FUTURE STRATEGIES IN THE TREATMENT OF AGGRESSIVE THYROID CANCER

Tania Pilli

Section of Endocrinology and Metabolism, Department of Internal Medicine, Endocrinology & Metabolism and Biochemistry, University of Siena, Italy.

Introduction. Radioiodine-refractory and anaplastic thyroid cancers have a poor prognosis that may not be significantly altered by the current treatment regimens. Therefore there is a compelling need to identify potential new therapies. TNF-related apoptosis inducing ligand (TRAIL) induces apoptosis selectively in tumor cells, the *IG20/MADD* (Insulinoma-Glucagonoma), a novel human gene, is over-expressed in cancer and confers TRAIL resistance. Therefore, we aimed to investigate the effects of *IG20/MADD* knockdown on TRAIL induced-apoptosis in different types of thyroid cancer. **Material and Methods.** A shRNA expressing lentivirus was used to selectively knockdown *IG20/MADD* in the human papillary thyroid cancer (PTC) cell lines: TPC1, KTC1 and BCPAP, the human follicular thyroid cancer (FTC) cell lines: WRO and FTC133 and the human anaplastic thyroid cancer (ATC): C643, CAL62 and HTh7. Apoptosis was assessed by propidium iodide or TMRM staining or detection of activated caspase-3 by flow-cytometry. **Results.** TPC1, BCPAP, WRO, C643, CAL62 and HTh7 cells were sensitive to TRAIL-induced apoptosis, while KTC1, FTC133, FRO and 8505C cells were resistant to even high dose of TRAIL (100 ng/ml). Upon knockdown of *IG20/MADD* all the sensitive cell lines cells had increased susceptibility to TRAIL-induced apoptosis and previously resistant 8505C and FTC133 cells became sensitive to TRAIL. Only FRO cells could not be rescued from TRAIL resistance because of caspase 8 deficiency, which is not a common finding in "in vivo" thyroid tumors. Moreover, the combination of TRAIL with select Akt/mTOR (perifosine and everolimus) and/or MEK inhibitors (PD-0325901) showed an antagonistic effect on TRAIL-induced apoptosis in ATC C643 cells, but had no effect on ATC 8505C cells.
Conclusions. *IG20/MADD* knockdown in TRAIL treated thyroid cancer cells enhanced apoptosis in sensitive cells and induced significant cytotoxicity in resistant cells. This effect was not enhanced significantly in the presence of various inhibitors of the MAPK/PI3K/Akt signaling pathways. TRAIL treatment combined with *IG20/MADD* knockdown may be a potential therapeutic modality for radioiodine-refractory and anaplastic thyroid cancer.

Keywords: Thyroid Cancer, TRAIL, Apoptosis, IG20

Thyroid carcinoma is the most frequently occurring endocrine cancer and is one of the most rapidly increasing human cancers in the United States and other countries (Davies et al 2006, Leenhardt et al 2004). A majority of patients with thyroid cancer who undergo appropriate treatment have an excellent outcome. However, in about 10% of patients with well-differentiated thyroid cancer (papillary thyroid cancer [PTC] and follicular thyroid cancer [FTC]), the tumor loses its ability to take up radioiodine, or becomes poorly differentiated or dedifferentiated, leading to recurrent disease and death (Pacini et al 2008). Further, patients with anaplastic thyroid cancer (ATC) have a very poor prognosis, with a mean survival time of less than 6 months from the time of diagnosis, an outcome that may not be significantly altered by current treatment regimens (Smallridge et al 2009). Therefore, there is a compelling need for better understanding the thyroid tumorigenesis and for improving the treatment of these cases.

Progress in identifying genetic/epigenetic alterations in thyroid cancer cells is rapidly offering several opportunities to develop new drugs directed to specific targets (e.g. tyrosine kinase inhibitors). The results of several phase II trials using molecular drugs are promising, however, none of the treated patients had a complete response, and only a minority of them had a partial response and the treatment associated toxicities may be significant (Schlumberger et al 2009, Smallridge et al 2009).

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF family and can induce apoptosis in a variety of cancer cells with little or no effect on normal cells (Wiley et al 1995, Walczak et al 1999, Ashkenazy&Dixit 1999). Because of this selectivity, TRAIL represents a promising target for anticancer therapy but the limitation of its use is represented by the development of resistance. Different mechanisms can inhibit TRAIL signaling in tumors (e.g. over-expression of FLICE inhibitory protein) (Thome et al 1997, Poulaki et al 2002).

Dr Prabhakar's group has, also, found that IG20 (insulinoma/glucagonoma) gene, is overexpressed in cancer cells and tissues and can confer resistance to TRAIL-induced apoptosis (Prabhakar et al 2008). It can encode, in nonneuronal tissues, at least four different splice variants (IG20-SVs), namely IG20pa, MADD/DENN, IG20-SV2 and DENN-SV (Al Zoubi et al 2001, Lim et al 2004, Li et al 2008), but only MADD has been shown

Correspondence to:
Tania Pilli
Section of Endocrinology and Metabolism, Department of Internal Medicine, Endocrinology & Metabolism and Biochemistry, University of Siena, Policlinico "Santa Maria alle Scotte", Viale Bracci 2, 53100 Siena, Italy.
email: luciabrilli@alice.it

to be necessary and sufficient for cell survival (Mulherkar et al 2006).

In a preliminary study (Subramanian et al 2009), we determined, by real-time PCR, the IG20 gene expression levels in thyroid normal and tumor tissues and in two cell lines derived from well differentiated (WRO) and undifferentiated (FRO) thyroid cancer. We found that IG20 levels were higher in benign and malignant thyroid tumors and in WRO and FRO cells relative to normal tissues. Predominantly, MADD and DENN-SV isoforms of IG20 gene were expressed. Using a short hairpin RNA (shRNA) that targets exon 15, expressed in all IG20 isoforms, we selectively knocked down IG20 resulting in increased spontaneous and TRAIL - induced apoptosis in WRO, but not FRO, cells.

TRAIL interacts with two cell-surface death receptors 4 & 5 (DR4/DR5) (Ashkenazy&Dixit 1999) and leads to activation of caspase-8 or caspase-10 which in turn activate downstream effector caspases (caspase-3 and -7) with an irreversible commitment to cell death (Kischkel et al 2000, Sprick et al 2000, Mitsiades et al 2000).

Since MADD is a negative regulator of caspase 8 (Mulherkar et al 2007), we expected an increased caspase-8 activation upon IG20 knockdown, therefore we determined the basal levels of caspase-8 in FRO cells and interestingly we found that these cells expressed much lower levels of caspase-8 compared to WRO follicular thyroid cancer-derived cell line (Subramaniam et al 2009). Therefore, we assumed that the resistance of FRO cells to apoptosis could be due to caspase-8 deficiency. To test this hypothesis, we re-expressed caspase-8 in FRO cells and we found that the cells became susceptible to TRAIL treatment; as expected, IG20 knockdown further increased the TRAIL-induced apoptosis (Prabhakar et al 2009).

We also determined the levels of caspase 8 in normal and tumor thyroid tissues but we did not find any deficiency or statistically significant difference in the expression of the protein. Since the caspase-8 defect appears to be rare in human thyroid cancers, it may not represent a limitation to the use of IG20 as therapeutic target in thyroid cancer (Prabhakar et al 2009).

Subsequently, we examined four additional thyroid cancer cell lines: BCPAP, TPC1, KTC1 and FTC133. Three out of them (BCPAP, TPC1, KTC1) derived from papillary thyroid cancer and FTC133 derived from follicular thyroid cancer. All cell lines expressed higher levels of IG20/MADD relative to normal thyroid tissues consistently with previous results (Prabhakar et al 2009). We tested sensitivity of all four thyroid cancer cell lines to TRAIL induced apoptosis and observed that among the PTC-derived cell lines: BCPAP cells and TPC1 cells were highly sensitive to TRAIL-induced apoptosis even when TRAIL was used at a lower concentration. In contrast, PTC-derived cell line KTC1 and the FTC-derived cell line FTC133 were only marginally susceptible even when a high concentration of TRAIL was used (Prabhakar et al 2009).

To see whether IG20/MADD was contributing to the observed resistance of KTC1 and FTC133 cells to TRAIL-induced apoptosis, we knocked down the IG20/MADD gene expression, and found that it could render KTC1 and FTC133 cells susceptible to TRAIL-induced apoptosis (Prabhakar et al 2009).

Recently, Dr Prabhakar's group has observed that MADD is a natural substrate for Akt phosphorylation and that only phosphorylated MADD (pMADD) can bind to DR4/DR5 and prevent activation of caspase-8 and protect cells from undergoing spontaneous and TRAIL-induced apoptosis (Jayarama et al 2008). In TRAIL susceptible cells, upon TRAIL treatment the non-phosphorylated Akt and MADD accumulate, which leads to MADD dissociation from and FADD association with DRs resulting in caspase-8 activation and cell death (Jayarama et al 2008).

Consistently with these findings, we found that upon TRAIL treatment the levels of both pAKT and pMADD were significantly reduced in the TRAIL sensitive BCPAP and TPC1 cells, while they remained unchanged in the TRAIL-resistant KTC1 and FTC133 cells (Prabhakar et al 2009). To see is if a reduction in the levels of pMADD can render TRAIL resistant KTC1 and FTC1 cells susceptible, we transfected these cells with either a empty vector or a vector capable of expressing DN-Akt and treated the cells with TRAIL. Over-expression of DN-Akt induced a significant dephosphorylation of pAkt and pMADD in KTC1 and FTC133 cells and rendered these cells more susceptible to spontaneous as well as TRAIL-induced apoptosis (unpublished data). These observations raised the possibility of using TRAIL in conjunction with either MADD knockdown or MADD dephosphorylation to enhance the therapeutic efficacy of TRAIL.

A reduction in pMADD levels upon TRAIL treatment could result from either up-regulation of PTEN or activation of a protein phosphatase; either of which can reduce the levels of pAkt with a resultant reduction in the levels of pMADD. However, the precise mechanism by which the levels of pAkt and pMADD are reduced in TRAIL sensitive thyroid cancer cell lines is unknown and is under active investigation.

Recently, we have selected four anaplastic thyroid cancer-derived cell lines (C643, HTh7, Cal62 and 8505C), three out of them with an activating mutation of RAS (CAL62, C643 and HTh7) and one of BRAF (8505C); HTh7 cells have also Akt copy gain. The use of cell lines with RAS mutation and Akt copy gain allows us to carry on studies in the context of an Akt constitutive activation that is commonly found in more advanced cancer stage and in more aggressive tumor histotypes, but also the cell line harbouring the BRAF mutation is functional to our investigation since some undifferentiated cancers derive from papillary thyroid tumors and have been shown to maintain the BRAF mutation.

We tested these cell lines for sensitivity to TRAIL-induced-apoptosis and 3 out of them, C643, CAL62 and HTh7, were found to be sensitive and one, 8505C, highly resistant even to the highest dose of the drug (100 ng/ml) (Pilli et al 2010).

In order to increase the sensitivity of these cell lines to TRAIL-induced apoptosis, we have combined TRAIL treatment with some Akt/mTOR (perifosine and everolimus) and/or MEK inhibitors (PD-0325901).

Surprisingly we have found that all the inhibitors, alone or in combination, had an antagonistic effect on TRAIL-induced apoptosis in C643 cells while in 8505C cells they did not show any significant addictive effect or synergy (Pilli et al 2010).

One of the mechanisms underlying this effect in C643 cells might be the induction of autophagy since both perifosine and everolimus are well known to be able to elicit this process. Indeed, we have demonstrate, that the expression of LC3-II, a protein required for the autophagosomes formation, upon TRAIL treatment was

significantly enhanced when these inhibitors are used in combination with it. Moreover, when C643 cells were treated with TRAIL plus the inhibitors in combination with chloroquine, an inhibitor of autophagy, their antagonistic effect was less strong (Pilli et al 2010).

On the other hand, upon IG20 knockdown, in C643 cells we were able to induce the same percentage of apoptosis using 50 ng/ml of TRAIL as compared to 100 ng/ml of the drug alone. In 8505C cells that are highly resistant to TRAIL the sensitivity to this drug improved significantly (Pilli et al 2010).

In summary, our data show that TRAIL alone might be sufficient to induce apoptosis in certain thyroid cancer cells, while in TRAIL resistant thyroid cancer cells, it should be used, in conjunction with down-modulation of IG20/MADD expression or phosphorylation to effectively induce apoptosis.

Moreover, since TRAIL treatment and down modulation of IG20/MADD expression or phosphorylation can selectively induce apoptosis primarily in cancer cells and not normal cells, combining these modalities of apoptosis induction might be a highly effective strategy to treat thyroid cancer.

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