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Interplay between Ret and Fap-1 regulates CD95-mediated apoptosis in medullary thyroid cancer cells

Valentina Nicolini [a], Giuliana Cassinelli [a,*], Giuditta Cuccuru [a,1], Italia Bongarzone [b], Giovanna Petrangolini [a,2], Monica Tortoreto [a], Piera Mondellini [b], Patrizia Casalini [c], Enrica Favini [a], Nadia Zaffaroni [a], Franco Zunino [a], Cinzia Lanzi [a]

[a] Molecular Pharmacology Unit, Department of Experimental Oncology and Molecular Medicine, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy
[b] Proteomics Laboratory, Molecular Mechanisms Unit, Department of Experimental Oncology and Molecular Medicine, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy
[c] Molecular Therapy Unit, Department of Experimental Oncology and Molecular Medicine, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy

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ABSTRACT

Emerging evidence suggests that Ret oncoproteins expressed in medullary thyroid cancer (MTC) might evade the pro-apoptotic function of the dependence receptor proto-Ret by directly impacting the apoptosis machinery. Identification of the molecular determinants of the interplay between Ret signaling and apoptosis might provide a relevant contribution to the optimization of Ret-targeted therapies. Here, we describe the cross-talk between Ret-M918T oncogenic mutant responsible for type 2B multiple endocrine syndrome (MEN2B), and components of death receptor-mediated extrinsic apoptosis pathway. In the human MEN2B-type MTC cell line MZ-CRC-1 expressing Ret-M918T, Ret was found associated with Fap-1, known as inhibitor of the CD95 death receptor trafficking to the cell membrane, and with procaspase-8, the initiator pro-form caspase in the extrinsic apoptosis pathway. Cell treatment with the anti-tumor Ret kinase inhibitor RPI-1 inhibited tyrosine phosphorylation of procaspase-8, likely inducing its local activation, followed by downregulation of both Ret and Fap-1, and translocation of CD95 into lipid rafts. According to the resulting increase of CD95 cell surface expression, the CD95 agonist antibody CH11 enhanced RPI-1-induced cell growth inhibition and apoptosis. *RET* RNA interference downregulated Fap-1 protein in MZ-CRC-1 cells, whereas exogenous *RET*-M918T upregulated Fap-1 in HEK293 cells. Overall, these data indicate that the Ret oncoprotein exerts opposing controls on Fap-1 and CD95, increasing Fap-1 expression and decreasing CD95 cell surface expression. The functional interplay of the Ret mutant with the extrinsic apoptosis pathway provides a mechanism possibly contributing to MTC malignant phenotype and a rational basis for novel therapeutic strategies combining Ret inhibitors and CD95 agonists.

1. Introduction

Medullary thyroid carcinoma (MTC) arises from thyroid C cells [1]. It represents 5% of thyroid cancers and may originate sporadically (75%) or as a component of the inherited multiple endocrine neoplasia type 2 (MEN2) syndromes including MEN2A, MEN2B and familial MTC [2]. MTC, which is the most common cause of death in MEN2 patients, is relatively unresponsive to radiotherapy and conventional chemotherapy. Thyroidectomy remains the standard treatment and the only curative therapy [3]. The lack of effective treatments for unresectable, recurrent, or metastatic MTC indicates the urgent need for new therapeutic options.

Activating mutations of the *RET* proto-oncogene have been implicated in the pathogenesis of hereditary MTC being present in almost 100% of MEN2A and MEN2B cases and in 30–50% of sporadic cases [4,5]. *RET* encodes a receptor tyrosine kinase (TK) widely expressed in neural crest-derived tissues including thyroid C cells. Germline *RET* mutations are recognized as disease causative events [6]. MEN2 is characterized by strong genotype/phenotype association, with specific *RET* mutations identified in each disease subtype [7]. MEN2B, the most aggressive form, is primarily associated with missense mutations in the intracellular TK domain. Most MEN2B patients harbor the M918T mutation, whereas <5% of patients have the A883F mutation. *RET* M918T, which is also the

* Corresponding author at: Molecular Pharmacology Unit, Department of Experimental Oncology and Molecular Medicine, Fondazione IRCCS, Istituto Nazionale dei Tumori, via Amadeo 42, 20133 Milan, Italy.
Tel.: +39 02 23902202; fax: +39 02 23902692.
E-mail address: giuliana.cassinelli@istitutotumori.mi.it (G. Cassinelli).
[1] Present address: Laboratory of Molecular Oncology, Center for Experimental Research and Medicine (CeRMS), Università di Torino, Turin, Italy.
[2] Present address: Indena S.p.A., Milan, Italy.

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most frequent somatic mutation in sporadic MTCs is prognostic of aggressive disease thus indicating a strong carcinogenic potential [3,7]. Substitution of methionine 918 with threonine results in increased Ret kinase activity, release of auto-inhibition, increased ligand-independent formation of activated monomers and dimers, and change in substrate specificity contributing to the receptor oncogenic activity [8,9]. Preclinical studies have provided evidence that Ret oncoproteins may represent a therapeutic target exploitable in subsets of thyroid tumors [5,10,11]. By the screening of a series of novel synthetic arylidene indolin-2-one compounds in a kinase assay, we identified the indolin-2-one named RPI-1 as a Ret inhibitor and reported its ability to inhibit kinase activity, expression and downstream signaling of Ret oncoproteins in thyroid cancer models [12,13]. The drug showed antitumor activity in mice carrying tumor xenografts from a human MTC cell line (TT) harboring the *RET* C634W MEN2A-type mutation [14]. Tumor regressions in treated animals were observed in association with apoptosis induction and angiogenesis inhibition [15]. Currently, several agents targeting Ret among other kinases are under clinical investigation in thyroid cancers [3,16].

Proto-Ret belongs to the family of receptors defined as "dependence receptors" because they depend on their respective ligands to prevent apoptosis [17]. This group of structurally unrelated receptors shares the common feature of inducing two distinct forms of signal transduction: proliferation, survival, differentiation and migration in the presence of ligand, apoptosis in the absence of ligand. Apoptosis requires the receptor cleavage by specific caspases and the release/exposure of proapoptotic peptides [18]. The proapoptotic function is apparently lost in oncogenically activated Ret receptors, whereas recent studies suggest that they may intercept apoptotic pathways by multiple mechanisms. Indeed, either selective signaling disruption by *RET* dominant-negative or expression knockdown by *RET* siRNA induce apoptosis in TT cells [19,20]. We found that activation of Akt and Erks was inhibited by RPI-1 in TT cells, with the consequent activation of Bad and caspase-9, resulting in apoptosis induction through the intrinsic pathway [15]. Recently, Fas-associated phosphatase-1 (Fap-1, PTPN13) has been identified as one of the RPI-1-sensitive components in the signaling network of *RET* mutant-expressing MTC cells [21]. Fap-1, a large non-receptor protein tyrosine-phosphatase (PTP), is the only protein known to associate with the C-terminal negative regulatory domain of CD95 (Fas/APO1) death receptor (DR) [22,23].

Elucidation of mechanisms and players of the cross-talk between Ret signaling and apoptosis pathways may provide a rational basis for the optimization of Ret-targeting approaches in MTC. The known function of Fap-1 as negative regulator of CD95 DR [24] prompted us to investigate the involvement of the extrinsic apoptosis pathway in RPI-1-induced cell death. Here, we examined the role and the molecular determinants of DR-mediated apoptosis in a cellular model of human MEN2B MTC, focusing on the interconnection between Ret and CD95-mediated apoptosis.

2. Materials and methods

2.1. Chemicals and reagents

Synthesis and chemical structure of RPI-1 (1,3-dihydro-5,6-dimethoxy-3-[(4-hydroxyphenyl) methylene]-H-indol-2-one) (Cpd1) were previously reported [12]. The agonistic anti-CD95 antibody, clone CH11, was purchased from MLB Co. (Nagoya, Japan). The caspase inhibitors: pan caspase inhibitor Z-VAD-FMK, caspase-8 inhibitor Z-IETD-FMK and caspase-9 inhibitor Z-LEHD-FMK were from Calbiochem (San Diego, CA). For cell treatments, RPI-1, dasatinib, sorafenib, and caspase inhibitors were dissolved in DMSO and diluted in cell culture medium (final solvent concentration 0.5%).

2.2. Cell culture, growth inhibition and transfection assays

The human MTC cell lines MZ-CRC-1, harboring the MEN2B-type RET M918T mutation and TT, harboring the MEN2A-type RET C634W mutation [25] were grown in DMEM and Ham's F12 medium (Invitrogen, Carlsbad, CA), respectively, supplemented with 15% FBS (HyClone Laboratories, Logan, UT), in a 5% CO_2 atmosphere. NIH3T3 mouse fibroblasts and NIH3T3^{MEN2B}, stably transfected with the *RET* M918T mutant (short isoform), were maintained in DMEM containing 10% and 5% calf serum (Colorado Serum Company, Denver, CO), respectively, in a 10% CO_2 atmosphere [26]. Human embryonic 293 T cells (HEK293) were grown in DMEM supplemented with 10% FBS and transiently transfected by calcium phosphate precipitation with pRc/CMV expression vector carrying the insert *RET*^{M918T} (short isoform) [27,28].

For cell growth inhibition assays, fibroblasts were treated with RPI-1 the day after seeding whereas MTC cells were exposed to RPI-1 4 days after plating because of their prolonged lag period. The drug antiproliferative effect was evaluated after 3 days by cell counting (fibroblasts) or after 7 days by the sulforhodamine B (SRB) colorimetric assay (MTC cells) as previously described [14]. Drug concentrations able to inhibit cell growth by 50% or by 80% (IC_{50}, IC_{80}) were calculated from dose–response curves. The effect of RPI-1/CH11 combination treatment on cell growth was evaluated by SRB assay. Cells were incubated with RPI-1 for 96 h or with CH11 for 48 h, alone or in combination (CH11 in the last 48 h). Drug interactions, expressed as synergistic ratio (SR) values, were calculated according to Kern et al. [29]. $SR > 1$ indicates synergy; $SR = 1$, or $SR < 1$, indicates absence of synergy.

2.3. RNA interference

To silence *RET* in MTC cells, the Hs-RET-9-HP–validated siRNA from Qiagen (Santa Clarita, CA) was used. The negative control siRNA (AllStars) was from Qiagen. The oligonucleotides (final concentration 200 nM for MZ-CRC-1 cells and 100 or 200 nM for TT cells) were transfected into the cells using the Lipofectamine 2000 transfection reagent in serum-free Opti-MEM I (Invitrogen). Cells were incubated for 4 h before complete medium addition. After 48 h, the transfection procedure was repeated. Cells were then lysed for Western blot analysis, after additional 48 h.

2.4. Biochemical analyses

Cells were processed for total protein extraction or immunoprecipitation, followed by Western blotting, as previously described [14,15]. The biochemical effects of CD95 stimulation were analyzed in MZ-CRC-1 cells treated with CH11 antibody for 48 h. Where indicated, cells were pre-treated with RPI-1, or vehicle, for 48 h before addition of the CH11 antibody. To study the effects of caspase inhibition, cells were pre-treated with 50 μM caspase inhibitors for 1 h, and then exposed to vehicle or RPI-1 for 48 h. Cells were treated with dasatinib and sorafenib for the indicated times at the concentrations corresponding to the IC_{80} and/or IC_{50} assessed by cell counting at 72 h.

For the isolation of lipid rafts-enriched cell fractions, cells were washed twice in ice-cold PBS containing sodium orthovanadate (0.1 mM), and lysed in 1% Triton X-100 lysis buffer as described [30]. Detergent-soluble and -insoluble (enriched in lipid rafts) fractions were separated by centrifugation (10,000 × *g* for 15 min). Equal volumes of each fraction were analyzed by Western blotting.

Mouse monoclonal antibodies used were: anti-phosphotyrosine, clone 4G10, from Upstate Biotechnology (Lake Placid, NY);