Hypoxanthine Concentrations in Normal Subjects and Patients with Solid Tumors and Leukemia

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ABSTRACT

Mean plasma hypoxanthine (Hyp) concentrations determined by high-pressure liquid chromatography were 0.56 μM (range, 0.2 to 1.9 μM) in 16 normal subjects, 0.68 μM (range, 0.1 to 1.1 μM) in 10 untreated acute leukemic subjects, and 0.89 μM (range, 0.3 to 2.6 μM) in 14 solid tumor patients. Despite large differences in Hyp concentration between patients, every 4-hr sampling, indicated that diurnal variation in individual patients was small (maximum, 2.3-fold). While the mean plasma and malignant effusion Hyp concentrations did not differ significantly, bone marrow plasma Hyp concentration averaged 4.0-fold greater than that of simultaneously drawn venous plasma. Allopurinol (maximum, 2.3-fold). While the mean plasma and malignant effusion Hyp concentrations did not differ significantly, bone marrow plasma Hyp concentration averaged 4.0-fold greater than that of simultaneously drawn venous plasma. Allopurinol 300 mg p.o. caused a mean 1.5-fold increase in plasma Hyp concentration within 3 hr. In 17 patients with acute leukemia, treatment with allopurinol at 300 mg daily plus initiation of chemotherapy caused a mean 7-fold increase in plasma Hyp to 4.6 μM (range, 1 to 12 μM). The ability of Hyp to modulate the toxicity of antimetabolites affecting purine synthesis (6-diazo-5-oxo-L-norleucine, 6-mercaptopurine riboside, 6-mercaptopurine, and 6-thioguanine) was determined in vitro using human B-lymphoblast (WI-L2) and promyelocytic leukemia (HL-60) cell lines. Hyp permitted growth of both cell lines in the presence of clinically achievable concentrations of all 4 drugs, but the initial culture concentrations of Hyp required were above those found in patients. Since Hyp was consumed rapidly during the culture period, the average Hyp concentrations required for the protection of cells were actually much lower. We conclude that, in patients with acute leukemia receiving allopurinol during chemotherapy, plasma Hyp concentrations are significantly elevated; the potential for antagonism of antimetabolite activity is uncertain.

INTRODUCTION

Antimetabolites which interfere with de novo purine synthesis constitute an important part of the armamentarium of drugs available for the treatment of leukemia and other cancers (4, 15, 26, 36). However, not all patients respond equally well to these drugs, and some eventually become refractory to the therapy. Resistance to purine analogues has been attributed to the deficiency of enzymes necessary to convert them to their nucleotide forms (7, 31). Other mechanisms have been observed in experimental tumors, such as increased degradation of drugs and inability of resistant cells to convert the ribonucleotide analogues to the deoxynucleotide analogue (10, 29).

This study was designed to examine the possibility that increased plasma Hyp concentrations during treatment may also be a mechanism of resistance to antimetabolites acting in the purine biosynthetic pathways. Extracellular Hyp is important for the activity of purine antimetabolites in 2 aspects: it can be utilized to restore purine nucleotide pools in cells the de novo synthesis of which has been blocked, and it potentially may compete with some of these antimetabolites for transport into the cell or for the enzymes necessary to convert the drugs to their active nucleotides (9).

Using a newly developed high-pressure liquid chromatographic method (43), we have measured plasma Hyp in groups of normal subjects, patients with solid tumors, and patients with acute leukemia. Frequent blood samples were obtained from some patients to assess diurnal variation, and Hyp concentration in bone marrow and malignant effusions was compared to that of venous plasma to determine the distribution of Hyp in different body compartments. We also examined the effect on plasma Hyp of treatment with HPP alone or the combination of HPP and chemotherapy. Finally, the effect of Hyp at concentrations encompassing the range observed in vivo was tested for its ability to modulate the toxicity of de novo purine synthesis inhibitors toward the HL-60 promyelocytic leukemia and the WI-L2 lymphoblast human cell lines.

MATERIALS AND METHODS

Subjects and Patients. Blood samples were obtained from 5 groups of subjects and patients for Hyp measurement: (a) normal laboratory personnel; (b) patients with acute lymphocytic leukemia or acute nonlymphocytic leukemia, either untreated or in relapse; (c) patients with advanced solid tumors of many types; (d) patients with malignant pleural or peritoneal effusions due to solid tumor involvement; and (e) patients with acute leukemia undergoing initial induction chemotherapy for either untreated or relapsed disease. No restrictions were placed on diet or activity in any group of subjects or patients, and samples were drawn at random times of day. For all groups except the normal subjects and acute leukemic subjects receiving induction chemotherapy, blood samples were obtained at least 3 weeks following any prior therapy and at a time when no toxicity attributable to prior therapy was evident.

High-Pressure Liquid Chromatography. A detailed procedure for simultaneous measurement of plasma Hyp, uric acid, HPP, and oxypurinol was developed in this laboratory and has been published elsewhere (43). Delay in separation of plasma from the formed elements of blood has been shown to increase plasma Hyp. Therefore, samples were collected in prechilled heparinized tubes, and plasma was separated immediately by centrifugation, precipitated with 0.1 volume of 4.4 N HClO₄, and neutralized with Alamine/Freon, as described by Khym (21). A high-pressure liquid chromatographic system (Waters Associates, Milford, MA) with a reverse-phase C₁₈-pBondapak column was used in this study. Peaks were identified by their retention times and their absorbance ratios at 254 and 280 nm. An aliquot of 20-μl sample was analyzed isocratically.

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3 The abbreviations used are: Hyp, hypoxanthine; DON, 6-diazo-5-oxo-L-norleucine; HPP, 4-hydroxy-3,4-pyrazolopyrimidine (allopurinol); HGPRT, hypoxanthine-guanine phosphoribosyltransferase; 6-MP, 6-mercaptopurine; MIMPR, 6-methylmercaptopurine riboside; 6-TG, 6-thioguanine.

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using phosphate eluent (50 mM KH₂PO₄, pH 4.60). The limit of detection for all compounds of interest was 0.1 μM.

Cell Culture and Growth Studies. A human B-lymphoblast (WI-L2) and a human promyelocytic leukemia (HL-60) cell line were used to determine the growth-inhibitory effect of various concentrations of antimetabolites and the blockade of this effect by Hyp in vitro. These cells were grown in RPMI 1640 medium (Cell Culture Facility, University of California, San Diego, La Jolla, CA) supplemented with 10% fetal calf serum (Irvine Scientific, Irvine, CA), 1% glutamine, and 1% antibiotics-antimycotics mixture (Grand Island Biological Co., Grand Island, NY). Growth assays were performed in Linbro multiwell tissue culture plates (Flow Laboratories, Inc., McLean, VA). The log-phase cells were washed with RPMI 1640 medium to remove any Hyp in the growth medium. Hyp and antimetabolites (6-MP, 6-TG, MMPR, and DON) were prepared in the same medium, except that fetal calf serum was replaced with bovine serum albumin (5 mg/ml) or 10% charcoal-treated fetal calf serum. Cells were cultured at 10³/ml in triplicate with 1 to 100 μM Hyp and 1, 4, and 10 μM of each antimetabolite at 37° for 3 days under 4% CO₂. The number of cells was counted electronically using a Model ZB Coulter Counter, and the percentage of control growth in antimetabolite-treated cultures was calculated by comparison with cells growing in Hyp-free and antimetabolite-free medium.

**RESULTS**

Means and Ranges of Plasma Hyp. Previous reports have indicated that, in acute leukemia, both the production and plasma concentration of uric acid were increased (29). Similar changes in plasma Hyp, which is the precursor of uric acid, have not been reported. A comparison of plasma Hyp concentrations in randomly collected blood samples from 16 normal subjects, 10 untreated acute leukemic subjects, and 14 untreated solid tumor patients is summarized in Chart 1. The geometric mean plasma Hyp concentration was 0.56 μM (range, 0.2 to 1.9 μM) in normal subjects, 0.68 μM (range, 0.1 to 1.1 μM) in acute leukemic subjects, and 0.89 μM (range, 0.3 to 2.6 μM) in solid tumor patients, and the differences between these groups were not significant. Included in Chart 1 are measurements made on malignant ascites or pleural effusions from solid tumor patients. The geometric mean effusion Hyp concentration was 0.4 μM (range, 0.1 to 1.0 μM), about one-half of that in plasma of solid tumor patients.

Mean plasma uric acid concentration in these acute leukemic patients was 410 μM (range, 110 to 610 μM) (data not shown), which is 1.5-fold greater than the mean of 276 μM (range, 210 to 340 μM) found in the normal subjects; plasma uric acid in the solid tumor patients was not significantly different from that in normal subjects (mean, 255 μM; range, 150 to 440 μM).

**Diurnal Variation of Plasma Hyp.** Four solid tumor patients were studied for diurnal variation of plasma Hyp. None of these patients was receiving chemotherapy or HPP at the time of sampling. Blood was collected at intervals of 4 hr for a period of 24 hr. No restriction was placed on food intake or physical activity during the sampling time. Chart 2 shows that, in each of these 4 patients, plasma Hyp concentration remained within relatively narrow limits over 24 hr compared to the wider range found between patients. The average variation in plasma Hyp concentration over 24 hr was 1.9-fold, and the range was 1.4- to 2.3-fold. The variation could not be related to meals, activity, or sleep.

**Effects of HPP and Chemotherapy on Plasma Hyp.** The effect of a single dose of 300 mg of HPP p.o. on plasma Hyp concentration and the pharmacokinetics of HPP were examined in the same 4 patients in whom diurnal variation was also studied. Chart 3 shows the plasma concentrations of Hyp, HPP, and oxypurinol, the major metabolite of HPP, during the 8 hr following HPP ingestion. In 3 of 4 patients, plasma HPP peaked at 2 hr and then fell rapidly, with an elimination half-life of 55 to 65 min. In the fourth patient, gastrointestinal absorption of HPP was delayed. Maximum plasma oxypurinol was reached about 2 hr after HPP ingestion, and it averaged 35 μM (range, 21 to 60 μM). This level remained steady for each patient over the next 6 hr. Other investigators have also reported a long half-life of 14 to 20 hr for oxypurinol (3, 23). Plasma Hyp concentration increased to a mean of 1.35 μM (range, 0.55 to 2.50 μM) during the 6-hr period following HPP ingestion. There was a 1.5-fold increase over the mean Hyp concentration during the preceding 24 hr before the administration of HPP.

Plasma Hyp was measured before and then again 48 hr after the initiation of treatment with HPP, daunorubicin, and 1-β-D-arabinofuranosylcytosine on 6 courses in 5 patients with acute leukemia (Chart 4). In addition, individual nonpaired measurements were made either before or 48 hr after initiation of treatment on 11 courses given to 6 other acute leukemic subjects. Whereas a single dose of HPP alone did not substantially increase plasma Hyp, the combination of HPP plus the initiation of chemotherapy did. Considering only the 6 paired observations, the plasma Hyp concentration increased by a mean of 8.6-fold (p < 0.01; t test for paired observations). When all measurements...
were included, the plasma Hyp concentration was 7-fold higher at 24 hr, with a mean of 4.6 μM (range 0.9 to 12.0 μM). A linear relationship was observed between plasma Hyp and oxypurinol concentrations measured in the same samples \( r = 0.71; p < 0.01 \).

**Hyp Concentration in Bone Marrow.** Hyp concentration in bone marrow was compared with venous blood plasma in 9 solid tumor patients, and the results are shown in Chart 5. In all patients, the marrow plasma Hyp concentrations were consistently greater than those of simultaneously drawn venous plasma. The mean difference between marrow plasma and venous plasma was 5.7 μM \( (p < 0.01; t \text{ test for paired observations}) \). The possibility that this difference was due to hemolysis in marrow specimens was excluded by the finding that there was no increase in Hyp concentration in marrow plasma when RBC were added and subjected to mechanical destruction.

**Effect of Hyp on Antimetabolite Activity.** Both WI-L2 and HL-60 cells are normally grown in medium containing 10% fetal calf serum, which itself contributes large amounts of Hyp to the culture. In order to avoid this problem, WI-L2 cells were grown in purine-free RPMI 1640 medium containing bovine serum albumin (5 mg/ml), transferrin (35 μg/ml), insulin (5 μg/ml), 2.5 nm selenium, and 20 μM ethanolamine. HL-60 cells were grown in RPMI 1640 medium containing charcoal-stripped fetal calf serum at a final concentration of 10%. The fetal calf serum contained less than 1 μM Hyp after treatment with charcoal (0.5 g per 10 ml of serum) as determined by high-pressure liquid chromatography. Both media were supplemented with known concentrations of Hyp in experimental cultures.

Chart 6 shows the effect of initial Hyp concentration between 1 and 100 μM on the proliferation of WI-L2 and HL-60 cells in the presence of 6-MP, 6-TG, MMPR, and DON. Antimetabolite concentrations from 1 to 10 μM were chosen as representative of the concentrations of these drugs that can be achieved clinically (25, 27). Increasing the Hyp concentration 10-fold from 1 to 10 μM resulted in increased growth at all 3 concentrations of all of the drugs; however, the magnitude of this effect was not large. A further 10-fold increase in initial Hyp concentration increased growth substantially, even in cultures treated with the highest antimetabolite concentrations, to 50% or more of the growth occurring in non-drug-treated control cultures for both cell lines. Thus, Hyp antagonized the antiproliferative effect of all 4 drugs, but a 50% reduction in antimetabolite effect required Hyp concentration above those observed even in patients with acute leukemia receiving HPP and induction therapy who had a marked increase in Hyp production.

It is important to emphasize that the data depicted in Chart 6
Plasma Hyp and Antimetabolite Activity

DISCUSSION

Little information is available on the concentration of Hyp in various human body fluids under physiological conditions, and the accuracy of concentrations reported previously has now been called into question by the finding that special handling of specimens is required to avoid leakage of Hyp from RBC and platelets (43). The release of Hyp during clotting probably accounts for the fact that the mean plasma Hyp values reported here are 10-fold less than are those reported for human serum by other investigators (20, 33, 34).

Although there was a wide variation in plasma Hyp concentrations measured at random times in individual subjects, a comparison of mean values in normal subjects, solid tumor patients, and acute leukemias revealed no significant differences among these groups. In addition, in individual subjects, plasma Hyp concentration varied by an average of only 1.9-fold during a 24-hr period. These observations suggest that plasma Hyp may be regulated by metabolic controls which must affect either the entrance of Hyp into the plasma or its clearance or both. Our measurements provide some information on how changes in the production or clearance of Hyp influence plasma Hyp concentrations. The finding of similar mean plasma Hyp concentrations in leukemic and normal subjects suggests that the increased production in leukemia (30) does not exceed the clearance capacity of xanthine oxidase or urinary excretion (8, 9, 14, 30). Under normal circumstances, the clearance of Hyp via xanthine oxidase is less significant than is that by other mechanisms, since inhibition of xanthine oxidase by HPP caused only a 1.5-fold increase in Hyp concentration. However, when HPP was combined with increased Hyp production resulting from initiation of chemotherapy in patients with acute leukemia, there was a 7-fold increase in plasma Hyp concentration. Thus, under conditions of increased catabolic flux of purines, the activity of xanthine oxidase does have a very significant effect on plasma Hyp, and this was reflected by the good correlation between plasma Hyp and oxypurinol, which is the active metabolite of HPP present in highest concentration in plasma after administration of HPP (14, 16).

The finding of a mean 4.0-fold higher concentration of Hyp in bone marrow than in venous plasma is of interest for several reasons. First, it suggests that, in contrast to liver, which clears large amounts of Hyp from plasma on a single pass (32), bone marrow is a net producer of Hyp. A number of investigators have concluded that bone marrow cells and gastrointestinal mucosa cells have a very limited capacity to carry out de novo purine synthesis, and they depend on preformed purine in plasma for synthesis of purine nucleotides via the salvage pathway (1, 24, 28, 32, 39, 40). However, Hyp itself is an important regulator of purine synthesis (17-19, 36, 38), and concentrations as low as 1 µM are sufficient to decrease de novo synthesis in fibroblasts (38) and marrow (22). All of the previous studies on de novo purine synthesis in marrow cells were performed under conditions in which the concentration of Hyp in the cell environment was probably quite high (1, 24, 28, 36, 39, 40). Thus, rather than reflecting an inability of marrow cells to synthesize purine de novo, the limited incorporation of [14C]formate used to measure the activity of this pathway may be due to the inhibitory action of the high local concentration of Hyp. If the rapid cell proliferation in the gut is also accompanied by high local concentrations of Hyp, then the hypothesis that gut cells cannot produce purines are based on the initial concentration of Hyp in the culture. Table 1 indicates that, while the concentration of Hyp remained stable in medium to which no cells were added, both WI-L2 and HL-60 cells consumed much of the Hyp present during the 3-day period of growth. Thus, the average concentration of Hyp was significantly lower than was the initial concentration, and this was more marked in cultures with the lower starting Hyp concentrations. The impact of considering average rather than initial Hyp concentrations on the curves depicted in Chart 6 would be to flatten their slopes somewhat but, on the other hand, it indicates that average Hyp concentrations even lower than 10 µM were capable of affecting purine antimetabolite activity.
via the de novo route may also be incorrect (28).

Several investigators have reported previously that Hyp can reverse the cytotoxicity of MMPR (2, 37). Our finding that Hyp reduces the activity of 6-MP, 6-TG, MMPR, and DON indicates that the effect is not merely due to competition for HGPR or for transport into the cell, since MMPR is phosphorylated by adenosine kinase rather than HGPR (5, 35) and since DON does not require HGPR for activation. The results of this study show that the concentrations of plasma Hyp in patients receiving HPP and chemotherapy are elevated into a range which is just below that which begins to interfere with the cytotoxic activity of clinically relevant concentrations of purine antimetabolites against human leukemia cells in vitro. It is important to emphasize that the curves in Chart 6 were constructed using the concentrations of Hyp present at the beginning of culture. Hyp concentration rapidly increased as cells grew and, at the end of the 3-day culture period, there was practically no Hyp left in the medium originally containing 10 μM Hyp, and there was a 30 to 60% reduction of Hyp in the cultures originally containing a 100 μM concentration. Thus, no matter what the mechanism of the Hyp effect, the degrees of modulation depicted in Chart 6 were actually achieved with average Hyp concentrations that were considerably below the initial concentrations indicated on the abscissa. Although the growth conditions of leukemia cells in vitro are quite different than those in vivo, this raises the possibility that the 7-fold increase in plasma Hyp that accompanied initiation of chemotherapy in acute leukemic patients receiving HPP may be sufficient to reduce the effectiveness of the antipurine antimetabolites during induction therapy. The increase in Hyp also provides a potential explanation of the observation in rabbits that, although HPP can block the catabolism of 6-MP (11-13) and increase the area under the plasma concentration times time curve (41), it does not increase the toxicity of 6-MP (42). Additional studies are required to evaluate completely the role of Hyp and HPP in modulating the activity 6-MP and the other antipurine antimetabolites in humans (6) and to validate the extrapolation that we have made between in vitro results and in vivo observations.

REFERENCES

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