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Activation of protein phosphatase causes alternative splicing of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL): Potential effect on immune surveillance

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Abstract

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) belongs to the TNF superfamily of proteins. It is highly expressed on natural killer cells, cytotoxic T lymphocytes, and monocytes after stimulation, and plays a critical role in immune surveillance. Two splice variants of TRAIL were identified recently that show no proapoptotic activity. Phosphorylation level in splicing factors, serine–arginine-rich (SR) and heterogeneous ribonucleoproteins (hnRNPs) govern the mRNA splicing of several apoptosis-related genes. We characterized the apoptotic stimuli-mediated alternative splicing pattern of TRAIL and investigated the possible underlying mechanism of alternative splicing. Etoposide and cycloheximide induced alternative splicing, whereas staurosporine (a broad kinase inhibitor) blocked both constitutive and alternative splicing. *De novo* ceramide synthesis and subsequent protein phosphatase-1 (PP-1) activation enhanced the alternative splicing, as did TNF-α but not interferon alpha (IFN-α) stimulation. We demonstrated that TRAIL alters gene expression through mRNA splicing and may change proapoptotic potential in response to cytokine stimulation.

Keywords: Alternative splicing; TRAIL; Immune surveillance; Signaling pathway; SR proteins; Phosphatase activation; Ceramide synthesis; Apoptosis; Lymphocyte; PBMC; Cytokine

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a rare molecule that kills transformed cells, but largely spares normal cells. It is therefore a promising anti-cancer agent [1]. TRAIL is highly expressed on natural killer cells (NK cells), cytotoxic T lymphocytes (CTLs), and monocytes after interferon alpha (IFN-α) and gamma (IFN-γ) stimulation, and is a key player in tumor surveillance [2–4]. A recent study identified two splice variants of TRAIL (TRAIL-β and TRAIL-γ), although the mechanism responsible for this alternative splicing has not been elucidated [5]. The lack of exon 3 in TRAIL-β and exons 2 and 3 in TRAIL-γ result in massive truncation of the TRAIL extracellular domain and a consequent loss of proapoptotic potential [5].

Post-transcriptional modification of gene expression through alternative splicing is emerging as an important mechanism for introducing significant protein diversity at a low genetic cost [6,7]. Alternative splicing generates functionally distinct products from the same apoptosis-related genes including Bcl-x, caspase-9, caspase-2, Fas, and caspase-8, probably providing distinct regulatory functions

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for apoptotic fine tuning [8,9]. Pre-mRNA splicing occurs within the spliceosome, a macromolecular complex comprising serine–arginine-rich (SR) and heterogeneous ribonucleoproteins (hnRNPs) [10]. RNA-recognition motifs (RRM) in the N-terminus and a variable-length arginine/serine-rich domain at the C-terminus (the RS domain) characterize SR proteins [10,11]. These molecules are also subjected to post-translational modification during apoptosis [12–14]. The serine residues within the SR domains undergo reversible phosphorylation, leading to modulation of splicing activity and finally to alteration of mRNA splicing. Exon skipping associated with alternative splicing can produce frame shifts, thereby generating a stop codon and splice variants with an opposite/dominant-negative function [15]. Recent studies provided several mechanistic insights into how alternative splicing might modulate the function of apoptosis-related genes. They suggest that *de novo* ceramide generation and subsequent phosphatase activation causes dephosphorylation of SR proteins leading to alteration of mRNA splicing, as observed for Bcl-x in A549 cells, as well as caspase-2 and Fas in U937 cells [16–18].

The present study examined the crosstalk between alternative splicing of TRAIL and intracellular signaling pathways. We examined proapoptotic stimuli-mediated splicing patterns and sought to elucidate the underlying mechanism. The results demonstrated that *de novo* ceramide generation and subsequent phosphatase activation induced alternative splicing of TRAIL mRNA, and that TNF-α stimulation also generated alternative splicing of TRAIL.

Materials and methods

Reagents. Reagents used were as follows: etoposide, staurosporine, cycloheximide, calyculin A, fumonisin B1, D-erythro-C6 ceramide, and PMA (Sigma Chemical Co., St. Louis, MO); human recombinant TNF-α (PeproTec, Rocky Hill, NJ); kinase inhibitors SB203580 and SB202190, and human recombinant IFN-α (Calbiochem, La Jolla, CA).

Cell culture. The local ethics committee approved all experimental protocols and a signed consent was obtained from each healthy volunteer. PBMC from healthy volunteers, as well as U937 and Jurkat cells were grown in 5% CO_2 at 37 °C in RPMI1640 medium (Invitrogen, San Diego, CA), supplemented with 9% heat-inactivated bovine serum (Invitrogen), penicillin, and streptomycin (Invitrogen). Cells were grown and then harvested at midlog phase.

Flow-cytometry analysis for the determination of apoptosis. We analyzed apoptosis by flow cytometry as described previously [18].

RNA isolation and RT-PCR analysis. Total RNA was isolated using the RNeasy Total RNA kit (Qiagen, Hilden, Germany) as described by the manufacturer [18]. One microgram of total RNA was reverse transcribed into cDNA using the Qiagen OneStep RT-PCR kit (Qiagen) and specific primer pairs were as described previously [5,18,19]. The resulting cDNA was used as the template for PCR amplification of TRAIL splice variants (sense 5′-GAA TCC CAT GGC TAT GAT GGA GGT CCA G-3′ and anti-sense 5′-GGA TTC GAG GAC CTC TTT CTC TCA CTA-3′ (GenBank Accession No. U37518)) or caspase-2 splice variant (sense 5′-AAC TGC CCA AGC CTA CAG AA-3′ and anti-sense 5′-GTC AAC CCC ACG ATC AGT CT-3′ (GenBank Accession No. U13021), or GAPDH (sense 5′-GCA GGG GGG AGC CAA AAG GG-3′ and anti-sense 5′-TGC CAG CCC CAG CGT CAA AG-3′ (GenBank Accession No. J04038), The PCR cycling conditions used were as follows: 35 cycles (94 °C, 2 min; 58 °C, 30 s; 72 °C, 1 min) for TRAIL, 35 cycles (94 °C, 30 s; 55 °C, 30 s; 72 °C, 1 min) for caspase-2, and 30 cycles (94 °C, 45 s; 67 °C, 30 s; 72 °C, 1 min) for GAPDH, as described previously [5,18,19]. The resulting fragments were subjected to electrophoresis and analyzed as described previously [18].

Sequencing of RT-PCR products. The RT-PCR products of TRAIL and caspase-2 mRNA were purified and analyzed in both directions using sequencing primers as follows and as described previously [18]: TRAIL forward 5′-TTC ACA GTG CTC CTG CAG TC-3′, reverse 5′-GAG GAC CTC TTT CTC TCA CTA-3′; Caspase-2 forward 5′-AAC TGC CCA AGC CTA CAG AA-3′, reverse 5′-GTC AAC CCC ACG ATC AGT CT-3′.

Results

Characterization of mRNA splicing patterns in response to multiple proapoptotic stimuli

The two primer pairs shown in Fig. 1A were used to distinguish normal transcripts (NT: constitutive splicing) from splice variants (SV: alternative splicing). Induction of alternative splicing in caspase-2 mRNA was carried out as a control experiment [18]. Multiple proapoptotic stimuli generated the larger fragments of caspase-2 and the smaller fragments of TRAIL (Fig. 1B). Sequence analysis confirmed that these fragments were identical in sequence to caspase-2S and TRAIL-β, respectively. Proapoptotic stimuli-mediated alternative splicing patterns were different between caspase-2 and TRAIL mRNA: staurosporine induced a strong alternative splicing of caspase-2 mRNA, but inhibited both constitutive and alternative splicing of TRAIL mRNA in U937 cells and PBMC (Fig. 1B). All treatments induced a loss of mitochondrial membrane potential ($\Delta\Psi_m$) and an increase in hypodiploidism, indicating induction of apoptosis (Fig. 1C).

Dose- and time-dependent induction of alternative splicing of caspase-2 and TRAIL

We chose etoposide for U937 cells and cycloheximide for PBMC because of their potent activity for inducing alternative splicing (Fig. 1B). RT-PCR revealed that alternative splicing of caspase-2 and TRAIL mRNA was dose-dependent (Fig. 2A). The bands of TRAIL-γ mRNA were not detectable (data not shown). Time-course experiments revealed prompt induction of alternative splicing of caspase-2 and TRAIL mRNA 1 h after stimulation (Fig. 2B), which was significantly time-dependent during apoptosis (Fig. 2C). These results indicated that this induction of alternative splicing occurs transcription-independently and is regulated via signaling pathways other than those controlling protein synthesis.

Phosphatase activation and subsequent ceramide synthesis causes alternative splicing of TRAIL mRNA

Ceramide activates serine/threonine protein phosphatases [20]. We next examined whether this mechanism could