A cross-talk between RNA splicing and signaling pathway alters Fas gene expression at post-transcriptional level: Alternative splicing of Fas mRNA in the leukemic U937 cells

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It is now widely accepted that alternative splicing is a mechanism that is responsible for generating protein complexity at low genetic cost. However, little is known about molecular mechanisms that govern alternative splicing of key apoptotic regulators. Here we investigate the effect of pro-apoptotic stimuli on alternative splicing of Fas mRNA by means of reverse transcription-polymerase chain reaction (RT-PCR). Exposure of U937 cells to etoposide, staurosporine, pacritaxel, or cyclohexamide promoted the appearance of the splice variant, which retained the 152-base-pair intron 5. Pretreatment with calyculin A, an inhibitor of protein phosphatase-1 (PP-1) as well as fumonisin B1, an inhibitor of ceramide synthase, prevented etoposide-induced alternative splicing of Fas mRNA. Our data demonstrate that cross-talk between RNA splicing and signaling pathways through endogenous ceramide synthesis and subsequent phosphatase activation is a mechanism that modifies Fas gene expression at the posttranscriptional level. (J Lab Clin Med 2005;146:184–191)

Abbreviations: ASF/SF2 = alternative splicing factor/splicing factor 2; CCD = charge coupled device; hnRNPA1 = heterogeneous nuclear ribonucleoprotein A1; JNK = c-Jun N-terminal kinase; MAPK = mitogen-activated protein kinase; MKK = mitogen-activated protein kinase kinase; RRM = RNA recognition motif; RT-PCR = reverse transcription-polymerase chain reaction; SAPK = stress-activated protein kinase; SD = standard deviation; SMase = sphyngomyelinase; snRNP = small nuclear ribonucleoprotein; SR protein = serine-arginine protein; $\delta\Psi m$ = loss of mitochondrial membrane potential

Pre-mRNA splicing occurs within the spliceosome, which consists of splicing factors including SR proteins and their antagonistic hnRNP proteins.[1–3] The SR proteins are nuclear phosphoproteins that play a critical role in constitutive and alternative pre-mRNA splicing. SR proteins have 1 or 2 RNA RRMs and an RS domain that is rich in alternating serine and arginine residues.[1–4] Reversible phosphorylation in serine residues is likely to regulate the functional activity of SR proteins by altering the ratio of positive and negative charges on SR proteins, which affects RNA binding activity and protein–protein interactions of SR proteins. A recent study showed that ceramide induces dephosphorylation of SR proteins, which leads to alternative splicing of Bcl-x(L) and caspase-9 mRNA.[5,6] In addition, we recently demon-

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Submitted for publication December 22, 2004; accepted for publication May 11, 2005.

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0022-2143/$ – see front matter

doi:10.1016/j.lab.2005.05.004

strated that etoposide-induced alternative splicing of caspase-2 mRNA occurs through endogenous ceramide synthesis and subsequent phosphatase activation.[7] This evidence suggests that cross-talk between RNA splicing and signaling pathways is a mechanism that regulates gene expression at the posttranscriptional level.

Splicing signals are a frequent target of mutations in genetic diseases, cancer, and autoimmune diseases.[8,9] In humans, it has been estimated that at least 15% of point mutations that result in genetic diseases cause RNA splicing defects.[10] Splice variants of Fas mRNA have been detected in hematopoietic as well as nonhematopoietic cancer cell lines. Distinct exon(s) is excluded in splice variants.[11] The encoded proteins lack the death domain or, less frequently, a transmembrane domain and act as dominant negative inhibitors of normal Fas, thereby inhibiting Fas-mediated apoptosis.[12]

In 13 of 22 patients (59%) with cutaneous T-cell lymphomas, a novel spliced transcript of the Fas gene was recently identified that displays retention of a 152-base-pair sequence corresponding to intron 5 and encodes a dysfunctional Fas protein.[13] Interestingly, this splice variant was not caused by splice site mutations because none were detected in intron 5 or in its boundaries. Intriguingly, a soluble form of Fas (sFas) is generated through alterative splicing: Exon 6 inclusion results in an mRNA encoding a membrane-bound Fas, whereas exon 6 skipping results in an mRNA encoding sFas that acts as an apoptosis inhibitor.[14] This study was designed to identify the regulatory mechanism that governs alternative splicing of Fas mRNA in response to pro-apoptotic stimuli.

MATERIALS AND METHODS

Drugs and chemical reagents. Etoposide, pacritaxel, staurosporine, cyclohexamide, calyculin A, fumonisin B1, and D-e-C_6 ceramide were purchased from Sigma-Aldrich (St. Louis, Mo). Kinase inhibitor SB203580 and SB202190 were purchased from Calbiochem (San Diego, Calif).

Cell culture and treatment. U937 cells of the human leukemic cell line were grown in 5% CO_2 at 37°C with RPMI 1640 (Invitrogen, Carlsbad, Calif) and supplemented with 9% heat-inactivated bovine serum (Invitrogen), penicillin, and streptomycin (Invitrogen). Cells were grown and harvested at mid-log phase. U937 cells were incubated in the absence or presence of 100 μmol/L etoposide, 100 μmol/L pacritaxel, 1 μmol/L staurosporine, and 355 μmol/L (100 μg/mL) cyclohexamide for the indicated time periods. For inhibition experiments, U937 cells were preincubated in 2 μmol/L SB203580, 40 μmol/L SB202190, 100 μmol/L fumonisin B1, or 0.5–5.0 nmol/L calyculin A for the periods indicated and then stimulated with 100 μmol/L etoposide for 4 hours. For treatments with D-e-C_6 ceramide, U937 cells were incubated in the absence or presence of 100 μmol/L D-e-C_6 ceramide for 24 hours.

Flow-cytometry analysis for the determination of apoptosis. We analyzed apoptosis by means of flow cytometry with $DiOC_6$(3,3′-dihexyloxacarbocyanine iodide; Lambda, Graz, Austria) to evaluate the loss of mitochondrial membrane potential ($\Delta\Psi m$) and with popidium iodide (100 μg/mL, Sigma-Aldrich) to evaluate DNA fragmentation, as described.[15] In brief, cells were stained for 15 minutes at 37°C with 40-nmol/L $DiOC_6$, washed, and analyzed with a flow cytometer (Epics XL, Beckman Coulter, Hialeah, Fla). DNA fragmentation was quantified by the percentage of cells with hypodiploid DNA. Treated U937 cells were fixed with 70% ethanol and treated with RNAase (100 μg/mL, Sigma-Aldrich) and then stained with popidium iodide (100 μg/mL, Sigma-Aldrich) for 30 minutes on ice. The stained cells were analyzed with a flow cytometer (Epics XL, Beckman Coulter).

Total RNA extraction and RT-PCR assays. Total RNA was isolated from treated or untreated cells through the addition of a 600 μL RT lysis buffer and purification in accordance with the instructions provided by the manufacturer (Rneasy Total RNA kit; Qiagen, Valencia, Calif). We used the Qiagen OneStep RT-PCR kit under the following conditions: 1× QIAGEN One Step RT-PCR buffer, 400 μmol/L of each deoxyribonucleoside triphosphate, and 0.6 μmol/L of each primer, 2 μL of Qiagen OneStep RT-PCR enzyme mixture, and 1 μg of template RNA in a total volume of 50 μL. RNA was reverse-transcribed for 30 minutes at 50°C; the initial PCR step was activated by means of heating for 15 minutes at 95°C before PCR. For evaluation of Fas splice variant expression, we used sense primer 5′-TCAAGGAATGCACACTCACC-3′ and antisense primer 5′-CCAAACAATTAGTGGAATTG-3′ (with reference to GeneBank accession number M67454), as reported.[13] Using these primers, we amplified the RT reaction for 35 cycles (94°C for 30 seconds, 58°C for 30 seconds, 72°C for 1 minute) For the evaluation of the caspase-2 splice variant expression, we used sense primer 5′-AACTGCCCAAGCCTACAGAA-3′ and anti-sense primer 5′-GTCAACCCCACGATCAGTCT-3′ (with reference to GeneBank accession number U13021), as reported.[7,16] Using these primers, we amplified the RT reaction for 35 cycles (94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute). For evaluation of GAPDH expression, we used sense primer 5′-GCAGGGGGGAGCCAAAAGGG-3′ and anti-sense primer 5′-TGCCAGCCCCAGCGTCAAAG-3′ (with reference to GeneBank accession number J04038). Using these primers, we amplified the RT reaction for 33 cycles (94°C for 45 seconds, 67°C for 1 minutes, 72°C for 1 minute). The PCR products were subjected to electrophoresis on 2.0% agarose gels. The amplified DNA fragments were visualized with ethidium bromide, and fluorescence was detected with a CCD digital camera (Kodak, Rochester, NY) and analyzed with *Quantity One* software (Bio-Rad, Hercules, Calif).

Sequencing of RT-PCR products. The RT-PCR products of Fas mRNA were purified with the GFX DNA purification kit (Amersham bioscience, Piscataway, NJ) before both strands were sequenced with a BigDye Termination Cycle Sequencing Ready Reaction kit (Perkin-Elmer, PE Applied