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Uridine homeostatic disorder leads to DNA damage and tumorigenesis

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ABSTRACT

Uridine is a natural nucleoside precursor of uridine monophosphate in organisms and thus is considered to be safe and is used in a wide range of clinical settings. The far-reaching effects of pharmacological uridine have long been neglected. Here, we report that the homeostatic disorder of uridine is carcinogenic. Targeted disruption (–/–) of murine uridine phosphorylase (*UPase*) disrupted the homeostasis of uridine and increased spontaneous tumorigenesis by more than 3-fold. Multiple tumors (e.g., lymphoma, hepatoma and lung adenoma) occurred simultaneously in some UPase deficient mice, but not in wild-type mice raised under the same conditions. In the tissue from *UPase* –/– mice, the 2′-deoxyuridine,5′-triphosphate (dUTP) levels and uracil DNA were increased and p53 was activated with an increased phospho-Ser18 p53 level. Exposing cell lines (e.g., MCF-7, RKO, HCT-8 and NCI-H460) to uridine (10 or 30 μM) led to uracil DNA damage and p53 activation, which in turn triggered the DNA damage response. In these cells, phospho-ATM, phospho-CHK2, and phospho-γH2AX were increased by uridine. These data suggest that uridine homeostatic disorder leads to uracil DNA damage and that pharmacological uridine may be carcinogenic.

Introduction

Uridine, a natural nucleoside in organisms, is considered safe and thus is widely used in a range of clinical settings. For instance, uridine is used for "rescue" from host toxicity of anti-metabolites in anticancer and anti-HIV therapies, for the treatment of developmental and genetic disorders, and even for *in vivo* studies in human subjects [1–12]. However, the far-reaching effects of pharmacological uses of uridine have long been neglected. This current study changed the paradigm of uridine safety.

In living cells, there exist two pyrimidine nucleotide biosynthesis pathways, i.e., the salvage pathway and the *de novo* pathway [13]. Uridine is the precursor of the pyrimidine salvage pathway, in which uridine is converted to uridine 5′-monophosphate (UMP) by uridine kinase (UK) [13,14]. UMP is a parental nucleotide for pyrimidine nucleotides [14]. In the presence of adenosine triphosphate (ATP), UMP is converted into uridine 5′-diphosphate (UDP) and uridine 5′-triphosphate (UTP) by nucleoside mono- and diphosphate kinases [15]. With glutamine as an amino donor, the carbonyl oxygen at C-4 of UTP is replaced by an amino group, forming cytidine 5′-triphosphate (CTP) [16]. Both UTP and CTP are building materials of RNA. Furthermore, ribonucleotide reductase catalyzes the conversion of cytidine 5′-diphosphate (CDP) to deoxycytidine 5′-diphosphate (dCDP), which is then converted into deoxycytidine 5′-triphosphate (dCTP) by deoxynucleoside diphosphate kinase [17,18]. In addition, under the catalysis of thymidylate synthase (TS), deoxyuridylate (dUMP) is methylated to deoxythymidylate (dTMP), which is then converted to deoxythymidine 5′-triphosphate (dTTP)

Abbreviations: ATM, ataxia telangiectasia mutated; ATP, adenosine triphosphate; ATR, ATM-Rad3-related; BER, base excision repair; CTP, cytidine 5′-triphosphate; DAPI, 4′,6-diamidino-2-phenylindole; dCDP, deoxycytidine 5′-diphosphate; dCTP, deoxycytidine 5′-triphosphate; DDR, DNA damage response; dTMP, deoxythymidylate; dTTP, deoxythymidine 5′-triphosphate; dUMP, deoxyuridylate; dUTP, 2′-deoxyuridine,5′-triphosphate; FLARE assay, fragment length analysis using repair enzyme assay; mCEC, mouse colon epithelial cells; PCE, polychromatic erythrocytes; PRPP, phosphoribosyl pyrophosphate; TS, thymidylate synthase; UDG, uracil DNA glycosylase; UDP, uridine 5′-diphosphate; UK, uridine kinase; UMP, 5′-monophosphate; UPase, uridine phosphorylase; UPase-2, uridine phosphorylase-2, a novel isoform; UTP, uridine 5′-triphosphate.

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by deoxynucleoside kinases [18,19]. Both dCTP and dTTP are the precursors of DNA synthesis. Via similar pathway, uridine can also be converted to deoxyuridine 5′-triphosphate (dUTP), which readily incorporates into DNA, resulting in uracil DNA damage [20]. Therefore, uridine is a critical nucleoside in pyrimidine metabolism.

In adult mammals, including humans, most normal tissues rely on the salvage of uridine from the plasma for pyrimidine nucleotide biosynthesis, while the liver and kidney remain active for *de novo* pyrimidine biosynthesis to supply circulating uridine for salvage [14]. For instance, uridine incorporation into human peripheral blood lymphocytes stimulated by phytohemagglutinin (PHA) is exponentially increased up to 30-fold compared to the resting status, but the *de novo* pyrimidine biosynthesis is increased by only 2–3-fold [21]. Uridine is also involved in purine nucleotide biosynthesis by providing phosphoribosyl pyrophosphate (PRPP) as a ribose moiety, which is required in both the *de novo* and salvage pathways [22]. Additionally, uridine participates in several other physiological and pathological processes, such as bio-membrane synthesis, carbohydrate metabolism, and the peripheral and central nerve activity [23,24]. Therefore, uridine concentration in the plasma is strictly regulated at 3–5 μM in various mammalian species, including humans [24,25]. However, the pathophysiological consequences of disruption of this rigid homeostasis are known.

Uridine phosphorylases are enzymes that reversibly convert uridine to uracil [26–28]. To date, two uridine phosphorylases have been identified, i.e., uridine phosphorylase (UPase) and a novel isoform uridine phosphorylase-2 (UPase-2). UPase is the classic uridine phosphorylase conserved throughout the evolutionary hierarchy of living organisms from bacteria to humans [29,30]. UPase-2 is a novel isoform, demonstrating its predominant expression in the kidney (in humans) or liver (in mice) [31,32]. In contrast, UPase is ubiquitously expressed in various tissues in mammals and thus is the main enzyme involved in regulating plasma and tissue uridine homeostasis. The intravenous administration of UPase protein rapidly removed plasma uridine and inhibited the activity of the pyrimidine salvage pathway by 65–92% upon tissues [33]; *UPase* knockout (–/–) in mice resulted in uridine accumulation and homeostatic disorder [34]. In *UPase* –/– mice, uridine is increased by 7-fold in the plasma and by 3–6-fold in tissues, such as the liver, lung and intestine. The current study found that *UPase* disruption and the resultant uridine accumulation led to uracil DNA damage and spontaneous tumorigenesis in mice. Our new data suggest that the homeostatic disorder of uridine is carcinogenic. The safety of the pharmacological use of uridine in human subjects needs to be re-evaluated.

Materials and methods

Animals and spontaneous tumorigenesis

UPase –/– (n = 78) and littermate wild-type (n = 76) mice were raised on a regular diet and water available *ad libitum* in the board-accredited animal facility at the Southern Illinois University School of Medicine. The housing environment is controlled at 24 ± 0.5 °C, 50 ± 10% humidity, and 12 hours of light from 08:00 to 20:00. At the age of 18 months, the mice were killed and grossly examined for palpable tumors. Necropsies were conducted if a mouse died or was killed for sickness before 18 months. Tumor masses and tissues were fixed in formalin and processed for H&E staining and histopathological analyses [35].

Cell culture

MCF-7, RKO, HCT-8 and NCI-H460 cells were purchased from American Type Culture Collection (ATCC; Manassas, VA). This is the most accredited cell bank worldwide, and all of the cell lines are characterized by a DNA short tandem repeat (STR) profile. The cells were maintained in the suggested medium containing 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C and in 5% CO_2. Primary colon epithelial cells from three male wild-type C57BL/6 mice (3 months old) were isolated and exposed to uridine (30 μM) for increasing times of 0, 12, 24, 36, 48 and 60 hours or for increasing uridine doses of 0, 10, 20, 30 and 40 μM for 48 hours.

Micronucleus body assay

Femur bone marrow cells were flushed out in fetal bovine serum (FBS) and collected at 100 × *g* at 4 °C for 5 min. The cells were re-suspended in 200 μl of 50% fetal bovine serum, spread on glass slides, air-dried overnight, and stained as follows: May-Gruenwald for 5 min; PBS for 2 min; and 1:20 diluted Giemsa for 15 min. The slides were briefly rinsed in water and then rinsed twice in 95% ethanol for 5 seconds, twice in 100% ethanol for 5 seconds, and twice in xylene for 5 seconds. After being air-dried, the slides were mounted with Cytoseal® 60 (Richard-Allan Scientific, MI), and the micronucleus bodies were examined under a microscope [36].

FLARE (fragment length analysis using repair enzymes) assay

Single cell suspensions were prepared with a Dounce homogenizer followed by filtration with a 50 μm nylon mesh. The cells were suspended in PBS at 1×10^6 cells/ml and mixed with an equal volume of 0.6% low melting agarose and added onto the top of a slide mounted with 0.6% regular agarose and covered with a cover slip. The cell membranes were disrupted at 4 °C for 1 hour in a buffer (2.5 M NaCl, 100 mM Na-EDTA, 10 mM Tris.Cl and 1% Triton X-100, pH 10.0). Uracil in the genome was excised by uracil-DNA glycosylase (Epicenter, WI) followed by treatment in a buffer of 300 mM NaOH and 1 mM Na_2-EDTA (pH 13.0) for 40 min on ice. Electrophoresis was conducted in the same buffer at 25 V (250 mA) for 30 min. The cells were neutralized in 0.4 M Tris.Cl (pH 7.5) and stained with 2.5 μg/ml propidium iodide (PI). DNA breaks in 50 comet cells/slide were evaluated for tail moment using Comet v.3.1 (Kinetic Imaging Ltd., UK) [37].

Apoptosis

The cells were grown on glass cover slips and exposed to 30 μM uridine at the indicated time followed by fixation with paraformaldehyde and DAPI staining; apoptotic cells were defined based on nuclear fragmentation and heterochromatin aggregation [38]. Apoptotic percentages were calculated as the number of apoptotic cells relative to the total number of cells (>100) from multiple random fields.

Extraction and measurement of dUTP

Tissues (50 mg) were homogenized in PBS. Proteins were precipitated in 60% methanol at –20 °C followed by centrifugation at 13,200 × *g* for 5 min at room temperature. The supernatants were extracted using a WAX SPE procedure, and the nucleotides in the extracts (50 μl per injection) were separated with a ThermoFinnigan Surveyor HPLC equipped with a Supelcogel ODP-50, 150 × 2.1 mm, 5 μM column (Sigma-Aldrich, MO). The HPLC effluents were introduced into a ThermoFinnigan (TSQ7000) triple stage quadrupole (TSQ) mass spectrometer equipped with electrospray ionization (ESI). Ion transitions at m/z 467.15 → 369.15 for dUTP were used in a multiple reaction monitor (MRM) mode.

Western blot

Tissues from wild-type and *UPase* –/– mice or cells were homogenized in cell lysis buffer containing protease inhibitors (Roche, IN). Soluble proteins were collected at 20,000 × *g* for 20 min, and 50 μg of the soluble proteins was subjected to western blotting as previously described [39].

Statistical analysis

Statistical analysis was performed using Student's *t* test with the INSTAT statistical analysis package (Graph Pad Software, CA). Significance was defined as $p < 0.05$.

Results

Spontaneous tumorigenesis increases in UPase –/– *mice*

Spontaneous tumor occurrence was estimated in *UPase* –/– and wild-type littermate control mice that were raised on a regular diet and with water available *ad libitum*. The animals were grossly examined for palpable masses at the age of 18 months, and necropsies were conducted if a mouse died or was killed for sickness before 18 months. As shown in Fig. 1, spontaneous tumors occurred more frequently and earlier in the *UPase* –/– mice than in the wild-type animals. The tumors appeared early as at 8 months in *UPase* –/– mice and at 13 months in wild-type mice. Table 1 summarizes the incidence and types of spontaneous tumors. The overall incidence of tumors was over 3 times higher in the *UPase* –/– mice than in the wild-type mice. More importantly, some of the *UPase* –/– mice simultaneously suffered from multiple types of tumors, such as mesenteric lymphoma, hepatoma, and lung adenoma (Fig. 2A). This