Life-Threatening Toxicity in a Dihydropyrimidine Dehydrogenase-deficient Patient after Treatment with Topical 5-Fluorouracil

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ABSTRACT

In humans, 80–90% of an administered dose of 5-fluorouracil (5-FU) is degraded by dihydropyrimidine dehydrogenase (DPD; EC 1.3.1.2), the initial rate-limiting enzyme in pyrimidine catabolism. Cancer patients with decreased DPD activity are at increased risk for severe toxicity including diarrhea, stomatitis, mucositis, myelosuppression, neurotoxicity, and, in some cases, death. We now report the first known cancer patient who developed life-threatening complications after treatment with topical 5-FU and was shown subsequently to have profound DPD deficiency. RT-PCR and genomic PCR methodologies were used to identify a G to A mutation in the GT 5’ splicing recognition sequence of intron 14, resulting in a 165-bp deletion (corresponding to exon 14) in this patient’s DPD mRNA. Immunoprecipitation and Western blot analysis were then used to demonstrate that the aberrant DPD mRNA is translated into a nonfunctional DPD protein that is ubiquitinated. We conclude that the presence of this metabolic defect combined with topical 5-FU (a drug demonstrating a narrow therapeutic index) results in the unusual presentation of life-threatening toxicity after treatment with a topical drug. These data further suggest that degradation by the ubiquitin-proteosome-mediated system plays a role in the elimination of the DPD protein.

INTRODUCTION

5-FU remains one of the most frequently used chemotherapeutic agents for the treatment of several different malignancies, including carcinomas of the breast, colon, and skin (1). The mechanism of 5-FU antitumor action (and most host toxicities) depends on anabolism of the drug to cytotoxic nucleotides, which in turn can act at several sites including inhibition of thymidylate synthase or incorporation into RNA and DNA (2). Studies from our laboratory and others have shown that 80–90% of an administered dose of 5-FU is converted into biologically inactive metabolites through the catabolic pathway (3). The initial and rate-limiting step in the catabolic pathway is DPD (EC 1.3.1.2; Ref. 4). Thus, DPD has a critical role in 5-FU pharmacokinetics by regulating the amount of 5-FU available for anabolism.

The role of DPD in 5-FU toxicity is best illustrated by patients with DPD deficiency. After administration of standard doses of 5-FU, these patients develop profound toxicity including mucositis, granulocytopenia, neuropathy, and death (5–8). The cause of this potentially life-threatening toxicity appears to be decreased catabolism, resulting in markedly prolonged exposure to 5-FU (6). Population studies in breast cancer patients have demonstrated that ~5% of patients were relatively DPD deficient, with enzyme activity below the 95th percentile of a control population (9).

All previous reports of severe toxicity in DPD-deficient patients have occurred after parenteral administration of 5-FU (5–8). This report describes the first known profoundly DPD-deficient cancer patient who developed life-threatening complications following treatment with topical 5-FU. RT-PCR and genomic PCR methodologies were used to identify the mutation responsible for DPD deficiency in this patient (10). In addition, immunoprecipitation and Western blot analysis were used to show that the aberrant DPD protein from this patient was ubiquitinated, suggesting that degradation by the ubiquitin-proteosome system may have a role in the elimination of the DPD protein.

PATIENT AND METHODS

Study Patient. The patient was a 76-year-old white male who began treatment for basal cell carcinoma with a 2-week course of topical 5-FU (5% efudex cream) applied twice daily to affected lesions on the scalp. After completion of the first week of topical 5-FU treatment, the patient complained of severe abdominal pain, associated with bloody diarrhea, vomiting, fever, and chills. The past medical of this patient demonstrated no history of inflammatory bowel disease. Physical examination revealed severe stomatitis, an erythematous skin rash, and abdominal pain on light palpation with guarding. The patient was admitted to the hospital and found to be neutropenic (WBC, 0.6 K/μl with 20% neutrophils and 16% bands) and thrombocytopenic (57 K/μl). The patient underwent colonoscopy with biopsy, revealing severe inflammatory colitis. An esophagastroduodenoscopy also revealed inflammatory changes in the esophagus, stomach, and small bowel.
A repeat colonoscopy 2 weeks later revealed persistent severe inflammatory changes, mainly in the area of the terminal ileum. The pathology was consistent with inflammatory bowel disease with no evidence of malignancy. After a period of 3 weeks, during which he required total parenteral nutrition and broad-spectrum antibiotics, the patient gradually improved, returning to his previous state of health. At the present time, he remains in good health with no evidence of recurrent cancer.

**Determination of DPD Activity.** DPD enzyme activity was determined in the cytosol of the patient’s PBM cells (11). In brief, blood (30 ml) was collected in heparinized tubes, and PBM cells were isolated using Histopaque (Sigma Chemical Co., St. Louis, MO). The cells were washed three times with PBS, resuspended with 4°C buffer A (35 mM potassium phosphate (pH 7.4), 2.5 mM magnesium chloride, and 10 mM 2-mercaptoethanol), and lysed by sonication (three times for 10 s with 30-s intervals). After centrifugation at 14,000 × g for 15 min at 4°C, the supernatant was removed, and the protein concentration was determined by the Bradford assay (12). The assay mixture (1 ml total, 37°C) contained 200 μM NAPDH, 8.23 μM [6-3H]-5-FU (56 mCi/mmol), buffer A, and 200 μg of cytosolic protein. At 5-min intervals (from 0 to 30 min), 130 μl of the reaction were transferred to a microfuge tube and vortexed with an equal volume of cold 100% ethanol. After a 10-min incubation at −70°C, the samples were centrifuged for 10 min at 14,000 × g and filtered (0.2-μm Acrodisc filter; Gelman Sciences, Ann Arbor, MI). Separation of 5-FU and its catabolites was performed by reverse-phase high-performance liquid chromatography using a Hewlett-Packard 1050 HPLC system equipped with an automatic injector and an on-line radioisotope flow detector (Radiomatic FLO-ONE Beta; Packard Instrument, Meriden, CT) as described (11). Uracil concentrations in plasma and urine were measured as described (13).

**RNA Isolation and cDNA Synthesis.** Total RNA was isolated from PBM cells using RNAzol (Biotex, Houston TX), following the manufacturer’s instructions. Random-primed cDNAs were prepared from 5 μg of total RNA using a Pharmacia first-strand cDNA synthesis kit (Piscataway, NJ). The reaction mix was treated with 1 μl of RNase H and incubated for 30 min at 37°C before PCR amplification.

**PCR Amplification of DPD cDNA.** The amplification of DPD cDNA was performed in a 50-μl reaction volume containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl2, plus dexoxygenucleotride triphosphates (0.2 mM each), 10 μmol of each primer, 5 μl of template cDNA, and 2.5 units of Taq polymerase. Samples were amplified in an MJ model PTC-100 thermal cycler (MJ Research, Inc., Watertown, MA) programmed for a temperature-step cycle of 95°C (1 min), 65°C (2 min), and 72°C (3 min). This cycle was repeated for five steps, after which the 65°C annealing temperature was decreased to 63°C, and the cycling continued for an additional 25 steps with a 10-min extension at 72°C after the final cycle. Three primer pairs were used to generate three overlapping human lymphocyte DPD cDNA fragments as shown in Fig. 1. PCR primer 1 was designed from 5′ rapid amplification of cDNA ends data obtained from the characterization of the DPD gene (14). Primers 2, 3, 4, and 5 were designed based on human lymphocyte DPD cDNA sequence (GenBank accession number: U20938). Primer 6 is a bifunctional primer composed of an oligo d(T) chain attached to an anchor domain, which contains a NotI restriction site.

**Genomic DNA Preparation and PCR Amplification.** Genomic DNA was prepared from PBM cells using the Easy-DNA kit (Invitrogen) following the manufacturer’s instructions. Two hundred ng of genomic DNA was used as a template in the PCR reaction. After denaturation at 95°C for 2 min, 35 cycles of denaturation at 95°C (1 min), annealing at 55°C (1 min), and extension at 72°C (1 min) were performed. The primers used (primer 7 sense, 5′-GTGAGAAGGACCTCATAAAATATTGAACACACAAGGATGAGGACACTCTG-3′ and Primer 8 antisense, 5′-GAATTGGATGTTATGAAATACCCATTCCATCTGGAACACGACGTCACACG-3′) corresponded to positions at the 3′ end of intron 13 and the 5′ end of intron 14, respectively (14). These primers were designed to amplify a 342-bp fragment containing the exon 14 including both the 5′ and 3′ splice sites. After amplification, the PCR product was purified on 2.3% low melting point agarose gel and subcloned into the pCR II vector (Invitrogen). The digoxigenin first-strand cDNA synthesis kit (Piscataway, NJ) was used for the synthesis of cDNA.

**DNA Sequencing.** DPD cDNA was subjected to double-stranded sequencing by the dideoxynucleotide chain termination method using Sequenase 2.0 and [α-35S]dATP. The 35S-labeled products were resolved on 6% polyacrylamide-urea gels. Reactions were repeated three times in each direction and analyzed using MacVector 4.1 Sequence Analysis software (IBI, New Haven, CT).

**Western Immunoblot Analysis of PBM Cytosol.** PBM cytosol (200 μg) from the DPD-deficient patient and a healthy control (with DPD activity in the normal range) were fractionated by SDS-PAGE on a 1.0-mm thick, 7% (w/v) polyacryl-
amide gel. Western immunoblot analysis was then performed using a purified rabbit polyclonal antibody against human liver DPD, as described previously (4).

**Immunoprecipitation of DPD from PBM Cytosol.** PBM cytosol (200 μg) from the DPD-deficient patient and a healthy control (with DPD activity in the normal range) were incubated for 2 h at 4°C with 2 μg of anti-rat liver DPD polyclonal antibody (15). Protein A/agarose (Sigma) was added (20 μl), and the sample was incubated for an additional hour at 4°C. Immunocomplexes were washed with 1 ml of TBS (700 mM NaCl, 13.0 mM KCl, and 125 mM Tris), and proteins were fractionated by SDS-PAGE as indicated above. Proteins were transferred to a nitrocellulose membrane as described previously (4, 16).

**Western Immunoblot Blot Analysis of Immunocomplexes Using Anti-DPD Polyclonal Antibody.** The membrane containing the immunoprecipitated proteins (described above) was blocked for 1 h with TBS containing 5% (w/v) nonfat dry milk, rinsed twice (5 mins each) with TTBS (TBS containing 0.25% Tween 20/v/v), and incubated overnight at 4°C in TTBS containing 1% dry milk and a 1:2500 dilution of rabbit anti-human DPD polyclonal antibody (4). Membranes were then washed four times (5 mins each) with TTBS and incubated for 1 h with TTBS containing 1% dry milk and a 1:20,000 dilution of goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (Southern Biotechnology Associates, Birmingham AL). After rinsing three times with TTBS (5 min each), immunostaining was visualized with LumiGLO (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

**Western Immunoblot Blot Analysis of Immunocomplexes Using Two Anti-Ubiquitin Monoclonal Antibodies.** Ubiquitin immunostaining was done as described previously using two monoclonal antibodies that recognize different epitopes (16). Briefly, the membrane containing the immunoprecipitated proteins (described above) was autoclaved in water for 30 min at 120°C and blocked with 0.5% dry milk. The membrane was then incubated overnight at 4°C in TTBS containing 0.1% dry milk and a 1:500 dilution of two mouse anti-human ubiquitin monoclonal antibodies (PanVera, Madison, WI). The membrane was washed in TTBS and incubated 1 h with TTBS containing 0.1% dry milk and a 1:20,000 dilution of goat anti-mouse secondary antibody conjugated to horseradish peroxidase (Southern Biotechnology Associates). After rinsing, immunostaining was visualized with LumiGLO (Kirkegaard & Perry Laboratories).

### RESULTS

**DPD Enzyme Activity in PBM Cells and Plasma/Urine Uracil Levels.** Three separate determinations (over a 6-month period) demonstrated no detectable DPD enzyme activity in the patient’s PBM cytosol. In addition, uracil levels were elevated in the patient’s plasma (2.33 nmol/ml; normal range, 0.33 ± 0.05; n = 5) and urine (285 nmol/ml; normal range < 2.5; n = 10; Ref. 7). These data show that this patient is profoundly DPD deficient.

**Identification of a 165-bp Deletion in the Patient’s DPD cDNA.** We performed RT-PCR on total RNA isolated from the patient’s PBM and obtained the full-length DPD cDNA in three overlapping fragments of 1604, 1688, and 1585 bp (Fig. 1). Resolution of PCR products on a 1% agarose gel revealed that the 1688-bp DPD cDNA amplicon (corresponding to bp 1322–3009 of the DPD cDNA) was slightly smaller than the normal amplicon from individuals with normal DPD activity (data not shown). Sequence analysis showed that this 1688-bp amplicon contained a 165-bp deletion (10), which was present in eight of eight analyzed clones. The recent characterization of the human DPD gene by our laboratory (14) revealed that this 165-bp deletion corresponds to exon 14. This deletion represents the only sequence difference identified in this DPD-deficient patients complete DPD mRNA (including 5’ and 3’ untranslated regions).

**Genomic DNA Analysis.** The identification of the exon 14 deletion in the patient’s DPD mRNA prompted us to examine this region of the DPD gene. Oligonucleotide primers (primers 7 and 8) were synthesized based on the sequence of the 3’ end of intron 13 and the 5’ end of intron 14 of the DPD gene (14). These primers enabled amplification of a 342-bp fragment containing exon 14 (including and both the 5’ and 3’ splicing sites) from the patient’s genomic DNA (Fig. 2). Sequence analysis showed that there was a G to A mutation in the 5’ donor splice consensus sequence of intron 14 (detected in eight of eight analyzed clones).

**Western Immunoblot Analysis Using Rabbit Anti-Human DPD Polyclonal Antibody.** Immunoblot analysis was carried out with the anti-human DPD polyclonal antibody as described previously (4). PBM cytosol from the DPD-deficient patient migrated as two broad bands with apparent molecular masses, larger (145 kDa) and smaller (90 kDa), than the typical 110-kDa band obtained from the PBM cytosol of individuals with normal DPD enzyme activity (Fig. 3).

**Western Immunoblot Analysis of the Immunoprecipitated Immunocomplexes with the Anti-Human DPD Polyclonal Antibody.** Immunocomplexes were precipitated from the cytosol of the DPD-deficient patient and a healthy control using the anti-rat DPD polyclonal antibody (15), fractionated by SDS-PAGE, and transferred to a nitrocellulose membrane. Immunoblot analysis with the anti-human DPD polyclonal antibody revealed the same unique pattern in the DPD-deficient patient as observed without immunoprecipitation (Fig. 4A). Both the 90- and 145-kDa bands were specifically immunoprecipitated by the anti-rat DPD polyclonal antibody and detected by the anti-human DPD polyclonal antibody.
Western Blot Analysis of the Immunocomplexes with Two Anti-Ubiquitin Monoclonal Antibodies. Immunoblot analysis of the immunocomplexes in the DPD-deficient patient using two anti-ubiquitin monoclonal antibodies revealed the same unique pattern as observed from PBM cytosol (Fig. 4B). Both the 90- and 145-kDa bands were specifically immunoprecipitated by the anti-rat DPD polyclonal antibody and recognized by the anti-ubiquitin monoclonal antibodies. The 110-kDa DPD from an individual with normal enzyme activity showed no detectable immunoreaction with the anti-ubiquitin antibodies (Fig. 4B). The 90- and 145-kDa bands are probably conjugates of DPD with multiple ubiquitins (8.5 kDa) because they were immunoprecipitated with anti-rat DPD antibodies and immunostained with anti-human DPD and anti-ubiquitin antibodies. The 90-kDa ubiquitin-DPD band appears to be a conjugate of ubiquitin with a proteolytic fragment of DPD. These findings suggest that ubiquitin-DPD conjugates accumulate in the patient’s PBM cells.

DISCUSSION

In the present study, we describe a 76-year-old white male diagnosed with basal cell carcinoma who, upon administration of standard doses of topical 5-FU to the scalp, developed severe gastrointestinal and hematological toxicity. Previous studies have demonstrated similar toxicities in DPD-deficient patients receiving i.v. 5-FU; however, there has never been a reported case of life-threatening toxicity from a patient receiving only topical 5-FU (5–9). Several earlier studies of topically applied 14C-labeled 5-FU report a cutaneous absorption rate of 10% (18–20). We estimate that application of 2 g of 5% 5-FU cream applied twice daily would result in a total exposure of ~20 mg/day of 5-FU (which translates into ~0.33 mg/kg for this patient). This amount is well below the typical 500–550 mg/kg i.v. bolus 5-FU dose administered for cancer chemotherapy (2).

The initial characterization of this patient involved three separate DPD enzyme determinations (over a 6-month period). These studies demonstrated no detectable DPD enzyme activity in this patient’s PBM cytosol. In addition, the patient exhibited 7- and 114-fold elevated uracil levels in plasma and urine, respectively. The severe toxicity observed in this patient required immediate cessation of treatment with 5-FU and prevented us from conducting any 5-FU pharmacokinetic studies. However, we conclude that the severe toxicity observed in this patient resulted from profound DPD deficiency secondary to treatment with topical 5-FU based on the following: (a) the gastrointestinal and hematological toxicities reported in this patient are similar to those reported in other DPD-deficient patients after parenteral administration of 5-FU; (b) the complete lack of measurable DPD enzyme activity in this patient’s PBM cytosol; (c) the elevated uracil levels in plasma and urine, which suggest abnormalities in pyrimidine catabolism; (d) the observed toxicity began after administration of topical 5-FU alone (no other chemotherapeutic agent was administered); and (e) the complete reversal of toxicity after cessation of treatment with topical 5-FU.

To determine the molecular basis for DPD deficiency in this patient, we performed RT-PCR on total RNA isolated from the patient’s PBM cells as described in “Materials and Methods.” The complete DPD cDNA was cloned in three overlapping fragments of 1604, 1688, and 1585 bp (Fig. 1). Resolution of PCR products on a 1% agarose gel revealed that the 1688-bp DPD cDNA amplicon (corresponding to bp 1322–3009 of the DPD cDNA) from the DPD-deficient patient was slightly smaller than the normal control (data not shown). Sequence analysis demonstrated that there was a homozygous 165-bp deletion in this 1688-bp amplicon. The recent characterization of the human DPD gene by our laboratory (14) has revealed that this 165-bp deletion corresponds to exon 14. Complete sequence analysis of the remaining DPD cDNA (including the 5’ and 3’ untranslated regions) demonstrated that this deletion represents the only sequence difference identified in this DPD-deficient patient’s DPD mRNA. An identical deletion was reported in 1995 by Meinsma et al. (17) in a Dutch pediatric patient with uracil and thyminuria presenting with growth and developmental abnormalities. Later studies by Wei et al. (10) identified the same 165-bp deletion in a DPD-deficient British family in which one of the family members had developed severe toxicity (grade IV pancytopenia and mucositis) after treatment with i.v. bolus 5-FU.

The identification of the exon 14 deletion in this patient’s DPD mRNA prompted us to examine this region of the DPD gene. Genomic DNA was isolated from the patient’s PBM cells and primers based on the sequence of the 3’ end of intron 13 (forward primer 7) and the 5’ end of intron 14 (reverse primer 8) of the DPD gene (14). Resolution of PCR amplification products demonstrated the expected 342-bp fragment (Fig. 2). Sequence analysis demonstrated a homozygous G to A mutation (10) in the 5’ donor splice sequence of intron 14.
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The identification of ubiquitinated forms of DPD in this study have given us the first insight into a biochemical mechanism involved in DPD deficiency. Recent studies by Tai et al. (22) report that enhanced proteolysis of mutant alleles of human TPMT (EC 2.1.1.67) as a mechanism for loss of TPMT protein and catalytic activity (22). Degradation half-lives were determined by pulse-chase experiments, which used heterologous expression of wild-type and mutant human TPMP cDNAs in yeast. Although our initial attempts to express active DPD in mammalian cells were unsuccessful, we are currently evaluating a number of different cell lines and expression systems so that experiments similar to those used to determine TPMT half-life can be performed.

This study represents the first characterization of a DPD-deficient patient who developed life-threatening toxicity after exposure to topical 5-FU. Considering the previously reported low cutaneous absorption rate (~10%) of topical 5-FU, we suggest that life-threatening toxicity in the population of patients receiving topical 5-FU will be limited to profoundly DPD-deficient patients (no measurable DPD enzyme activity). The high degree of vascularization in the scalp may also have played a contributory role in the toxicity observed in this patient by enhancing absorption through the scalp, ultimately resulting in higher plasma 5-FU levels. The molecular basis for DPD deficiency in this patient was identified in both the mRNA and genomic DNA. Western immunoblot analysis of this patient’s PBM cytosol revealed a unique pattern of DPD bands that appear to contain ubiquitinated DPD. These data suggest that the ubiquitin-proteasome system contributes to the loss of DPD protein in patients lacking exon 14. Furthermore, the combination of this metabolic defect and a drug with a narrow therapeutic index results in the unusual presentation of life-threatening toxicity after administration of a topical drug and emphasizes the quantitative importance of drug absorption through the skin.

REFERENCES