CHAPTER VI

DISCUSSION

Cholangiocarcinoma, carcinoma that arises from biliary duct epithelium, is rare compared to the other cancer types. However, it has a high incidence rate in the northeastern part of Thailand. The etiology of cholangiocarcinoma is still unclear. Inflammation and secreted death receptor ligands, TNF-alpha, are particularly implicated in the pathogenesis of cholangiocarcinoma, along with dysplasia, an intermediate step in the development to cholangiocarcinoma. Dysregulation of apoptosis plays an important role in pathogenesis of tumor development. Apoptosis is an active process initiated by either external or internal stimuli that lead to cell death. Apoptosis can be classified to two major signaling pathways that activate caspases: the extrinsic death receptor pathway and the intrinsic mitochondrial cytochrome c/Apaf-1 pathway. The mitochondrial death pathway can be activated by various stimuli such as irradiation, anti-cancer drugs and prostaglandins. Activation of the extrinsic apoptosis pathway by stimulation of death receptors such as CD95 (Apo-1/Fas), TRAMP, TRAIL-RI, TRAIL-R2 and TNF-R1 also leads to the activation of apoptosis. TNF-alpha is a death ligand that can trigger apoptosis by more than one pathway through its receptors; the most widely accepted pathway involves TNF-alpha associated death domain protein (TRADD), Fas-associated death domain protein (FADD), and caspase-8. TNF-alpha-activated caspase 8 subsequently activates caspase-3, which in turn cleaves multiple cellular proteins, resulting in apoptosis. In recent years, the roles of TNF-alpha/TNFR and the apoptosis signaling pathway in carcinoma have been increasingly investigated. However, knowledge about effects of apoptosis of cholangiocarcinoma by TNF-alpha is scarce.

In this study, we investigated the apoptotic sensitivity/resistance of cholangiocarcinoma cell lines after prolonged treatment of TNF-alpha, using KKU-100 and KKU-213, poorly differentiated and well-differentiated cholangiocarcinoma cell lines, respectively. Both cell lines were investigated for TNFR expression by RT-PCR. The results showed that both of them expressed TNFR1 and TNFRII. To investigate anti-tumor effects, we determined the cytotoxicity and apoptosis of cholangiocarcinoma after TNF-alpha administration. Cytotoxicity testing was performed by MTT assay and apoptotic sensitivity/resistance testing was performed by DAPI staining. Both methods included exposure to 160 ng/ml TNF-alpha and incubation for 24 h.

Both cytotoxicity and DAPI staining confirmed the resistance of TNF-alpha to induce apoptosis in cholangiocarcinoma. In DAPI staining, TNF-alpha-treated KKU-100 and KKU-213 cell lines did not show characteristic DNA fragmentation and chromatin condensation compared to positive control. When correlated with the MTT assay, these results revealed no significant difference of cytotoxicity with or without TNF-alpha administration to KKU-100 and KKU-213 cell lines. These results are contrary to previous reports, where high dose of TNF-alpha (100 ng/ml) can induce apoptosis after it is administrated for 24 h in ameloblastoma (AM-1 cell line). In addition, analysis of the TNF-alpha action on many other tumor cell lines such as breast carcinoma (BT-20), epidermal carcinoma (HepG-2) and breast adenocarcinoma (MCF-7) demonstrated that TNF-alpha induced cytotoxicity but not apoptosis in cells that expressed only TNF-RI (Higuchi M. 1994). On the other hand, these investigators showed that apoptosis occurred only in cells that expressed both type of receptors (Sarin A. 1995).

Other cholangiocarcinoma cell lines, HuCCA-1 and HuCCA-1Nu, were exposed to high concentration of TNF-alpha (100 ng/ml) but apoptosis could not be induced in either cell line. In the same cell lines, apoptosis was induced by 760 pg/ml TNF-alpha for HuCCA-1 and 100 pg/ml TNF-alpha for HuCCA-1 Nu, in combination with 1 ug/ml actinomycin D. Moreover, only TNF-RI was found to participate directly in the TNF-alpha induced apoptosis of these cholangiocarcinoma cell lines (Utaisincharoen P. 1999).

TNF-alpha alone was unable to kill KKU-100 and KKU-213 cell lines. These results indicate that KKU-100 and KKU-213 are similar to several other cancer cell lines, such as ovarian tumor cell line (SKOV-3) and hepatoma (Hep G2), that are resistant to the toxic action of TNF-alpha (Morimoto H. 1991, Wong GHW. 1994, Wang CY. 1996).

The above-mentioned cholangiocarcinoma cell lines respond to apoptosis with an upregulation of apoptosis inhibitor bcl-2, a protein that protects the cells from the toxic effects of TNF-alpha. (Terada T. 1996, Harnois DM. 1997). Overexpression of Mcl-1, a potent antiapoptotic member of the Bcl-2 family, plays a role in apoptosis resistance in human cholangiocarcinoma cells (Taniai M. 2004 and Justin L. Mott. 2008). Further, Mcl-1 is overexpressed in 70% of primary tumor samples as measured by immunohistochemistry (Kobayashi S. 2005). Mcl-1 blocks apoptosis by preventing the mitochondrial dysfunction that results from activation of Bcl-2 family, Bax and Bak (Michels J. 2005). Activated Bax and/or Bak form homo-oligomers within the outer mitochondrial membrane that cause membrane permeabilization and release of apoptogenic, intermembrane and in the cytosol, these polypeptides act through caspase-9 to promote the activation of the effector caspase-3 and caspase-7, which induce cell death (Deng Y. 2002).

Additionally, the ability of TNF-alpha to induce cell death is prevented by it signal through NF-ΚB which results in activation of several anti-apoptotic genes.

Another signaling molecule that is involved apoptosis selection is the TRAF family of proteins. The TRAF family of proteins (6 members, TRAF 1-6) is likely to be a critical element in the choice. These proteins react with several members of the TNF receptor superfamily. The proteins of this family lack a death domain, and are characterized by their unique but conserved C terminal domain, the TRAF domain, which is divided between the TRAF N and TRAF C sub domain. TRAF2 to 6 contain an N-terminal zinc finger region, a ring finger, and five tandem zinc fingers. The function of the zinc fingers is still not clear, but it is believed to activate downstream signaling, in particular for NK- ΚB action. As for the TRAF domain (C terminal region), it interacts with TRADD and triggers the proapoptotic pathway (TNFR1- TRADD-FADD-Caspase). TNFRI and TNFRII can engage another pathway (TNFR- TRADD-TRAF- NF- ΚB) and this pathway is believed to be involved in cell survival.

The TRADD-TRAF association may provide a molecular mechanism for selection between the two pathways; apoptosis or NF-ΚB activation. If so, it is conceivable that sites of control for divergence from the activation of apoptosis to the transcription factor NF-ΚB might be discovered. For example, molecular control sites might exist at the level of the receptor by selection of TNFRII over TNFRI1. Or molecular controls may stimulate TRADD to bind with different TRAFs or (FADD), depending on which pathway needs to be entered (apoptosis or NF-ΚB).

Another antiapoptotic pathway to be described was signaling via the PI3K2/Akt module (Dudek, H. 1997, Kulik, G. 1997 and Kauffmann-Zeh, A. 1997). The constitutive activation of PI3K signaling due to the loss of the lipid phosphatase PTEN has been observed in various cancers, including advanced

prostate cancer (Simpson, L. 2001). Akt1 has been identified as the most prominent kinase downstream of PI3K. It inhibits apoptosis by phosphorylation of the proapoptotic protein BAD; transcription factors of cAMP-responsive element-binding protein, YAP, and FOXO families; and by affecting glucose metabolism (Thompson, J. E. 2004 and Datta, S. R. 1999). Other targets of Akt connected with regulation of apoptosis include the IkB, Mdm2, Par-4, GSK3, and mTOR kinases (Goswami, A. 2005 and Song, G. 2005). In addition to the constitutive activation of PI3K signaling in PTEN-deficient cells, growth factors, chemokines, and neuropeptides can protect prostate cancer cells from apoptosis via PI3K-dependent (Shulby, S. A. 2004, Lin, J. 1999, Dolloff, N. G. 2005 and Sumitomo, M. 2001) as well as PI3K-independent mechanisms (Kulik, G. 2001).

However, further studies may require determining which molecular controls are correlated to the resistance of TNF-alpha mediated apoptosis in KKU-100 and KKU-213 cholangiocarcinoma cell lines.

PROBLEM

Degradation of DNA in other apoptotic systems is known to be associated with the impairment of the DNA repairing system (PARP). This zinc finger DNA-binding protein detects DNA strand breaks and recruits a DNA repair complex to the damage site. This cleavage occurs before the appearance of DNA fragmentation. Damage to the formation of this enzyme (PARP) leads to chromosome instability, cell cycle arrest and cell death. The data presented in this study cannot be interpreted for PARP, because intact PARP band intensities were absent. This may be caused by many problems, including mutation or a problem of antibody properties.