Hypoxanthine Transport in Human Glioblastoma Cells and Effect on Cell Susceptibility to Methotrexate

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Purpose. Cancer cells may circumvent the cytotoxic effect of antimetabolite drugs that inhibit *de novo* nucleotide synthesis via the uptake of extracellular preformed nucleobases or nucleosides. The goal of this study was to investigate the nucleobase transport mechanism in human U-118 glioblastoma cells and to determine whether the purine nucleobase hypoxanthine affects cell susceptibility to methotrexate.
Methods. Uptake experiments were performed using ^{3}H-labeled hypoxanthine. RT-PCR was used to determine the expression of nucleoside transporters. Methotrexate-induced apoptosis was analyzed using annexin V staining and FACScan analysis.
Results. Hypoxanthine transport in U-118 cells involved both carrier-mediated (K_m = 10.5 ± 6.3 μM, V_{max} = 1.45 ± 0.69 pmol/10^5 cells/60 s) and simple diffusion processes (K_d = 0.36 ± 0.009 μm/10^5 cells/60 s). Uptake was sensitive to Na^+ and inhibited by nucleobases but not nucleosides or nucleoside transport inhibitors. In contrast, uptake of a nucleoside, uridine, was inhibited by nucleosides but not nucleobases. RT-PCR analysis suggested the presence of hENT1, hENT2, and hCNT1 nucleoside transporters in U-118 cells. In the absence of hypoxanthine, methotrexate inhibited U-118 cell proliferation and induced apoptosis. These toxic effects were diminished when hypoxanthine was present at physiologically relevant concentrations.
Conclusions. Hypoxanthine transport in U-118 cells involves a Na^+-dependent, high-affinity nucleobase transport system functionally distinct from nucleoside transporters. At physiologic concentrations, hypoxanthine protects glioblastoma cells from the cytotoxicity of methotrexate.

KEY WORDS: nucleobase transport; hypoxanthine; glioblastoma; methotrexate; nucleoside transporters.

INTRODUCTION

Antimetabolite drugs targeting key enzymes of *de novo* nucleotide synthesis are an important class of drugs for cancer chemotherapy. Nucleobase and nucleoside transporters play important roles in tumor response to antimetabolite drugs. On one hand, they are essential for cellular entry of anticancer nucleoside analogues such as cytosine arabinoside and 5-fluorouracil (1,2). On the other hand, nucleobase or nucleoside transporter-mediated uptake of physiologic nucleobases or nucleosides can compromise the activity of antimetabolite drugs such as methotrexate. These drugs exhibit their cytotoxic effect by inhibiting *de novo* nucleotide synthesis but do not themselves rely on nucleobase or nucleoside transporters to enter cells. Increasing evidence suggests that circumvention of *de novo* inhibition through the salvage pathways plays an important role in tumor response to antimetabolites (3,4). Indeed, nucleoside transport inhibitors have been explored to potentiate the cytotoxicity of antimetabolites (5–7).

Mammalian nucleoside transporters have been well characterized and identified at the molecular level (1,2,8). These transporters belong to two classes: the equilibrative and the concentrative nucleoside transporter families. Two major equilibrative nucleoside transporters, ENT1 and ENT2, have been identified and characterized. ENT1 and ENT2 are inhibitable by classic nucleoside transporter inhibitors such as dipyridamole (DP) and nitrobenzylthioinosine (NBMPR) but differ in their relative sensitivities to NBMPR (1,2). Additionally, several Na^+-dependent concentrative nucleoside transporters (CNT1–3) have been cloned and characterized (8,9). In contrast to nucleoside transporters, transporters for nucleobases in mammalian cells are much less understood. In a number of cells such as human erythrocytes, a high-affinity equilibrative nucleobase transporter was functionally characterized (1,10). Specific Na^+-dependent nucleobase transporters were also described in rat jejunal tissue, pig renal epithelial cell line LLC-PK1, opossum kidney epithelial cell line (OK), and rabbit choroid plexus (1,11–13). To date, genes encoding these specific mammalian nucleobase transporters have not been identified. In addition, the equilibrative nucleoside transporters ENT1 and ENT2 (or *es* and *ei*) have been historically implicated in transporting nucleobases in some cell types (1). Recently, using *Xenopus* oocytes expressing recombinant hENT1 and hENT2, Yao *et al.* demonstrated that hENT2, but not hENT1, was able to transport various nucleobases with low affinities (K_m in the millimolar range) (14).

The primary physiologic role of nucleobase and nucleoside transporters is to transport nucleobases or nucleosides into cells for nucleotide synthesis in the salvage pathways. In most mammals including humans, the purine nucleobase hypoxanthine is the principal salvageable purine in the bloodstream (15). Hypoxanthine is particularly abundant in the cerebral spinal fluid (CSF), where its concentration is maintained at micromolar range (average 5 μM) as a result of ATP metabolism and carrier-mediated uptake at the blood–brain barrier (15–17). In mammalian cells, hypoxanthine is transported by various nucleobase transporters as well as the equilibrative nucleoside transporter ENT2 (1,14). Although these transporters are critical for salvaging hypoxanthine in normal cells, several studies suggest that expression of hypoxanthine transporters in tumor cells may greatly affect tumor sensitivity to antimetabolite drugs that target *de novo* pathways (6,18,19).

In the present study, we investigated the mechanism of hypoxanthine transport in human U-118 glioblastoma cells and determined the effect of hypoxanthine on cell susceptibility to a classic antifolate antimetabolite, methotrexate (MTX). Tumors of the central nervous system (CNS) are among the most frequent childhood cancers and are also most difficult to treat (20,21). In recent years, MTX-based chemotherapy is gaining a significant role in the treatment of both childhood and adult brain tumors (22,23). Among various CNS tumors, glioblastomas are one of the most common malignancies and also appear less responsive to antimetabolite chemotherapy (23,24). Because of the abundance of hypoxan-

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ABBREVIATIONS: DP, dipyridamole; MTX, methotrexate; NBMPR, nitrobenzylthioinosine.

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thine in the CNS, glioblastoma cells may circumvent the *de novo* inhibition effect of antimetabolite drugs through effective uptake and utilization of hypoxanthine via the salvage pathways. Currently there is little information on hypoxanthine transport in glioblastoma cells. Accordingly, the first goal of our study is to characterize the mechanism of hypoxanthine uptake in human U-118 cells, a cell culture model for glioblastomas. Our second goal is to determine whether hypoxanthine has a protective effect on glioblastoma susceptibility to MTX.

MATERIALS AND METHODS

Materials

Human glioblastoma (astrocytoma) cell line U-118 was purchased from ATCC (Manassas, VA). High-glucose DMEM medium and fetal bovine serum were obtained from Invitrogen (Carlsbad, CA). Dialyzed FBS was from Hyclone (Logan, UT). [^{3}H]Hypoxanthine (27 Ci/mmol) and [^{3}H]uridine (13.7 Ci/mmol) were from Moravek Biochemicals (Brea, CA). Methotrexate, hypoxanthine, adenine, cytosine, uracil, adenosine, inosine, uridine, dipyridamole, papaverine, and NBMPR were purchased from Sigma (St. Louis, MO).

Cell Culture and Uptake Assays

U-118 cells were cultured in high-glucose DMEM medium containing 10% FBS at 37°C in a CO_2 incubator. All experiments were performed using cells between passages 449 and 461. To minimize the interference of intracellular metabolism on transport assay, a rapid filtration method that allowed us to perform uptake assays within short time periods (seconds to minutes) was used. Briefly, exponentially growing U-118 cells were trypsinized, washed with D-PBS, and resuspended in Waymouth buffer (135 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.2mM $MgCl_2$, 0.8 mM $MgSO_4$, 28 mM Glucose, 13 mM Hepes, pH 7.4) at a density of 4×10^5/ml. In the Na^+-free uptake assay, NaCl was replaced with choline chloride in the uptake buffer. Uptake was initiated by addition of 50 μl of cell suspension to 50 μl of uptake mixture containing 0.25 μCi [^{3}H]hypoxanthine or [^{3}H]uridine at room temperature. After incubation, uptake was terminated by transferring samples to 5 ml of ice-cold D-PBS. Cells were filtered on 0.3 μm nitrocellulose filters (Millipore, Bedford, MA) and washed three times with 15 ml of ice-cold D-PBS. The filters were then air-dried and dissolved in 3 ml of scintillation cocktail, and the radioactivity was determined by liquid scintillation counting. Nonspecific binding of radioactive materials to the blank filters was minimal and negligible. For each data point, uptake was performed in triplicates, and each experiment was repeated two to four times. Data were expressed as mean ± SD. The kinetic parameters (apparent K_m and V_{max} values) were determined by nonlinear least-squares fits of substrate/velocity profiles to the following equation: $V = V_{max} \cdot S/(K_m + S) + K_d \cdot S$, where V is the initial rate of uptake, V_{max} is the maximal transport rate, S is the hypoxanthine concentration, K_m is the concentration at half-saturation (Michaelis-Menten constant), and K_d is the rate constant for the nonsaturable diffusional component. In inhibition studies, various inhibitors were added to the uptake mixtures at concentrations specified in the figures. Differences between mean values were analyzed for statistical significance using an unpaired Student *t* test; $p < 0.05$ was considered statistically significant.

RNA Extraction and RT-PCR

Total RNA was extracted from U-118 cells by Trizol reagent, and the integrity of total RNA was checked by agarose gel electrophoresis. We used 0.5 μg of total RNA in cDNA synthesis using the Superscript cDNA First Strand Synthesis kit (Invitrogen, Carlsbad, CA) as described previously (25). The expression of human equilibrative nucleoside transporters (hENT1-2) and Na^+-dependent nucleoside transporters (hCNT1-2) was analyzed using gene-specific primers listed in Table I. PCR amplification was performed for 35 cycles (94°C, 1 min; 57°C, 1 min; and 72°C 2 min) with a final elongation for 10 min at 72°C. The PCR products were analyzed by electrophoresis on 1% agarose gel.

Growth Inhibition Studies

In these studies, dialyzed FBS with no detectable hypoxanthine was used at 10% for cell culture. U-118 cells in logarithmic growth phase were seeded on 24-well plates (5×10^4 cells/well). MTX and hypoxanthine at various concentrations were added into the wells either alone or in combinations. Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2. After 48–72 h incubation, cell morphology was examined under an inverted phase light microscope. For protein determination, cells were washed gently with D-PBS, and protein contents were measured using a BCA protein assay kit (Pierce, Rockford, IL).

FACScan Analysis

Phosphatidylserine expression on early apoptotic cells was determined using a commercial annexin V-FITC apopto-

Table I. Primers Used in RT-PCR Analysis of hENT1-2 and hCNT1-2

Transporter	Primers	Expected size
hENT1	Sense: 5′-acatgtcccagaatgtgtccttgg-3′ Antisense: 5′-ctcaggatcacccctggcagaag-3′	1320 bp
hENT2	Sense: 5′-atcaccgccatcccgtacttccagg-3′ Antisense: 5′-ccgcagcactccaagtggatgaag-3′	1448 bp
hCNT1	Sense: 5′-gcgtgctgctctggtttaag-3′ Antisense: 5′-tagtagggtacctcactgtgcacagatcgtgt-3′	1513 bp
hCNT2	Sense: 5′-cttgtgctctcgcctcatcaaagc-3′ Antisense: 5′-ggccatagcactgaactccttatc-3′	821 bp

The expected size of the PCR products are shown.

sis detection kit (BD Biosciences, San Jose, CA) following the manufacturer's instruction. Briefly, cells were seeded on 24-well plates (5×10^4 cells/well), and MTX and hypoxanthine at various concentrations were added either alone or in combinations. After 48–72 h of incubation, cells were harvested by trypsinization and washed twice with ice-cold D-PBS. Cells were resuspended in 1× binding buffer (10 mM Hepes, 140 mM NaCl, 25 mM $CaCl_2$, pH 7.4) at 1×10^6 cells/ml. Then, 5 μl of annexin V-FITC and propidium iodide were added, and the mixture was incubated for 15 min at room temperature in the dark. Analysis was performed on a Becton-Dickinson FACScan flow cytometer (BD Biosciences, San Jose, CA). Twenty thousand events were acquired for each analysis, and the data were analyzed using the CellQwest program.

RESULTS

[^{3}H]Hypoxanthine Uptake

To determine the transport mechanism of hypoxanthine in U-118 cells, we performed [^{3}H]hypoxanthine uptake experiments using a rapid filtration method. The effects of temperature and sodium ion were first determined. Compared to room temperature (RT), uptake of ^{3}H-labeled hypoxanthine (10 μM) was significantly decreased at 4°C (~60% reduction) (Fig. 1A). In the absence of extracellular sodium, hypoxanthine uptake was also significantly reduced (45% reduction) (Fig. 1A). These data suggest that hypoxanthine transport in U-118 cells involved a temperature-sensitive and Na^+-dependent process. We then determined the time course of hypoxanthine uptake, and our data suggested that uptake of hypoxanthine was linear up to 5 min (data not shown). Previous studies demonstrated in a number of cell lines that hypoxanthine metabolism is insignificant at 60 s (11,19); thus, we chose to use 60 s as the initial rate period for the concentration dependence study. Hypoxanthine uptake was measured at concentrations ranging 0–50 μM (Fig.1B). Kinetic parameters were determined by fitting the data to a Michaelis-Menten equation consisting of a saturable and a diffusion component. The obtained K_m and V_{max} values were 10.5 ± 6.3 μM and 1.45 ± 0.69 pmol/10^5 cells/60 s, respectively. The diffusion constant K_d was 0.36 ± 0.009 μm/10^5 cells/60 s. At lower substrate concentrations (<10 μM), the carrier-mediated process contributed more than 70% to the total hypoxanthine uptake in U-118 cells.

Relationship to Nucleoside Transport

Previously, the ENT1 and ENT2 nucleoside transporters were reported to transport hypoxanthine (1,14). To determine whether the observed carrier-mediated hypoxanthine transport in U-118 cells was caused by ENTs or an independent nucleobase transport system, we investigated the effect of nucleobases, nucleosides, and classic nucleoside transport inhibitors on [^{3}H]hypoxanthine (10 μM) uptake in U-118 cells. At 1 mM, the purine nucleobases hypoxanthine and adenine and the pyrimidine nucleobase uracil significantly inhibited hypoxanthine uptake (58%, 51%, and 45% of control, respectively) (Fig. 2A). In contrast, nucleosides (adenosine and inosine) had no inhibitory effect. Furthermore, the classic equilibrative nucleoside transporter inhibitors, DP (10 μM) and NBMPR (10 μM) exhibited no inhibitory effect (Fig. 2A). Interestingly, NBMPR even increased hypoxanthine influx. The reason for this observation is unknown. Cytosine and papaverine appeared to have no inhibitory effect. To further investigate if there was a shared transport system for both nucleobases and nucleosides, we examined the effect of nucleobases, nucleosides, and nucleoside transporter inhibi-

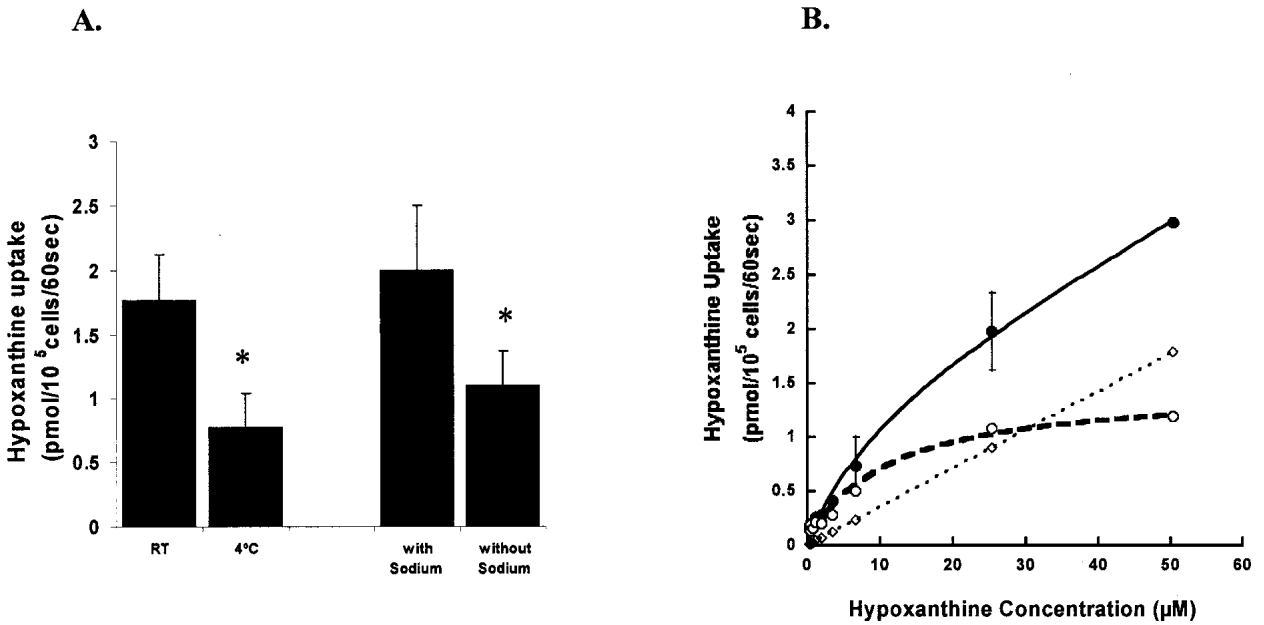

Fig. 1. (A) Temperature and sodium dependence of [^{3}H]hypoxanthine (10 μM) uptake in U-118 cells. *$p < 0.05$. (B) Concentration dependence of [^{3}H]hypoxanthine uptake in U-118 cells. The kinetic parameters were obtained by fitting the total uptake data (●) to a Michaelis-Menten equation described in Materials and Methods. Carrier-mediated (○) uptake and the linear diffusional component (◊) were calculated from the obtained kinetic parameters. Uptake was performed using a 60-s incubation time period. Each data point represents the mean ± SD of data obtained in triplicates (n = 3) from one representative experiment of 2-4 experiments of similar results.

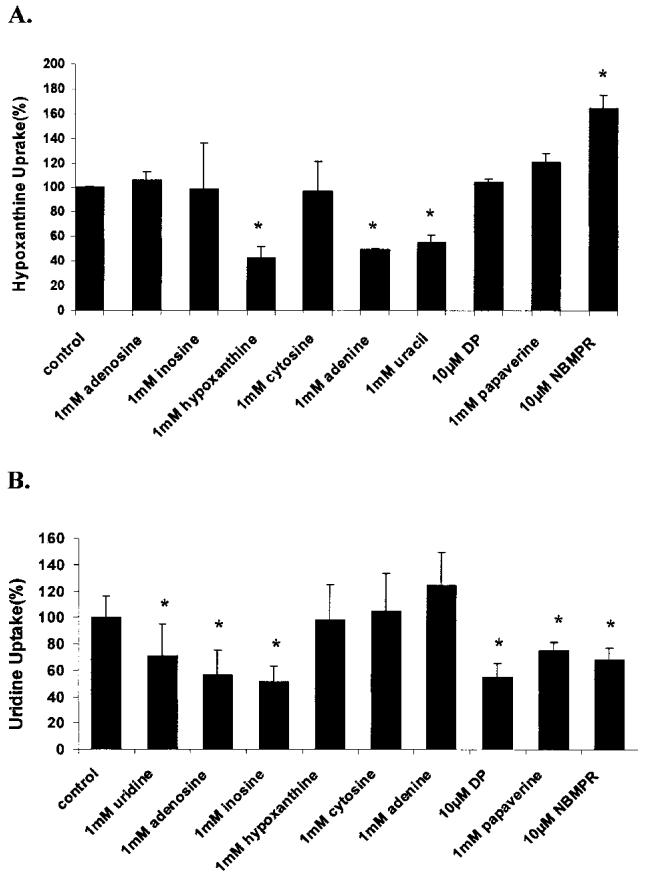

Fig. 2. Inhibition effect of various nucleobases, nucleosides, and nucleoside transporter inhibitors on (A) [^{3}H]hypoxanthine (10 μM) and (B) [^{3}H]uridine (10 μM) uptake in U-118 cells. Uptake was at 5 min intervals and expressed as percentage of control. Each data point represents the mean ± SD of data obtained in triplicates (n = 3) from one representative experiment of at least two experiments of similar results. *$p < 0.05$

tors on the uptake of [^{3}H]uridine, a common substrate for all of the known nucleoside transporters. As shown in Fig. 2B, uridine uptake was significantly inhibited by nucleosides (uridine, adenosine, inosine) and the ENT inhibitors DP and NBMPR. In contrast, hypoxanthine, cytosine, and adenine had no inhibitory effect (Fig. 2B). Together, these data demonstrated that hypoxanthine uptake in U-118 cells was mediated by a specific nucleobase transport system distinct from the nucleoside transporters.

Expression of Nucleoside Transporters in U-118 Cells

Our uridine uptake studies (Fig. 2B) suggested carrier-mediated nucleoside transport activity in U-118 cells. To elucidate the molecular identity of these nucleoside transporters, we performed RT-PCR analysis with primers that specifically amplify major nucleoside transporters in humans. As shown in Fig. 3, strong bands were observed for the equilibrative nucleoside transporters hENT1 and hENT2. A moderate band of the Na^+-dependent nucleoside transporter hCNT1was also detected. No band was detected for hCNT2.

Effect of Hypoxanthine on Cell Susceptibility to MTX

Our hypoxanthine transport study demonstrated the presence of a specific transport system for hypoxanthine in

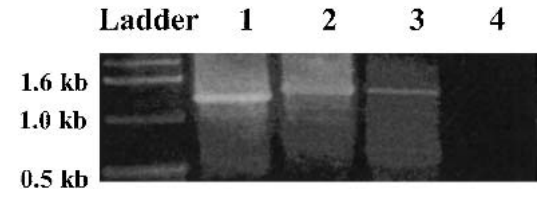

Fig. 3. RT-PCR analysis of human equilibrative and concentrative nucleoside transporters in U-118 cells using gene-specific primers. Lane 1, hENT1; lane 2, hENT2; lane 3, hCNT1; lane 4, hCNT2.

U-118 cells. Because a significant amount of hypoxanthine is present in the CSF (~5 μM), salvage of this freely available purine base by tumor cells may greatly attenuate the activity of antimetabolite drugs. To test this hypothesis, we examined whether the presence of hypoxanthine at physiologically relevant concentrations altered cell susceptibility to MTX. To eliminate the carryover of hypoxanthine from normal FBS serum, dialyzed FBS containing no detectable hypoxanthine was used in the cell culture medium. U-118 cells grew normally in medium containing dialyzed FBS and were sensitive to MTX at submicromolar concentrations (data not shown). To examine the effect of hypoxanthine, cells were seeded in medium containing various combinations of MTX, hypoxanthine, and the nucleoside transport inhibitor DP. After 72 h, total protein levels were determined to estimate cell growth rates. Compared to the control cells, addition of hypoxanthine (30 μM) alone did not alter cell growth rate (Fig. 4). When cells were treated with 0.2 μM MTX, significant growth inhibition was observed (Fig. 4). Addition of 30 μM hypoxanthine to MTX-treated cells significantly improved their growth rates.

Although at 2.5 and 10 μM, hypoxanthine did not significantly improve the growth rates of MTX-treated cells as determined by protein measurement (Fig. 4), there was a non–statistically significant trend in growth improvement at these concentrations. Furthermore, we noticed that at these concentrations hypoxanthine exhibited dramatic effects on MTX-treated cells as reflected by their morphology. Non-treated cells or cells cultured in medium containing hypoxan-

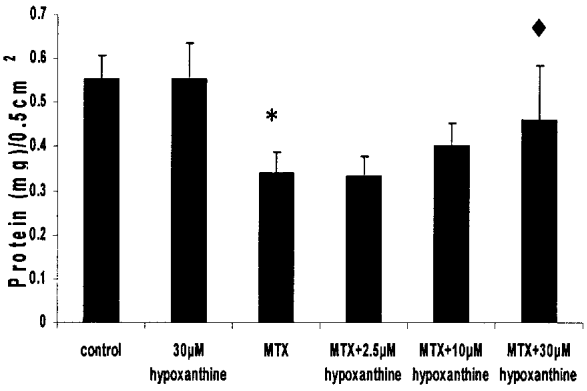

Fig. 4. Total protein after 72 h cultivation. Proliferation of U-118 was estimated by measuring total protein of cells cultured under various conditions. Control cells were cultured in medium containing no drugs. MTX was used at 0.2 μM. *Significantly different from control ($p < 0.05$). ♦ Significantly different from the MTX-treated cells ($p < 0.05$). Each data point represents the mean ± SD of data obtained from 2–4 wells (n = 2–4) from one representative experiment. The experiments were performed twice. *$p < 0.05$.

thine alone are healthy looking with extended cell bodies (Fig. 5A–C). In contrast, cells treated with MTX (0.2 μM) became rounded with shrunken cytoplasm, indicating the onset of apoptosis (Fig. 5D). When MTX-treated cells were coincubated with hypoxanthine at 2.5 and 30 μM, cells became healthy looking again with broader cell bodies (Fig. 5E,F). These data suggest that hypoxanthine at 2.5–30 μM had a protective effect on MTX-induced toxicity. To confirm that MTX induces apoptosis in U-118 cells and to quantify the protective effect of hypoxanthine, we performed FACScan analysis using an annexin V-FITC apoptosis detection kit. Increased annexin V staining of the redistributed phosphatidylserine is a general feature of apoptosis and has been used as a standard measurement of apoptosis (26). As shown in Fig. 6A, significantly increased annexin V staining was observed for cells treated with 0.2 μM MTX, suggesting the onset of apoptosis in these cells. Addition of 30 μM of hypoxanthine to the control cells had no effect on annexin V staining (data not shown). However, addition of hypoxanthine to MTX-treated cells returned the annexin V staining signal to the control level (Fig. 6A). These data suggest that MTX-induced apoptosis was completely suppressed by the presence of hypoxanthine. Addition of the nucleoside transport inhibitor DP to the MTX-treated cells increased annexin V staining (Fig. 6B), suggesting a synergistic potentiation effect of DP on MTX toxicity. However, addition of hypoxanthine (30 μM) to cells coincubated with DP and MTX again returned the annexin V staining signal to that of the control cells that received no drug treatment. Similar results were obtained when hypoxanthine was used at 10 μM (data not shown). These data strongly suggested that hypoxanthine alone can protect cells from MTX toxicity regardless of whether or not DP is present.

DISCUSSION

Brain tumors are among the most frequent childhood cancers and are also most difficult to treat. In recent years, chemotherapy equipped with advanced delivery technologies is gaining a significant role in the treatment of various brain tumors (21,23). For example, MTX, a dihydrofolate reductase (DHFR) inhibitor that targets *de novo* purine and pyrimidine syntheses, has been used in the treatment of primary CNS lymphoma and neuroectodermal tumor (22,23). Earlier studies suggest that tumor cells can circumvent the inhibition of *de novo* nucleotide syntheses imposed by MTX through the salvage of extracellular preformed nucleobases or nucleosides (3,4,18). Because the purine nucleobase hypoxanthine is present at great abundance in the CSF, transporter-mediated

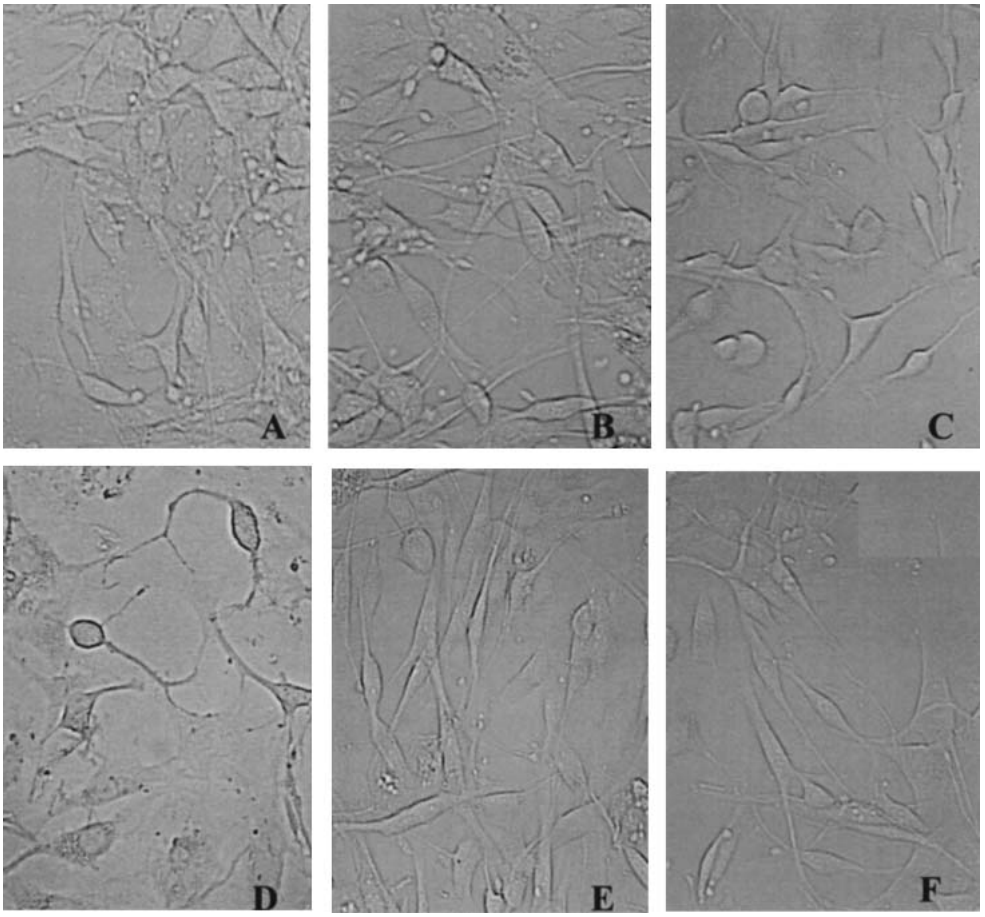

Fig. 5. Morphology of U-118 cells under various culture conditions: (A) 10% dialyzed FBS; (B) 10% normal FBS; (C) dialyzed FBS plus 2.5 μM hypoxanthine; (D) dialyzed FBS plus 0.2 μM MTX; (E) dialyzed FBS plus 0.2 μM MTX and 2.5 μM hypoxanthine; and (F) dialyzed FBS plus 0.2 μM MTX and 30 μM hypoxanthine. Cells were cultured for 72 h, and the pictures were taken under an inverted phase light microscope.

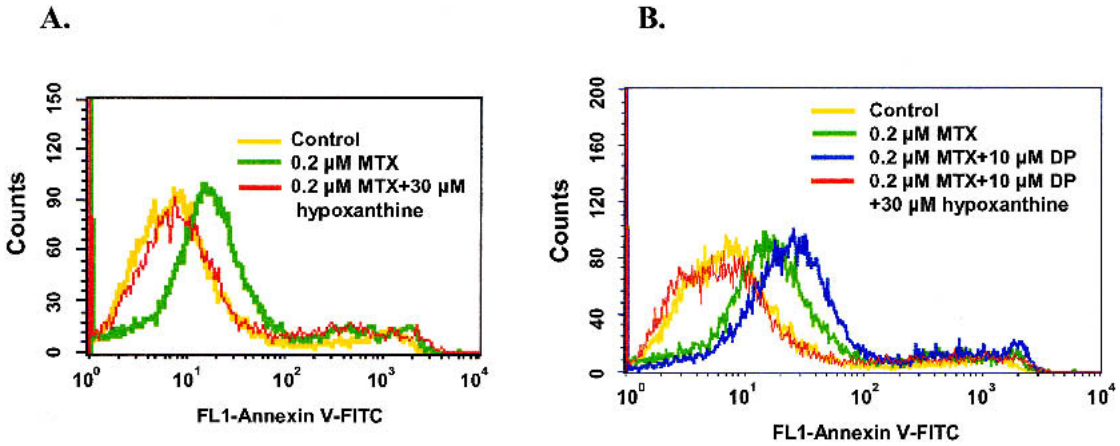

Fig. 6. Annexin V staining of U-118 cells cultured under various conditions as specified in the histogram. (A) Effect of hypoxanthine on MTX-treated cells. (B) Effect of hypoxanthine and DP on MTX-treated cells. Phosphatidylserine expression on early apoptotic cells was determined using an annexin V-FITC apoptosis detection kit and FACScan analysis.

hypoxanthine uptake may have an important impact on cell sensitivity to MTX. In this study, we tested this hypothesis in U-118 human glioblastoma cells by determining the mechanism of hypoxanthine transport and the effect of extracellular hypoxanthine on cell susceptibility to MTX.

Using uptake assays, we demonstrated that hypoxanthine transport in U-118 cells involved both carrier-mediated and simple diffusion processes. At lower concentrations (<10 μM), hypoxanthine uptake in U-118 cells was mainly (>70%) through the carrier-mediated process (Fig. 1B). This transport system was sensitive to Na^+ and had an apparent K_m of ~10.5 μM for hypoxanthine. The nucleobases, adenine and uracil, inhibited hypoxanthine uptake, whereas nucleosides (adenosine and inosine) and nucleoside transport inhibitors (DP and NBMPR) had no inhibitory effect. Specific Na^+-dependent nucleobase transporters have been previously described in a number of mammalian tissues and cells. In LLC-PK1 and OK cells, a Na^+-dependent nucleobase transporter interacting with hypoxanthine and uracil has been described (11,12). This transporter is sensitive to DP (K_i = 4.6 and 0.9 μM for LLC-PK1 and OK, respectively) but does not interact with adenine and nucleosides. Because adenine potently inhibited hypoxanthine transport in U-118 cells and DP had no inhibitory effect, the hypoxanthine transporter in U-118 cells seemed to differ from those in LLC-PK1 and OK cells. Instead, this transporter appeared more similar to the Na^+-dependent hypoxanthine transporter in the rabbit choroid plexus in that both are sensitive to adenine inhibition (Fig. 2A) (13). However, cytosine, which inhibits the choroid plexus transporter, did not inhibit hypoxanthine transport in U-118 cells, suggesting that there might be some substrate selectivity difference between the human and rabbit transporters. Previously, Marshman *et al.* showed that in human breast adenocarcinoma cells (MCF7 and T-47D) and non–small cell lung carcinoma cells (A594 and COR-L23), transport of hypoxanthine appeared to occur via Na^+-independent equilibrative transporters that were either sensitive or insensitive to DP (19). In U-118 cells, transport of hypoxanthine is Na^+-dependent, suggesting that glioblastoma cells may use a different mechanism to transport hypoxanthine. However, because the removal of Na^+ did not reduce the uptake to the level measured at 4°C (Fig. 1A), it is likely that some Na^+-independent nucleobase transporters are also expressed in U-118 cells.

We also demonstrated high expression of the equilibrative nucleoside transporters, hENT1 and hENT2, in U-118 cells (Fig. 2B and Fig. 3). The Na^+-dependant nucleoside transporter hCNT1 is detected at a lower level and may not play a significant role in nucleoside transport since we did not see a Na^+-dependent effect of uridine uptake in U-118 cells (data not shown). Previously it has been shown that rENT1 and rENT2 are the major nucleoside transporters in a rat glioma cell line C6 (27). When expressed in *Xenopus* oocytes, hENT2 has been shown to transport hypoxanthine with a K_m of 0.8 mM (14). In the present study, hypoxanthine uptake (10 μM) in U-118 cells was not affected by nucleosides and the ENT2 inhibitor DP (Fig. 2A), suggesting that the hENT2 transporter does not play a significant role in transporting hypoxanthine at low substrate concentrations in U-118 cells.

Several studies showed that in cultured peripheral cancer cell lines, the presence of hypoxanthine in cell culture media can abolish the antitumor activity of antimetabolite drugs (19,28). In the brain where hypoxanthine is present at micromolar concentrations, hypoxanthine uptake by glioblastoma cells may protect these cells from the toxicity of antimetabolite drugs. We tested this hypothesis by examining the response of U-118 cells to MTX in the absence and presence of hypoxanthine. We found that when cultured in media lacking hypoxanthine, U-118 cells were very sensitive to MTX at concentrations as low as 0.2 μM. MTX toxicity is manifested in two aspects. First, MTX-treated cells exhibited retarded growth (Fig. 4), as expected from the pharmacologic action of MTX. Second, cells exposed to MTX became apoptotic as reflected in their morphologic changes and increased annexin V staining of plasma phosphatidylserine (Figs. 5 and 6). MTX was recently shown to induce apoptosis in activated T cells (29). Our study provided the evidence that MTX can also induce apoptosis in human glioblastoma cells in the absence of hypoxanthine. When hypoxanthine was present at low micromolar concentrations (2.5–30 μM), cells became insensitive to MTX (Figs. 4–6). These data may provide an explanation of why glioblastomas are less sensitive to MTX-based

chemotherapy. The nucleoside transport inhibitor DP has been previously explored to potentiate MTX cytotoxicity toward tumor cells clinically and experimentally (5,30). Our data showed that DP potentiated the toxic effect of MTX only in the absence of hypoxanthine, probably by blocking the hENT1- and hENT2-mediated transport of residual nucleosides derived from the serum or released by dead cells. However, in the presence of hypoxanthine, the potentiation effect of DP is completely abolished (Fig. 6B). These data clearly demonstrated that DP cannot potentiate MTX toxicity in the presence of hypoxanthine if DP does not inhibit hypoxanthine transport. Therefore, DP may not be effective in increasing glioblastoma sensitivity to MTX *in vivo*.

From Fig. 1A and B, it is estimated that at low concentrations, ~70% of hypoxanthine uptake in U-118 glioblastoma cells occurs via specific nucleobase transporters, which are mainly Na^+-dependent concentrative transporters. Additionally, Na^+-dependent nucleobase transporters generally exhibit higher substrate affinity than equilibrative nucleobase transporters (1,10). Therefore, *in vivo* where hypoxanthine is present at low micromolar concentrations, the Na^+-dependent concentrative nucleobase transporters may play a more prominent role in regulating glioblastoma cell sensitivity to MTX. Currently, there are no specific inhibitors available for the Na^+-dependent nucleobase transporters (1,10). The development of high-affinity inhibitors toward these transporters may represent a useful strategy to enhance tumor sensitivity to antimetabolite drugs *in vivo*.

In summary, we demonstrated that hypoxanthine uptake in U-118 human glioblastoma cells is mediated by a specific nucleobase transport system. In the absence of hypoxanthine, U-118 cells are sensitive to the antifolate drug MTX. However, the presence of hypoxanthine at physiologically relevant concentrations protects cells from MTX, and this protection effect cannot be reversed by the nucleoside transporter inhibitor DP. Our studies highlight the importance of salvage pathways in mediating tumor resistance to antimetabolite drugs. Identification of the nucleobase transporters in glioblastoma cells and development of high-affinity inhibitors toward these transporters may represent a useful strategy to enhance tumor sensitivity to antimetabolite drugs.

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