Human Carboxylesterase 2 Is Commonly Expressed in Tumor Tissue and Is Correlated with Activation of Irinotecan

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ABSTRACT

The prodrug irinotecan is an active agent for the treatment of advanced colorectal cancer and a number of other solid tumors. Irinotecan is converted in vivo to SN-38 (7-ethyl-10-hydroxy-camptothecin), the active metabolite that causes cell death, by human liver carboxylesterases. Previous studies suggest that human carboxylesterase 2 (CES2) is the key activating isoform. Although conversion of irinotecan to SN-38 by liver carboxylesterase is an inefficient process, clinical data indicate that irinotecan has significant antitumor activity. This scenario raises the possibility that local conversion of irinotecan to SN-38 by CES2 in tumor tissues might occur. The expression profile of CES2 protein in human tumor tissues was evaluated in a tissue array of 18 different types of human cancer and in a panel of normal human liver samples by immunohistochemistry and Western blot, respectively. Cytosolic CES2 expression was observed in 101 of 154 tumors (66%) and 55 of 60 normal tissues (92%). Among the 18 types of tumors analyzed, 2 types (gallbladder tumor and lymphoma) did not express CES2, 5 types expressed weak CES2, and 11 types expressed moderate to intense CES2. In functional studies, CES2 protein was highly variable among liver samples, with a 15-fold range in cytosol and a 3-fold range in microsome fractions. Liver microsomal CES2 protein expression was significantly correlated with irinotecan activation to SN-38 (R² = 0.70; P = 0.007). This study confirms that CES2 is a key enzyme for irinotecan activation. Tumor CES2 expression may contribute to variable response to irinotecan chemotherapy for solid tumors.

INTRODUCTION

Irinotecan, a semisynthetic derivative of the natural alkaloid camptothecin, is one of the most active drugs in the treatment of colorectal cancer, with promising activity against other solid tumors (1–4). Irinotecan is activated by human liver CES to generate SN-38 (7-ethyl-10-hydroxy-camptothecin), a topoisomerase I inhibitor that is 100-1000 times more potent than irinotecan in vitro and in vivo (5–10).

CES are a family of serine-dependent esterases involved in the metabolism of endogenous lipids and drugs (8, 11–13). CES have been purified from membrane-rich fractions of mammalian cells and tissues (8, 12). The mammalian CES are localized in the endoplasmic reticulum and cytosol of many tissues, with the highest activity observed in the microsomal and lysosomal fractions of the liver (11–13). Multiple forms of CES have been identified in human tissues. In human livers, two major isoforms, CES1 and CES2, have been identified (12, 14, 15), whereas four different types of CES have been recognized in human brain extracts (16). In term placentae, three isoforms of CES were documented (11, 17). CES activity was also observed in human intestinal biopsies (10).

Using purified enzyme, the CES responsible for the activation of irinotecan in humans has recently been identified as CES2 (5, 18). CES2 (also called hCE-2) had a 12.5-fold higher affinity for irinotecan and a 5-fold higher maximal rate of irinotecan hydrolysis than CES1 (5). CES2 was 26-fold more active than CES1 and was 65% as active as rabbit liver CES, the most active irinotecan-hydrolyzing enzyme known (18). In cytotoxicity assays, incubation of irinotecan (1 μM) with purified CES2 resulted in 60% reduction in human cancer cell survival, compared with no significant reduction in cell survival after incubation with CES1 (5), indicating that CES2 is likely to be a key enzyme in irinotecan activation in human liver. Irinotecan bioactivation by human liver CES (CES1 and CES2) is an inefficient process in vitro (18, 19). Clinical studies also indicated that systemic concentrations of SN-38 after i.v. administration of irinotecan are very low (SN-38:irinotecan area under the concentration-time curve ratio = 0.02–0.07), and only 2–5% of the injected dose of irinotecan in people is converted to SN-38 (18), even though irinotecan has significant antitumor activity in cancer patients (19–23). This scenario raises the possibility that human CES may be expressed in tumor tissues, where local conversion of irinotecan to SN-38 might occur and contribute to the antitumor activity of irinotecan. Indeed, SN-38 formation has been demonstrated after incubation of irinotecan in tumor homogenates (9). To enhance the efficiency of drug usage, intratumoral activation of irinotecan by delivering CES

The abbreviations used are: CES, carboxylesterase(s); CES1, human carboxylesterase 1; CES2, human carboxylesterase 2; HPLC, high-pressure liquid chromatography.
genes into tumors via gene therapy has been proposed (18, 24). Therefore, there is a need to understand the CES2 expression pattern in common human tumors.

Currently, very limited information is available regarding the expression profile of CES2 in tumor tissues. Therefore, this study was conducted to evaluate the profile of CES2 in a tissue array of human tumors from 18 anatomical sites. A distinct pattern of CES2 expression was observed by immunohistochemistry. A significant variation in CES2 protein was also observed in human livers. We further identified that there is a statistically significant correlation between irinotecan activation to SN-38 and CES2 protein level in human liver microsomes, establishing that this observation is not restricted to purified enzyme studies (5, 18).

**MATERIALS AND METHODS**

**Chemicals and Reagents.** Enhanced chemiluminescence Western blotting detection reagents were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). The secondary antibody was peroxidase-conjugated donkey antirabbit IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Tissue arrays of common human cancers and normal tissues were purchased from Imgenics (San Diego, CA). Reagents for immunohistochemistry (Dewax, peroxide block, power block, link, horseradish peroxidase, 3,3'-diaminobenzidine tetrahydrochloride, hematoxylin, and buffers) were purchased from BioGenex (San Ramon, CA). For HPLC analysis, solvents and reagents were of the highest commercially available grade from Sigma (St. Louis, MO) and Fisher Scientific (Pittsburgh, PA). Irinotecan and SN-38 were gifts from Dr. J. Patrick McGovren (Pharmacia Corp., Kalamazoo, MI).

**Antibody Preparation.** A polyclonal antibody for CES2 was generated by Genemed Synthesis Inc. (South San Francisco, CA; Ref. 25). PCGENE software (Oxford Molecular, Hunt Valley, MD) was used to select a COOH-terminal peptide within CES2 for generation of antipeptide antibodies. The sequence of the peptide was H2 N-KIQELEEPEERHTEL-COOH. Peptides were synthesized and conjugated with keyhole limpet hemocyanin which were injected into rabbits for the generation of the antibody. Characterization of this antibody has been presented previously (25).

**SDS-PAGE and Western Analysis.** Cytosolic and microsomal fractions from 13 human donor livers were purchased from XenoTech LLC (Kansas City, KS) and included 9 Caucasian, 2 African American, and 2 Hispanic subjects (10 males and 3 females; age, 21–70 years). More donor information is available from XenoTech LLC. Human liver cytosols (200 μg) or microsomes (40 μg) in 50 mM Tris-HCl (pH 7.4), 150 mM KCl, and 2 mM EDTA were mixed with an equal volume of 2× Laemmli sample buffer [0.25 M Tris-HCl (pH 6.8), 20% β-mercaptoethanol, and 4% SDS; Ref. 26]. The mixtures were heated at 100°C for 3 min. Heated mixtures were then centrifuged at 10,000 × g for 5 min to remove insoluble materials, and supernatants were subjected to SDS-PAGE (10%). The proteins were transferred to nitrocellulose membrane in transfer buffer (96 mM glycine, 12 mM Tris base, and 15% methanol) using a Semiphar Transphor Unit (Amersham Pharmacia Biotech, San Francisco, CA) for 1 h. An immunoblot was performed using the rabbit anti-CES2 and donkey antirabbit IgG as primary and secondary antibodies, respectively. Detection was done using enhanced chemiluminescence reagents from Amersham Pharmacia Biotech. CES2 was quantitated using a Personal Densitometer Scanning Instrument (Molecular Dynamics, Sunnyvale, CA).

**Common Cancer Tissue Array Set.** The distribution of CES2 in human tumors was evaluated using the SuperBioChips Laboratories tissue array purchased from Imgenics. Each tissue array slide contained 60 samples, each of which was 2 mm in diameter and 4-μm thick. Tissue array slides were mounted on siliconized glass slides and purchased ready for use in immunohistochemistry. Slides containing no parenchymal cells were not used for evaluation. Final analysis yielded 154 evaluable cases of tumor tissues, originating from 18 human tissues. Matched normal tissues were available for 7 of 18 tumor types (n = 60).

**Immunohistochemistry.** Immunohistochemistry was performed using the streptavidin biotin complex method on a BioGenex i6000 automated staining system based on the protocol provided by the manufacturer (BioGenex). For negative controls, slides were incubated either in the absence of the anti-CES2 antibody (PBS only) or with the primary antibody plus the peptide (1 mg/ml) used to generate the anti-CES2 antibody. Slides were scanned with Nikon Eclips (E800) using the MetaMorph Imaging System and printed with a Tektronix Phaser 740.

**Semiquantitative Assessment of CES2.** The expression of CES2 in situ was evaluated by a semiquantitative scoring system. The intensity of staining was scored as 0 (negative), 1 (weak), 2 (medium), and 3 (strong). The extent of staining was scored as 0 (0%), 1 (1–25%), 2 (26–50%), 3 (51–75%), and 4 (76–100%), according to the percentage of cells staining positive for CES2. The sum of the intensity and extent scores was used as the final staining score (0–7) for CES2. Tissues with a final staining score > 2 were considered to be positive. A final staining score of 2–3 was considered +, a final staining score of 4–5 was considered ++, and a final staining score of 6–7 was considered +++.

**SN-38 Production in Human Liver Cytosols and Microsomes.** Irinotecan (5 μM) was preincubated in 0.1 M sodium phosphate buffer (pH 7.4) at 37°C for 3 min. Reactions were initiated by adding prewarmed human liver cytosolic or microsomal proteins (1 mg protein/ml) to the prewarmed drug mixture, followed by mixing in a shaking water bath at 120 rpm for 30 min. A sample of the incubation mixture (100 μl) was extracted in duplicate with 200 μl of cold methanol/acetonitrile mixture (50:50, v/v), vortexed, centrifuged at 14,000 rpm for 5 min, and evaporated to dryness. The dried samples were reconstituted in 95 μl of HPLC mobile phase (see below) and 5 μl of 1 M HCl. A final volume of 80 μl was injected onto the HPLC. HPLC analysis of irinotecan and SN-38 used modifications of the method by Dodds et al. (27). Briefly, HPLC pump LC-10ADVP and controller SCL-10AVP (Shimadzu Scientific Instruments, Columbia, MD) were used to deliver a mobile phase of 75 mM ammonium acetate (pH 6.0):acetonitrile (77:23, v/v) isocratically at a flow rate of 1.0 ml/min to a Waters symmetry C8 column (3.9 × 150 mm; id, 5 μm) and precolumn [3.9 × 29 mm; id, 5 μm (Milford, MA)]. Samples were injected.
with a Shimadzu SIL-10ADVP autosampler. Fluorescence detector RF 10AXL (Shimadzu Scientific Instruments) was used to quantitate total SN-38 concentrations using excitation and emission wavelengths of 355 and 530 nm, respectively. SN-38 production was linear with protein concentration (0.25–1 mg/ml) and incubation time (15–60 min). Enzyme activities of CES in the microsomal and cytosolic fractions were determined for each donor liver in three independent experiments. Enzyme activity was calculated as the rate of SN-38 production/mg protein/min of incubation. As a negative control, irinotecan was incubated in the same way as stated above, except in the absence of microsomal or cytosolic fraction. The trace SN-38 production from negative control was subtracted out, and the difference was used to calculate SN-38 production. A second negative control omitted irinotecan from the microsomal or cytosolic incubations.

**Statistical Analysis.** The correlation between CES enzyme activities and protein CES2 concentrations in the cytosolic and microsomal fractions and the relationship between CES activity or CES2 level in the cytosolic and microsomal fractions were assessed using Spearman’s rank test. The difference in CES2 expression between the tumor tissues and their matched normal tissues was assessed using the chi-square test, as was the relationship between CES2 and differentiation status. All comparisons were considered significant at \( P < 0.05 \).

**RESULTS**

**Relationship between CES2 Protein Level and Irinotecan Activation.** CES2 protein expression in human liver samples was determined by Western blot analysis (Fig. 1). CES2 protein level was highly variable among the 13 liver samples, with a 15-fold range in cytosol and a 3-fold range in microsomes, as determined by densitometry data. Median CES enzyme activity in liver microsomes and cytosols was 17.5 units/mg/min (range, 9.8–30.5 units/mg/min) and 1.3 units/mg/min (range, 0.5–2.4 units/mg/min), respectively. The ratio of microsomal:cytosol CES activity ranged from 5 to 35 (median, 19.5) in the 13 liver specimens. There was no correlation between cytosolic CES activity and microsomal CES activity (\( R_s = -0.13; \ P = 0.66 \)). There was also no correlation between CES activity and CES2 protein concentration in the cytosolic samples (\( R_s = -0.01; \ P = 0.97 \)). In contrast, a statistically significant correlation was found between SN-38 production and CES2 protein concentration in the microsomal fraction (\( R_s = 0.70; \ P = 0.007 \); Fig. 2).

**Immunohistochemical Analysis of CES2 Expression in Common Human Tumors.** A total of 154 tumors from 18 types of human tissues and 60 cases of normal tissues corresponding to 7 of 18 types of tumors were examined by immunohistochemistry using the CES2-specific antibody described above. CES2 was only observed in the cytoplasm of positively stained tissues. There was a significant variation in CES2 expression in both tumor tissues and their matched normal tissues, ranging from negative (−) to intense staining (+++). CES2 signal could be completely blocked by preadsorption with the cognate peptide, confirming the specificity of the antibody (data not shown). Overall, CES2 staining was observed in 101/154 tumors (66%) and 55 of 60 normal tissues (92%).

Analyzing the 18 types of tumors individually, we found that 2 types of tumors did not express CES2, 5 types of tumors expressed only weak CES2, and 11 types expressed weak to intense CES2 (Table 1). All of the thyroid papillary carcinomas (\( n = 10 \)) expressed CES2, with 50% of cases showing moderate to intense immunostaining (Fig. 3A). Weak to moderate CES2 immunostaining was observed in pancreatic ductal adenocarcinoma, esophageal squamous carcinoma (Fig. 3B), renal adenocarcinoma (Fig. 3C), colon adenocarcinoma (Fig. 3D), liver hepatocellular carcinoma (Fig. 3E), uterine cervix squamous carcinoma and adenocarcinoma (Fig. 3F), ovarian cystadenocarcinoma (Fig. 3G), skin melanoma (Fig. 3H), endometrial...
two-enzyme model best describes the formation of SN-38 in human liver, including both low and high CES forms (28). Several forms of CES and butyrylcholinesterases were initially thought to be responsible for this reaction, but $K_m$ values ($\sim 100 \mu M$) above that pharmacologically achievable made them unlikely to be responsible for irinotecan activation in patients (6, 29–31). Recently, purified human liver CES2 was demonstrated to have a similar $K_m$ (3.4 \mu M) as the low $K_m$ form (1.4–3.9 \mu M) observed in the enzyme kinetic studies (15). The current study provides additional evidence for an important role of CES2 in the activation of irinotecan.

SN-38 production occurred in all evaluated human liver microsomes, with a 3-fold range of activity. In addition, SN-38 production significantly correlated with CES2 protein concentration in human liver microsomes. This was in the context of a tissue preparation that contains many possible CES, P450, and related enzymes, unlike the previous study with purified CES2 protein (5, 18). This identifies a putative tool for prospectively predicting the variability in drug activation (i.e., SN-38 formation) for both tumor efficacy and normal tissue toxicity.

Currently, no information is available regarding the expression profile of CES2 in human cancer tissues, where local conversion of the prodrug irinotecan to the active metabolite, SN-38, might occur. In this study, the presence of CES2 was evaluated in different human tumor tissues. We found that 2 types of tumors did not express CES2, 5 types of tumors expressed only weak CES2, 11 types of tumors expressed weak to high CES2, and 1 had moderate to intense staining. These results may provide guidance for the future development of this agent. For example, gallbladder tumors and lymphomas had no

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Identifying the CES enzyme responsible for irinotecan activation is complicated by the ubiquitous nature of the CES family and substantial differences in substrate specificity. A two-enzyme model best describes the formation of SN-38 in human liver, including both low and high CES forms (28). Several forms of CES and butyrylcholinesterases were initially thought to be responsible for this reaction, but $K_m$ values ($\sim 100 \mu M$) above that pharmacologically achievable made them unlikely to be responsible for irinotecan activation in patients (6, 29–31). Recently, purified human liver CES2 was demonstrated to have a similar $K_m$ (3.4 \mu M) as the low $K_m$ form (1.4–3.9 \mu M) observed in the enzyme kinetic studies (15). The current study provides additional evidence for an important role of CES2 in the activation of irinotecan.

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CES2 expression, suggesting that they may not be able to locally activate irinotecan to the cytotoxic metabolite. Indeed, irinotecan did not have significant antitumor activity in Phase II studies of these tumor types (32–37). In addition, substantial variability in CES2 protein was found with each tumor type. This may represent an unrecognized influence on irinotecan activity in clinical practice (32–37). Comparing our findings with literature data, all of the tumor types with frequent CES2 expression had evidence of single agent irinotecan activity in the Phase II setting (>20% objective response rate). In contrast, tumor types in which CES2 expression was less common had evidence of irinotecan activity in only half of the Phase II studies (32–37).

General CES activity has been identified in several human normal tissues in addition to the liver (9, 10, 12, 13, 38, 39). Although bioactivation of i.v. administered irinotecan by CES occurs predominantly in the liver, intestinal CES can also activate irinotecan to generate SN-38 (10). It was shown that CES activity could be detected in colorectal carcinomas and liver metastases and that the total CES activity in the tumors was only 2–3-fold lower compared with normal liver, suggesting that tumors might be another site where local conversion of irinotecan to SN-38 could occur (9). Kinetic properties of irinotecan activation in human normal liver and colon tumors found that $K_m$ values were very similar (3.4 $\mu$M in liver and 3.8 $\mu$M in colon tumors), but $V_{max}$ was higher in liver (2.7 pmol/min/mg protein).
than in colon tumor (1.7 pmol/min/mg protein; Ref. 40). The current study also observed a higher degree of protein expression in normal liver (44% with moderate/intense staining) than in colon tumor (25% with moderate/intense staining; Table 1). However, the relationship between CES2 immunostaining and enzyme kinetics has yet to be defined.

In our study, there is a significant variation in the CES2 protein level, as determined by Western blot, in human liver samples. Our results are consistent with previous reports. Hosokawa et al. (41) reported that there was a significant variation in CES activity of human liver microsomes. They determined microsomal CES activities in 12 human livers, using 10 different CES substrates. They found that CES content in liver showed large individual differences, with an 8-fold range. Recently, it was reported that CES activity was widely variable in the cancer and normal tissue of patients with colorectal carcinoma, with an 8-fold range in colon tumor and a 10-fold range in normal colon tissue (9). This is consistent with the 5–12-fold range in the SN-38:irinotecan area under the curve ratio observed in cancer patients and may have an important influence on drug toxicity and antitumor activity (19–23).

Although CES activity was present in both the cytosolic and microsomal fractions of the liver, the cytosolic CES activity was only 3–20% of that in the microsomes. Higher CES2 protein concentrations in the liver microsomes as compared with the cytosol from the same specimen confirmed this finding. This is consistent with the previous studies of general CES, where activity predominantly occurred in the endoplasmic reticulum (11–13). The CES activity varied about 3-fold in microsomal fractions from the human livers. This is consistent with a recent report in which a 3-fold variability in overall plasma CES activity was also observed in 20 cancer patients (42).

CES has been used as a determinant of irinotecan activity in several human cancer cell lines (43, 44). The results were conflicting, due in part to different cell systems and different enzyme assay methods. A significant correlation was observed between general CES activity and chemosensitivity to irinotecan in human lung cancer cells (43). In contrast, CES activity did not relate to the sensitivity to irinotecan in human colon cancer cell lines (44). In this study, CES2 expression was evaluated in a static array of human tumors obtained by biopsy or surgical resection. Therefore, it is premature to conclude that the CES2 level would predict the chemosensitivity of irinotecan in those tumors. Investigation evaluating CES2 expression in human tumor tissues, in conjunction with clinical response data, is expected in future studies.

In summary, we have identified the presence of CES2 in liver samples and a panel of 154 human tumors. We found that there is a significant variation in CES2 expression in both tumor tissues and normal liver and a statistically significant correlation between specific irinotecan CES activity and CES2 protein in the microsomal fraction of normal liver. Because CES2 is more likely to be the key enzyme to activate the prodrug irinotecan, our results may represent a source for variable response to irinotecan therapy. These data may also provide support for exogenous CES2 in molecular therapeutic strategies for targeted activation of irinotecan.

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