Mechanisms of TRAIL-resistance

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Chapter 1

Introduction and scope of the thesis

Programmed cell death is a critical and active process and it involves complex molecular factors and signaling pathways, thereby maintaining tissue homeostasis and controlling potentially harmful cells. Currently, cell death is fundamentally divided into accidental cell death (ACD) or regulated cell death (RCD) based on functional aspects[1]. ACD is a biologically uncontrolled process that is triggered by unexpected attack and injury, whereas RCD is executed by a set of precise signaling cascades and therefore can be predicted and manipulated. Due to the comprehension of various molecular mechanisms leading to RCD, it can be classified into multiple subroutines including apoptosis, lysosomal cell death, pyroptosis, NETosis, immunogenic cell death, necroptosis, entosis, pathanatos, ferroptosis, autosis, oxeiptosis and alkaliptosis[2]. Apoptosis was the first process to be described[3]. Morphological characterizations of apoptosis include cell shrinkage, membrane blebbing, apoptotic body formation, DNA fragmentation and chromatin condensation. There are two apoptotic pathways: the extrinsic and the intrinsic. Extrinsic pathways (also called death receptor pathways) are triggered by ligands that interact with cell surface receptors. For example, the binding of TRAIL (TNF-related apoptosis-inducing ligand) to death receptors (DRs) stimulates apoptosis[4]. DRs refer to the members with death domains in the TNFR superfamily, which includes TNF receptor 1 (TNFR1), Fas (also called CD95 or Apo-1), death receptor 3 (DR3), death receptor 4 (DR4, also called TRAILR1), death receptor 5 (DR5, also called TRAILR2). The intrinsic pathways (also called mitochondrial apoptotic pathways) are usually initiated in an autonomous manner, for instance DNA damage or endoplasmic reticulum stress, and these result in leakage of cytochrome C from the mitochondrion.

The basic molecular mechanism of apoptosis has been well studied nowadays. In extrinsic pathways, the critical step is the activation of apoptotic initiators. Caspase-8 and -9 exist as inactive procaspase monomers that are activated by dimerization and this dimerization process is generalized as an "induced proximity model"[5]. It is functional in facilitating autocatalytic cleavage between large and small subunits to create a stable active dimer. Executioner caspases including caspase-3 or -7 exist as inactive dimers, which are activated by cleavage of initiator caspases. Once a single executioner caspase is activated, it cleaves and actives other executioner caspases leading to acceleration of caspase activation[6]. In contrast, in the intrinsic pathways, the formation of the apoptosome is the essential step. In this multiprotein complex cytochrome C, apoptotic protease-activating factor 1 (APAF1) and caspase-9 are assembled. Normally, cytochrome C exists in the inner membrane of mitochondria. It can be released in the early stage of apoptosis through a process called mitochondrial outer membrane permeabilization (MOMP), which is induced by proapoptotic proteins from the Bcl-2 family, such as Bax and

Bak. In the cytosol, the binding of cytochrome C to APAF1 triggers oligomerization of APAF1, leading to the recruitment and activation of caspase-9. Finally, executioner caspases are activated and they cleave downstream proteins[7].

TRAIL is widely considered as a potent anti-tumor therapeutic due to its selective apoptosis-inducing character. TRAIL binding to DR4 or DR5 triggers recruitment of Fas-associated death domain (FADD) and procaspase-8, forming a death-inducing signal complex (DISC) and leading to stimulation of caspase-dependent apoptosis[8,9]. Initially, TRAIL was recognized as an apoptosis-inducing ligand only via extrinsic pathways. However, more recent studies showed that Bid can be cleaved by active caspase-8 in the Fas-induced apoptosis pathway, thereby acting as a mediator between extrinsic and intrinsic pathways[10]. Later on, the same fate of Bid was also discovered in TRAIL-induced apoptosis pathway[11]. In type I cells, active caspase-8 efficiently induces sufficient executioner caspases to induce apoptosis. In contrast, in type II cells not enough executioner caspases are being activated and these cells require the additional involvement of mitochondrial activation to induce apoptosis via the intrinsic pathway. Therefore, cleavage of Bid by caspase-8 is required to stimulate intrinsic apoptosis pathway in type II cells[12].

Dulanermin, a recombinant human soluble protein corresponding to 114–281 amino acids of TRAIL, has been developed as a clinical anti-cancer drug. An early clinical Phase I study showed that Dulanermin was safe in patients with advanced cancer. However, only 2 patients (3%) with chondrosarcoma had partial treatment responses longer than 6 months[13]. This may be related to TRAIL-resistance phenomena, which were observed in many cancer cell lines, such as colorectal cancer cells[14], lung cancer cells[15] and hepatocellular cancer cells [16].

Here in this thesis, we aim to investigate the underlying molecular mechanism related to TRAIL-resistance phenomena using two specific variants, DR4-specific variant 4C7 and DR5-specific variant DHER. 4C7 contains mutations of G131R, R149I, S159R, N199R, K201H and S215D, while DHER contains mutations of D269H and E195R. Besides an enhanced affinity to either DR4 or DR5, 4C7 or DHER show a high apoptosis-inducing activity against cancer cell lines, such as COLO 205, DLD-1 and A2780[17,18]. We also aim to overcome TRAIL-resistance using combination strategies in different tumor cancer cells.

Colorectal neoplasia causes around 880,000 death worldwide every year[19]. It is estimated to be the third leading cancer types for new death in 2019 in United States[20]. One case presentation of a patient with BRAF mutant colon carcinoma enrolled in a phase 1b open-label clinical study showed the promising result that the disease remained stable during the treatment with FOLFIRI plus Dulanermin [21]. This implies that TRAIL shows anti-tumor effects on

colorectal cancer cells. Fucosylation is one of the important types of post-translational modification in colon cancer[22]. A positive correlation between TRAIL sensitivity and mRNA levels of fucosyltransferase enzymes FUT3 and FUT6 in a panel of colon adenocarcinoma cells was reported[23]. Taken together, this indicates that FUT3 and FUT6-induced fucosylation may be related to TRAIL-mediated apoptosis. Therefore, in **Chapter 2** we aimed to investigate a more precise role of fucosylation on DR4 and DR5-mediated apoptosis respectively, using TRAIL receptor-specific variants 4C7 and DHER[18,24]. We have found that low FUT3 or FUT6-expressing cells are insensitive to DR5 but not DR4-mediated apoptosis, while high FUT3 or FUT6-expressing cells are sensitive to TRAIL via both death receptors. This insensitivity to DR5 can be restored by upregulation of FUT3 or FUT6 as shown in FUT3 and FUT6 transfected cells. Moreover, increased association of death receptors was observed on FUT3 or FUT6 overexpression cells, which leads to more DISC formation and enhanced activation of caspase-8. Interestingly, we showed an improved sensitivity to TRAIL by external administration of L-fucose to colon cancer cells.

Many studies including our **Chapter 2** focus on the factors directly involved in the TRAIL-induced apoptosis signaling axis to elucidate TRAIL-resistance phenomena, such as dysregulation of TRAIL receptors, formation of DISC, activation of caspase-8, inhibition of anti-apoptotic protein c-FLIP or XIAP. It is also noteworthy to pay attention to communications between cells via extracellular signals. Nowadays, increasing evidence shows that extracellular vesicles (EVs) secreted by cancer cells influence tumor microenvironment and determine the therapeutic responses[25]. In **Chapter 3**, we for the first time showed that DR5, but not DR4, is expressed on the surface of EVs, leading to decreased sensitivity to TRAIL. Moreover, both long and short isoforms of DR5 are indicated to be displayed on EVs and they contribute to TRAIL sensitivity.

Taken together, above studies demonstrate two potential explanations to understand TRAIL-resistance phenomenon. Next, we move our attention to improve TRAIL-induced apoptosis by combination treatment. Epigenetic studies focus on the alterations of chromatin changes independent of DNA sequence and regulation of epigenetics is increasingly being investigated in cancers[26]. In **Chapter 4**, we focus on the inhibition of histone modifying enzymes and we discuss the aberrant regulation of histones in cancer. We highlight the current understanding of epigenetic mechanisms that drive the resistance to TRAIL-induced apoptosis. We also touched upon the improvement of TRAIL-induced apoptosis by selective histone inhibitors. At last, we suggest novel drug targets and using combination treatment to overcome TRAIL-resistance phenomenon.

The previous chapter demonstrates the importance of epigenetic regulation in TRAIL-induced apoptosis signaling and indicates combination treatment as an effective therapy for improving TRAIL sensitivity. In **Chapter 5**, we combined histone deacetylase (HDAC) inhibitors with TRAIL variants, DR4-specific TRAIL 4C7 and DR5-specific TRAIL DHER, to overcome TRAIL-resistance. We show that TRAIL-mediated apoptosis is largely improved in the colon cancer cell line WiDr by pretreatment of Entinostat, a HDAC 1, 2, 3-specific inhibitor. We also found that HDAC3-specific inhibitor RGFP966 and HDAC-8 specific inhibitor PCI34051 improve TRAIL sensitivity on a DLD-1 cell line. To confirm our observations, we silenced HDAC 1, 2 and 3 respectively using siRNA and followed by the treatment of TRAIL. In concert with our previous results, the data show an increased number of apoptotic cells. Furthermore, we established a 3D spheroid model to investigate the apoptosis-inducing effect of the combination treatment and we found improvement of apoptosis by detecting caspase 3/7 activity.

Since we showed that HDAC inhibitors enhance TRAIL-sensitivity, we are also interested in the role of histone acetyltransferase (HAT) inhibitors in apoptosis signaling. In **Chapter 6,** we used a novel p300 and CBP-selective inhibitor, A485, which was shown to be more potent than other inhibitors[27] and studied non-small-cell lung cancer cells. Firstly, we silenced EP300 and CREBBP, respectively, followed by the treatment with TRAIL. We found that TRAIL-induced apoptosis is largely increased in EP300 and CREBBP downregulated cells. This result implies that p300 and CBP are potential targets for improving sensitivity to TRAIL. Next, we showed that A485 on its own does not induce apoptosis and this may due to the upregulation of both pro- and anti-apoptotic proteins at the mRNA level. However, combining A485 and TRAIL significantly increased apoptosis of cells via the caspase cascade. This result indicates that A485 augments TRAIL-induced apoptosis. Furthermore, we showed a synergistic effect for the combination of A485 and TRAIL on cell proliferation in short and long-term. More importantly, we generated EGFR-TKI-resistant cell lines to explore the application of A485-TRAIL combination for this clinically relevant genotype. Interestingly, this combination also synergistically increased cell death in short and long-term. The volume of 3D spheroids generated from EGFR-TKI-resistant cells obviously decreased more by the combination treatment compared to single treatment.

The studies presented in this thesis are summarized and discussed in **Chapter 7.**