Irinotecan Activation by Human Carboxylesterases in Colorectal Adenocarcinoma Cells

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ABSTRACT

Carboxylesterases play a critical role in the bioactivation of the anticancer prodrug irinotecan [7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxy-camptothecin; CPT-11] into its active metabolite SN-38 (ethyl-10-hydroxy-camptothecin). We reported recently that human carboxylesterase-2 (hCE-2) is a higher-affinity, higher-velocity enzyme for irinotecan hydrolysis when compared with hCE-1. To further investigate the role of these isoforms, we cloned both cDNAs into the human colorectal adenocarcinoma cell line HT29. Extracts of HT29 cells transfected with hCE-2 exhibited significantly higher irinotecan hydrolysis (5.2 pmol/mg protein/hr) than hCE-1 (1.0 pmol/mg protein/hr). HT29 cells over-expressing hCE-2 were more sensitive to the toxic effects of irinotecan than cells expressing hCE-1 (EC₅₀ = 0.3 μM and 6.8 μM, respectively). Our data further support the notion that hCE-2 plays a substantial role in irinotecan activation in human tissue at relevant pharmacologic concentrations.

INTRODUCTION

Irinotecan [7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxy-camptothecin; CPT-11], a water-soluble semisynthetic derivative of the plant alkaloid camptothecin, is a potent antitumor prodrug with strong activity against lymphoma, lung cancer, colorectal cancer, gastric cancer, ovarian cancer, and cervical cancer (1). The in vivo bioactivation of irinotecan to its active metabolite SN-38 (ethyl-10-hydroxy-camptothecin) is catalyzed by carboxylesterases (EC 3.1.1.1; Ref. 2). SN-38 is a potent inhibitor of topoisomerase I (3) and is eliminated mainly through conjugation by hepatic uridine glucuronosyltransferase into SN-38 glucuronide (SN-38G). SN-38G is 100-fold less active as a topoisomerase I inhibitor compared with SN-38 and is actively secreted into the bile by a canalicular-multispecific organic anion transporter. A second major polar metabolite of irinotecan in vivo is the less potent APC, the product of CYP3A4-mediated oxidation of the terminal piperidine ring of irinotecan (4). APC is 100-fold less active than SN-38 as a topoisomerase I inhibitor. A rabbit carboxylesterase has been shown to catalyze the conversion of APC to SN-38 in vitro, although the in vivo conversion of APC to SN-38 in humans remains to be investigated (5). In addition to carboxylesterases from various species, irinotecan can also be activated by the human and equine butyrylcholinesterases (acylcholine acylhydrolase; EC 3.1.1.8; Ref. 6).

In a recent study, we reported the kinetic characterization of irinotecan conversion to SN-38 by two distinct human carboxylesterase isoforms, hCE-1 and hCE-2 (7). hCE-2 is a higher-affinity and higher-velocity enzyme when compared with hCE-1. The Kₘ values are 3.4 μM and 43 μM for hCE-2 and hCE-1, respectively, and the catalytic efficiency of hCE-2 is 60-fold higher than that of hCE-1. At pharmacologically relevant concentrations, hCE-2 converts irinotecan to SN-38 at a 25–30-fold higher rate than hCE-1. In addition, incubation of SQ20b cells in the presence of low, pharmacologically relevant concentrations of irinotecan and purified hCE-2 results in increased cytotoxicity when compared with that seen in the presence of irinotecan and hCE-1 (7).

Both the human and rabbit carboxylesterases have been transfected with several tumor cell lines and are developed in enzyme/prodrug combinations with irinotecan (8, 9). Overexpression of hCE-1 in human tumor cell lines results in increased activation of irinotecan to SN-38 and enhanced cytotoxicity (8). However, the irinotecan concentrations used in these studies were significantly higher than pharmacologically relevant plasma concentrations observed after irinotecan administration to patients. Transfection with the rabbit liver carboxylesterase, an enzyme 100-1000-fold more efficient than hCE-1 at converting irinotecan to SN-38 in vitro, enhanced cell sensitivity by 12–55-fold to irinotecan. More recently, Khanha et al. (10) reported that the cloned human small intestine carboxylesterase (hiCE) efficiently activated irinotecan in COS-7 cells and conferred sensitivity of cells carrying this gene. This suggests a role of this isoform in rendering the human small intestine to irinotecan induced toxicity. Interestingly, hiCE (11) and hCE-2 (12) are identical along the entire coding region except for a stretch

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4 The abbreviations used are: APC, 7-ethyl-[4-N-(5-aminopentanoic acid)-1-piperidino] carbonyloxy-camptothecin; hCE, human carboxylesterase; IEF, isoelectrical focusing; HPLC, high performance liquid chromatography.
of nine amino acids immediately after the first methionine, which is not present in hCE-2.

To further investigate the role of these carboxylesterase isoforms, we cloned both hCE-1 and hCE-2 cDNAs from a human cDNA library and transfected the human colorectal adenocarcinoma cell line HT-29. Hydrolysis of irinotecan by the transfected cell lines and the effect of gene expression on sensitivity to irinotecan have been determined.

MATERIALS AND METHODS

Cloning and Transfection of hCE-1 and hCE-2. The pIRES-neo expression vector was purchased from Clontech Laboratories, Inc. (Palo Alto, CA). The Concert plasmid purification kits, restriction enzymes, and other reagents used for cloning were from Life Technologies, Inc. (Gaithersburg, MD). AmpliTaq Gold polymerase was from Applied Biosystems (Foster City, CA). The HT29 cell line was from the American Type Culture Collection (Manassas, VA). X-VIVO 10 (with 2 mM L-glutamine) was from BioWhittaker, Inc. (Walkersville, MD). All primers used in this study were synthesized by Genosys Biotechnologies, Inc. (The Woodlands, TX). The hCE-1 and hCE-2 cDNAs were amplified from the human liver cDNA library (Life Technologies, Inc., Gaithersburg, MD) using the following primers: hCE-1 5'-TTGCCGCCACCATGTGGCTC-3' (upper) and 5'-CCAGGCTCCCAATGATCACTGGATCTTCATTCACAGCTCT-3' (lower); hCE-2, 5'-TTGCCGCCACCATGATCACTGGATCTTCATTCACAGCTCT-3' (upper) and 5'-CCAGGCTCCCAATGATCACTGGATCTTCATTCACAGCTCT-3' (lower).

The cDNAs were then cloned into pIRES-neo for expression in mammalian cells. PCR reactions were performed on the GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA). Cycling parameters for both hCE-1 and hCE-2 were as follows: 95°C for 10 min followed by 30 cycles of 94°C for 1 min, 68°C for 1 min, with a final hold at 72°C for 5 min.

The PCR products were cloned into pIRES-neo and propagated in Escherichia coli for plasmid purification. HT29 cells were cultured to 40–60% confluence and electroporated on the Electo Square Porator T820 (Genetronics, Inc., San Diego, CA). Parameters used for electroporation were as follows: 500 V/cm, 10 pulses of 1 ms each in X-VIVO 10 + 1% human serum albumin. Electroporated cells were plated, cultured for 24 h, and then cultured for 12–14 days in the presence of 250 μg Geneticin. Individual colonies were then grown in Geneticin-containing medium for preparation of cell extract. Cloned hCE-2 cDNA was sequenced on the 377XL DNA sequencer (Applied Biosystems, Foster City, CA). The junctures between the 5' ends of inserts and the vector were confirmed by sequencing using primers on the vector upstream to the inserts (T7 primer: 5'-TAATACGACTCACTATAGGG-3'). Sequence data were analyzed using the MegAlign II module in the LaserGene package (DNASTAR, Inc., Madison, WI).

Preparation of Cell Extracts. HT29 cells, untransfected or transfected, in log-phase growth were trypsinized, washed in PBS, and resuspended in 50 mM potassium phosphate containing 10% Triton X-100 and 1% DTT. The cells were then sonicated on ice (3 x 10 s) each on a Microson Ultrasonic Cell Distributor (Heat Systems, Inc., Farmingdale, NY), with power output set at 30%. Particulates were removed by centrifugation at 8000g for 10 min at 4°C.

IEF, Native-PAGE, and Western Blot. The precast IEF ready gels (pH 5–8) and Tris-HCl ready gels (10%), running buffers, Ready Gel Cell, and the Trans-blot SD Transfer Cell were from Bio-Rad Laboratories (Hercules, CA). ECL Plus was from Amersham Life Science, Inc. (Arlington Heights, IL). Tris-buffered saline with Tween [50 mM Tris, 138 mM NaCl, 2.7 mM KCl, 0.05% Tween 20 (pH 8.0)] was from Sigma Chemical Co. (St. Louis, MO). Primary antibodies against hCE-1 and hCE-2 were raised in New Zealand rabbits as described previously (13). The horseradish peroxidase-conjugated secondary antibody was from Amersham Life Science Inc. (Arlington Heights, IL). Ten micrograms each of the extracts prepared from HT29 cells transfected with pIRES-neo, pIRES-hCE-1, or pIRES-hCE-2 were electrophoresed at 100 V for 1 h and 250 V for 1 h, followed by 500 V for 30 min. Native-PAGE was run at 150 V for 1 h. As a quantitative reference, a mixture containing 20 ng each of purified hCE-1 and hCE-2 was loaded on each gel. Immediately after electrophoresis, proteins in the gel were transferred onto polyvinylidene difluoride membrane using the Trans-Blot Transfer Cell with 0.7% glacial acetic acid as transfer buffer for 45 min at 12 V constant. The membranes were then blocked in 5% BSA for 30 min to 1 h, followed by sequential incubation in primary and secondary antibodies in Tris-buffered saline with 0.05% Tween 20 for 1 h, with four washes, 10 min each, after each incubation. The blot was then stained with ECL Plus and the image captured on the ChemiDoc Imaging system (Bio-Rad Laboratories).

Irinotecan Hydrolysis. Protein extracts (2 mg/ml) prepared from HT29 cells, untransfected or transfected with hCE-1, hCE-2 or the parental vector (pIRES-neo) were incubated with the designated concentrations of irinotecan at 37°C in 0.25 ml of 50 mM potassium phosphate buffer (pH 7.4). At selected incubation times, the reaction was stopped by the addition of 4 volumes of ice-cold methanol and placement of the solution on ice. One hundred microliters of internal standard (camptothecin, 1 μg/ml in 0.1 N HCl) was added. Samples were evaporated to dryness under nitrogen and reconstituted in 200 μl of HPLC mobile phase. SN-38 was quantitated by HPLC.

Quantitation of SN-38 Production. Irinotecan and SN-38 concentrations were determined by HPLC as modified from Gupta et al. (14). Briefly, irinotecan and SN-38 were separated using a Partisphere 10 μ-C18 column (4.5 x 250 mm); Whatman Inc., Clifton, NJ) with a mobile phase consisting of 27% acetonitrile, 73% 0.1 M potassium dihydrogen phosphate containing 3 mM sodium heptane sulfonate (pH 4.0). Detection was monitored using a Hitachi F1050 fluorescence detector (Hitachi Instruments, Naperville, IL) with λex = 375 nm and λem = 566 nm. The standard curves of irinotecan and SN-38 were linear (r = 0.99) within the range of 15–2500 ng/ml and 2–250 ng/ml, respectively.

Colony-forming Cytotoxicity Assay. HT-29 cells plated 16–18 h earlier at a density of 5 x 104 cells/25 cm² in culture medium (3:1 DMEM, Hank’s F-12K, 20% FBS, and 100 μg/ml penicillin-streptomycin) were washed and placed in serum-free culture medium. Irinotecan (1% volume) was added to the cell-culture medium to achieve the designated concentrations. After a 4-h incubation at 37°C, the cells were washed with fresh
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**RESULTS**

**Transfection and Expression of hCE Genes in HT29 Cells.** The cDNAs for hCE-1 and hCE-2 were amplified from the human liver expression library and subcloned into the bicistronic expression vector pIRES-neo. Clones with the highest rate of irinotecan hydrolysis were used in subsequent experiments. The inserts in the clones were sequenced to ensure no additional mutations were unintentionally generated during the cloning process.

To determine the expression levels for these clones, extracts were subjected to IEF followed by Western blot. As shown in Fig. 1A, the two proteins were well separated from each other by their isoelectric points (pH 5.8 and 4.9 for hCE-1 and hCE-2, respectively; Ref. 12, 15). The proteins were also separated by nondenaturing PAGE (native-PAGE), as shown in Fig. 1B, because hCE-1 is a trimer and hCE-2 is a monomer under native conditions (12, 15). From these data, no significant differences were observed in the expression levels of hCE-1 and hCE-2 in the two clones. These results also provide evidence that any difference in irinotecan hydrolysis and cell sensitization by irinotecan is attributable to the kinetic properties of the enzymes.

**Timed Comparison of Irinotecan Hydrolysis in Transfected HT29 Cells.** The conversion of irinotecan (10 μM) to SN-38 by hCE-1 and hCE-2 cell extracts was compared at 37°C.

**DISCUSSION**

We determined previously that, in purified form, hCE-2 is a higher-affinity, higher-velocity enzyme than hCE-1 for irinotecan hydrolysis (7). In this study, we report the activation of irinotecan by these two carboxylesterases when expressed in the human adenocarcinoma cell line HT29. The rate of irinotecan conversion to SN-38 was much greater in HT29 cells overexpressing hCE-2 than cells overexpressing hCE-1. As a result, HT29 cells overexpressing hCE-2 were significantly more sens-
sitive to the cytotoxic effect of irinotecan than hCE-1 cells, as evidenced by an EC_{50} of 0.3 μM for hCE-2 and 6.8 μM for hCE-1. An EC_{50} of 0.5 μM CPT-11 has been reported recently for the African green monkey cell line COS-7 carrying the human small intestine carboxylesterase gene (10), albeit from transiently transfected cells.

In human liver, there are at least four different enzymes/isoforms known to convert CPT-11 to SN-38: hCE-1, hCE-2 (7), hCE-10, and the human butyrylcholinesterase (17). In addition, the human placenta was shown to contain three isoforms of carboxylesterases, two of which were essentially identical in sequence to those from the liver (18). The human brain carboxylesterase may be a new isoform (19), although hCE-1 was shown to be present in brain tissues (20). Irinotecan conversion to its active metabolite may result from any combination of these isoforms, and possibly others yet to be identified, with contributions from each individual enzyme dependent on their concentration within human tissues. In this study, we report the expression of individual carboxylesterase isoforms in a relevant human colon cancer cell line with nondetectable levels of enzyme capable of irinotecan activation.

The topoisomerase I inhibitors represent a promising new class of anticancer drugs. Among the topoisomerase I inhibitors, irinotecan in particular has shown activity against colorectal cancers (1). As a means to further improve the therapeutic index of irinotecan, enzyme prodrug combinations with irinotecan are currently being developed using either hCE-1 or a purified human liver carboxylesterase that catalyzes the production of benzoylecgonine and dihydroecgonine (19). Irinotecan glucuronidation with diarrhea. Cancer Res., 54: 3723–3729, 1994.

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