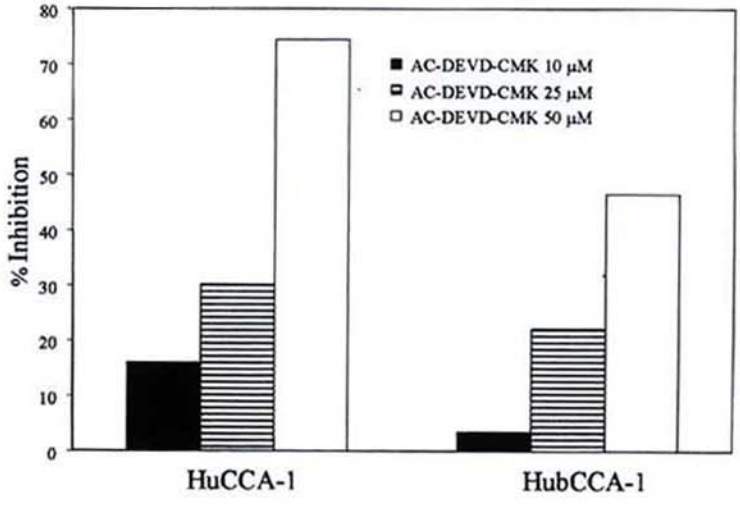

Fig. 5 AC-DEVD-CMK inhibits cytotoxic activity of the combined treatment of TNF-α and triptolide. HuCCA-1 and HubCCA-1 cells were preincubated with AC-DEVD-CMK at concentrations of 10, 25, 50 μM for 2 hours before exposured to the combination of 10 ng/ml TNF-α and 50 ng/ml triptolide. The percentage of inhibition was calculated and found to be statistically significant at 5% level for all 3 inhibitor concentrations.

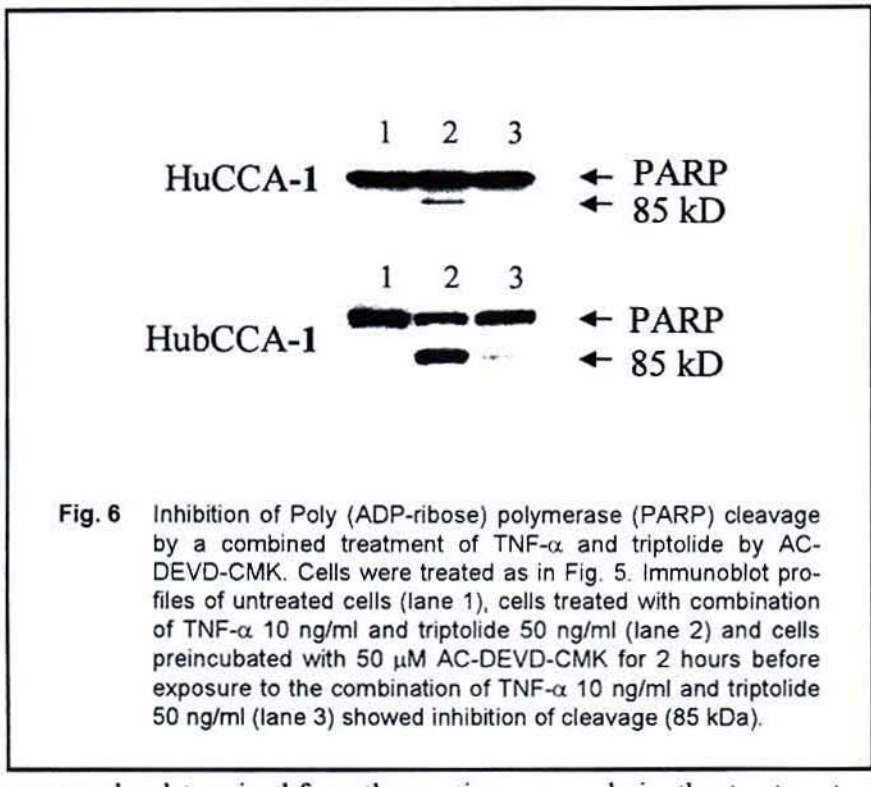

Fig. 6 Inhibition of Poly (ADP-ribose) polymerase (PARP) cleavage by a combined treatment of TNF-α and triptolide by AC-DEVD-CMK. Cells were treated as in Fig. 5. Immunoblot profiles of untreated cells (lane 1), cells treated with combination of TNF-α 10 ng/ml and triptolide 50 ng/ml (lane 2) and cells preincubated with 50 μM AC-DEVD-CMK for 2 hours before exposure to the combination of TNF-α 10 ng/ml and triptolide 50 ng/ml (lane 3) showed inhibition of cleavage (85 kDa).

age was also determined from these two cell lines by Western blot. The cells pretreated with this caspase inhibitor showed a significant reduction of degradation of PARP induced with the combined treatment of TNF-α and triptolide (lane 3, Fig. 6). The caspase 3 enzyme was involved in the PARP cleavage of apoptotic cells. As to be expected the Western blot analysis of tumor cell lysate treated with the combination of TNF-α and triptolide with antibody to procaspase 3 (32 kDa) showed degradation of this enzyme between 6-10 hours (data not shown). These results therefore confirm that TNF-α synergizes with triptolide in cytotoxicity and apoptosis induction against human cholangiocarcinoma cells. Lee *et al.*[13] previously reported that triptolide synergized with TNF-α in the induction of apoptosis in breast cancer, non-small cell lung cancer and fibrosarcoma cell lines and exhibited to enhance the cytotoxicity and apoptosis induction. The synergistic action between TNF-α and triptolide should provide an alternative approach in the treatment of cholangiocarcinoma and warrant further investigation.

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