Regulation of Enterocyte Apoptosis by Acyl-CoA Synthetase 5 Splicing

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Background & Aims: **The constant renewal of enterocytes along the crypt–villus axis (CVA) of human small intestine is due to cell-inherent changes resulting in the apoptotic cell death of senescent enterocytes. The aim of the present study was to examine underlying molecular mechanisms of the cell death at the villus tip.** ***Methods:*** **Characterization of human acyl-coenzyme A (CoA) synthetase 5 (ACSL5) was performed by cloning, recombinant protein expression, biochemical approaches, and several functional and in situ analyses.** ***Results:*** **Our data show that different amounts of acyl-CoA synthetase 5-full length (ACSL5-fl) and a so far unknown splice variant lacking exon 20 (ACSL5-Δ20) are found in human enterocytes. In contrast with the splice variant ACSL5-Δ20, recombinant and purified ACSL5-fl protein is active at a highly alkaline pH. Over expression of ACSL5-fl protein is associated with a decrease of the anti-apoptotic FLIP protein in a ceramide-dependent manner and an increased cell-surface expression of the death receptor TRAIL-R1. Expression analyses revealed that the ACSL5-fl/ACSL5-Δ20 ratio increases along the CVA, thereby sensitizing ACSL5-fl–dominated cells at the villus tip to the death ligand TRAIL, which is corroborated by functional studies with human small intestinal mucosal samples and an immortalized human small intestinal cell line.** ***Conclusions:*** **Our results suggest an ACSL5-dependent regulatory mechanism that contributes to the cellular renewal along the CVA in human small intestine. Deregulation of the ACSL5-fl/ACSL5-Δ20 homeostasis in the maturation and shedding of cells along the CVA might also be of relevance for the development of intestinal neoplasia.**

The fundamental pattern of epithelial differentiation in adult human small intestine is the crypt–villus axis (CVA), consisting of the proliferative cell pool located in the crypts, followed by differentiated enterocytes, which are finally eliminated by apoptosis at the luminal parts of the villus. The formation of this pattern is due to a well-balanced homeostasis of cellular proliferation, differentiation, migration, and apoptosis along the CVA that is controlled by a variety of signaling cascades including the Wnt, Notch, and Hedgehog pathways, and by proteins such as LKB1 and BMP.[1] In addition, epithelial differentiation is further regulated by minor (eg, iron, vitamins) and major (lipids, amino acids, carbohydrates) factors, which are able to regulate gene expression and cellular functions.[2] Among the lipids, long-chain fatty acids and especially their coenzyme A (CoA)-thioester derivatives are considered to play an important role in epithelial differentiation and maintenance of the CVA.

In mammalians, long-chain fatty acid CoA-thioesters are synthesized by the isoforms of the long-chain acyl-CoA synthetase (ACSL) gene family, the most important step in cellular long-chain fatty acid metabolism for the generation of bioactive acyl-thioesters.[3] Recent studies have indicated that the ACSL isoform 5 (ACSL5; fatty acid CoA ligases, AMP forming; E.C. 6.2.1.3.) is highly expressed in human small intestinal mucosa with an increasing gradient along the CVA, and these studies have suggested that ACSL5 synthesis is an indicator for the state of villus architecture.[4,5] The *ACSL5* gene is located on chromosome 10q25.1-q25.2, spans approximately 46 kb, and comprises 21 exons (GeneBank accession nos. AB033899, AB033920).[6] At present, 3 transcript variants are known differing in the 5′ untranslated region; *ACSL5_v1* encodes the protein ACSL5a, which contains an additional 56 amino acids at the amino-terminal end compared with the predominantly found ACSL5b protein (SwissProt accession No. Q9ULC5) encoded by both *ACSL5_v2* and *ACSL5_v3*.

Abbreviations used in this paper: ACSL5-fl, acyl-CoA synthetase long chain isoform 5-full length; ACSL5-Δ20, acyl-CoA synthetase long chain isoform 5 lacking exon 20; CoA, coenzyme A; CVA, crypt–villus axis; DAB, diaminobenzidine; ER, endoplasmic reticulum; FLIP, FLICE-inhibitory protein; FPLC, fast protein liquid chromatography; mAb, monoclonal antibody; PNS, postnuclear supernatant; TFA, tri-fluoro-acetic acid; TRAIL, TNF-related apoptosis inducing ligand.

0016-5085/07/$32.00
doi:10.1053/j.gastro.2007.06.005

The purpose of this study was to investigate the impact of the gradient pattern of the ACSL5 expression along the CVA for the differentiation and apoptosis of enterocytes in human small intestine. Our study revealed that the ratio of the ACSL5-full length (ACSL5-fl) and a so far unknown splice variant ACSL5-Δ20 increases along the CVA, which is assumed to be of functional relevance for death ligand-induced apoptosis of enterocytes at the villus tip in a ceramide-dependent manner. Alterations in the ACSL5-fl/ACSL5-Δ20 ratio along the CVA might also be involved in the pathogenesis of several intestinal disorders such as intestinal neoplasia.

Materials and Methods

Patients

The use of human tissues for study purposes was approved by each patient and by the local ethics committee at University Hospital RWTH Aachen or at Heidelberg University. Specimens of human normal terminal ileum ($n = 17$; mean age, 62 years; range, 48–80 years) or duodenum ($n = 13$; mean age, 68 years; range, 53–77 years) from surgical resections ($n = 30$) were used. Normal mucosa was mechanically dissected and then freshly used or immediately frozen in liquid nitrogen and stored at −80°C until use. Intestinal biopsies of 11 patients were used for the in vivo studies with tissue cultures ($n = 11$; mean age, 47 years; range, 21–66 years).

Protein Purification From Mucosa

Total protein from human small intestinal mucosa isolated according to Chomczynski's method[7] was separated by 7.5% curtain gels followed by electrophoretic elution of molecules between 80 and 50 kDa, accumulation by chloroform/methanol (1:4, vol/vol) precipitation, extraction with 0.1% *tri*-fluoroacetic acid (TFA), and purification by ultracentrifugation (430,000 ×*g* at 4°C for 15 minutes). The supernatants were separated by reversed phase chromatography on a fast protein liquid chromatography (FPLC) system (Resource RPC column, 3 mL; Amersham Pharmacia, Little Chalfont, England). A flow rate of 1 mL/min at 20°C with a stepwise linear gradient of the following solvent mixtures was used: solvent A, 0.1% TFA in water; solvent B, 0.1% TFA in acetonitrile.

Isolation of Enterocytes Along the Crypt–Villus Axis

Sequential isolation of small intestinal enterocytes along the CVA has been previously described[8] and was performed with slight modifications using mechanically dissected and further purified small intestinal mucosa of 5 different patients ($n = 5$). Briefly, a mucosa flap of about 9 cm^2 was washed in phosphate-buffered saline (PBS), incubated at 37°C for 10 minutes in 27 mmol/L sodium citrate and 1 mmol/L DTT (pH 7.3) followed by incubation with 1.5 mmol/L EDTA/0.5 mmol/L DTT (pH 7.3 at 37°C for 2, 3, 5, 10, 13, 15, 20, 30, and 58 minutes). At each time point, the isolated cells were pelleted and immediately further processed using Chomczynski's method.[7] To control cellular isolation, mucosal specimens were collected from the mucosa flap at each time point, formalin fixed, and prepared for histologic examination following routine protocols.

SDS-PAGE, Western Blot, and Immunostaining

Proteins solubilized in Laemmli's buffer were separated by SDS-PAGE (7.5% or 10%) and then either silver stained according to the Shevchenko procedure[9] with slight modifications or transferred to a PVDF Immobilon-P membrane (Millipore, Bedford, MA) by semidry blotting. The BioRad assay reagent was used for protein quantification (BioRad, München, Germany). The following antibodies were used: monoclonal antibody (mAb) KD7[4] for immunostaining of ACSL5-fl and ACSL5-Δ20, antibodies specific for FLIP (clone NF6) obtained from Axxora (Grünberg, Germany), antibodies against TRAIL-R and β-actin as well as all secondary horseradish peroxidase–conjugated antibodies (final dilution 1:10,000) from Santa Cruz Biotechnology, Santa Cruz, CA. The immunoreactions were visualized with the ECL substrate (Amersham Pharmacia). Negative controls included blots in which the primary antibody was omitted.

Reverse Transcription, Polymerase Chain Reaction, and Sequencing

Reverse transcription of RNA was carried out with a first-strand cDNA synthesis approach (Invitrogen Life Technologies, Carlsbad, CA). PCR runs were performed on a LightCycler system (Roche Diagnostics, Mannheim, Germany) as previously described.[4] For detection of ACSL5-fl and ACSL5-Δ20 expression the following sets of primers were used: 5′-TGC CAA AAC CAA GTT GTA AGG-3′, 5′-GAA AAA TGG CTT TGA CCT GTT C-3′; 5′-CAA AAC CAA GTC AAA GCC ATT-3′, 5′-TGG AAA GCT CTC CTC GCT TT-3′. For amplification of TRAIL-R1, TRAIL-R2, or c-FLIP, published primer sequences with the adapted PCR conditions were used.[10,11] Sequencing reactions were performed with an ABI 3700 capillary sequencer and the Big Dye Terminator kit (ABI, Weiterstadt, Germany).

Cloning of ACSL5-fl and ACSL5-Δ20

ACSL5 cDNAs were amplified by PCR using the following set of primers: 5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC TAC CAT GCT TTT TAT CTT TAA CTT TTT GTT TTC CCC ACT TCC-3′ and 5′-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC ATC CTG GAT GTG CTC ATA CAG GCT GT-3′ and cloned into the pENTRY vector of the GATEWAY cloning system (Invitrogen Life Technologies). Cytomegalovirus (CMV) expression constructs were synthesized by recombination into the pcDNA_DEST40 vector with a C-terminal fusion to a 6xHis-tag. For high-level expression of