Incorporation of Iododeoxyuridine into DNA of Granulocytes in Patients

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Abstract

Iododeoxyuridine (IdUrd) competes with thymidine for incorporation into DNA. In order to measure the incorporation of the drug in vivo, granulocytes were isolated from peripheral blood of patients at various times during and after 9- to 14-day IdUrd i.v. continuous infusions. DNA was extracted and enzymatically hydrolyzed. Both thymidine and IdUrd were separated and measured by high-performance liquid chromatography. The percentage substitution by IdUrd was less than 1% prior to the fifth day of infusion and then increased rapidly to achieve a maximal value between 7 and 17% at the end of the infusion. In our protocol, thrombocytopenia was the most frequent dose-limiting systemic toxicity. For our group of patients, there was a clear overall relationship between the extent of substitution by IdUrd and the hematological toxicity. To our knowledge, these data provide the first direct quantitative determination of substitution by a drug into DNA in vivo without the use of radiolabeled compounds. The ability to directly monitor drug incorporation into DNA may provide the basis for monitoring and improving the selectivity of therapy.

Introduction

IdUrd2 is a dThd analogue synthesized by Prusoff as a potential antineoplastic agent (1). Although IdUrd has demonstrated some cytotoxic activity when tested against animal tumors (1, 2), it has also been found that exposure of cells to IdUrd (or other halogenated pyrimidines) may result in significant radiosensitization, i.e., lower radiation doses to achieve the same cytotoxicity (3-10). The exact mechanism of radiosensitization by IdUrd is not completely understood, but the degree of radiosensitization is dependent upon the extent of IdUrd incorporation into DNA (8, 9). IdUrd must be phosphorylated intracellularly to compete with dThd nucleotides for incorporation into DNA.

At our institution, IdUrd is administered by prolonged (9 to 14 days) continuous i.v. infusion in association with hyperfractionated radiation therapy to patients with high-grade gliomas, sarcomas, or other selected tumors. The pharmacology of IdUrd in plasma has been reported elsewhere (11). In order to obtain a more direct measure of drug effects, we developed a technique to measure the percentage of dThd substitution by IdUrd in DNA. To our knowledge, this is the first report of quantitative determination of substitution by a drug into DNA in vivo without the use of radiolabeled compounds.

Granulocytes were chosen for in vivo monitoring of IdUrd incorporation into normal host tissue because these cells: (a) are readily obtained from peripheral blood; (b) are target cells for IdUrd toxicity; and (c) have been exposed to IdUrd during their mitotic phase, prior to release into the circulation. Under normal circumstances, the DNA in circulating granulocytes was synthesized approximately 6 to 10 days earlier, when these cells were formed within bone marrow (12).

Pulse labeling with [3H]dThd has been used to investigate marrow kinetics in vivo (12). As a dThd analogue, IdUrd offers a nonradioactive alternative for labeling of dividing cell populations. In cell culture, BrdUrd is widely used in place of [3H]dThd (13). Continuous infusion of the drug for 9 to 14 days simplifies the interpretation of the cytokinetic results. Thus, monitoring of the appearance of circulating granulocytes which contain IdUrd is a novel approach to the study of marrow cytokinetics.

Patients and Methods

Clinical Setting. Samples were obtained from patients included in an IdUrd continuous infusion Phase I study at the Clinical Center, NIH. These patients had histologically confirmed high-grade gliomas, sarcomas, or locally advanced non-central nervous system tumors with no conventional curative treatment option. Other eligibility criteria included peripheral blood counts (WBC > 3 x 10⁹ cells/ml; platelets > 200 x 10⁹ cells/ml), normal renal function (serum creatinine < 1.2 mg/100 ml), and life expectancy of at least 2 mo.

IdUrd (NSC 39661) was supplied by the Developmental Therapeutics Program of the National Cancer Institute. IdUrd was given by i.v. infusion at a constant rate of 1000 mg/m²/day [maximum tolerable dose, 1200 mg/m²/day (11)] with a portable infusion pump for 24 h per day for up to 14 consecutive days. Radiation therapy was started within 5 to 7 days from the beginning of the IdUrd infusion. After 2 wk off therapy, the cycle was repeated once. Peripheral blood was collected in heparinized tubes at various times during and after the IdUrd infusion. Starting with 10 ml of peripheral blood, triplicate determinations were made.

DNA Isolation. Granulocytes were isolated from peripheral blood using the Ficoll-Hypaque technique (14-16). The cells were disrupted, and the nucleic acids were precipitated with TCA following the procedure described by Karle et al. (17). DNA was hydrolyzed in alkaline solution and discarded. Then, the DNA precipitate was rinsed twice with phosphate-buffered saline to remove excess TCA and incubated at 37°C for 18 h with phosphodiesterase I (type VII from Crotalus atrox venom; Sigma Chemical Company, St. Louis MO) and DNaase I (type II from bovine pancreas; Sigma Chemical Company) in potassium phosphate buffer (0.05 M, pH 7.45). After this prolonged incubation, it was verified that all nucleic acids were present as nucleosides. These hydrolyzed DNA samples were used for HPLC analysis. To monitor changes in enzymatic activity, Escherichia coli DNA was used as control for DNA digestion on each day of analysis.

HPLC Analysis of DNA Nucleosides. Samples (200 μl) of the hydrolyzed DNA (approximately 10 to 50 μg) were analyzed by HPLC (Waters Associates) equipped with a Waters Z-Module containing a C₁₆ Radial-Pak cartridge with a 5-μm particle size (Waters Associates, Inc., Milford, MA). The nucleosides were detected simultaneously at 254 nm and 280 nm with a Waters Model 440 absorbance detector and with a Schoeffel UV variable wavelength Spectroflow monitor (SF770, Schoeffel Instruments Corp.) set at 300 nm. The samples were eluted at 3 ml/min with 0.1 M acetate buffer (pH 5.4) containing 4% acetonitrile. Peak height ratios at the 3 wavelengths were used to verify the purity of the IdUrd and dThd peaks. The lower limit of quantitation is 0.2% substitution by IdUrd.

Calculations. Using external standards for IdUrd and dThd, the percentage of substitution was calculated as follows.

\[
\text{% of substitution by IdUrd} = \frac{\text{mol of IdUrd}}{\text{mol of IdUrd} + \text{mol of dThd}} \times 100
\]

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2 The abbreviations used are: IdUrd, iododeoxyuridine; dThd, thymidine; BrdUrd, bromodeoxyuridine; TCA, trichloroacetic acid; HPLC, high-performance liquid chromatography.
RESULTS

Forty-one DNA samples were isolated from peripheral granulocytes obtained from 12 patients at various times during and after IdUrd infusion. Distinct peaks were separated by HPLC for nucleosides after hydrolysis of DNA extracted from granulocytes. In DNA samples isolated from cells collected before the start of the IdUrd infusion (as in the E. coli DNA control analysis), the place where IdUrd eluted was clean of interfering peaks. In the DNA samples isolated from cells collected after administration of IdUrd, a peak with retention time and wavelength ratio corresponding to IdUrd was detected (Fig. 1).

In 3 patients, dThd substitution by IdUrd in circulating granulocytes was serially monitored during and after 12 days of continuous IdUrd infusion (Fig. 2). Substitution by IdUrd was less than 1% prior to the fifth day of infusion and then increased rapidly to achieve a maximum at the end of the infusion. Approximately 1 wk later, there was no IdUrd detectable in the DNA of granulocytes. For the 12 patients studied, the range of substitution by IdUrd at the end of the infusion was between 7 and 17% (Table 1). The coefficient of variation observed for 19 duplicate measurements was 6%. No significant difference was found in values after the second course of infusion (4 patients).

In one patient, the relationship between incorporation of IdUrd into DNA and circulating blood cell counts was investigated. For both granulocyte counts and platelet counts, there were clear temporal relationships with the percentage of substitution by IdUrd in DNA of granulocytes (Fig. 3). The nadir of the platelet counts corresponded with the time of the maximum substitution by IdUrd, and the nadir of granulocyte counts occurred approximately 6 days later. After the end of the

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<th>Patient</th>
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<tr>
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Fig. 1. Chromatogram for hydrolyzed DNA of granulocytes. Granulocytes were isolated from a patient after 14-day continuous i.v. infusion of IdUrd (1000 mg/m²/day). Detection was at 300 nm with an attenuation of 0.01 absorbance units full-scale. The relative positions of dThd and IdUrd are noted.

Fig. 2. Percentage of dThd substitution by IdUrd. Substitution was measured in DNA of granulocytes of 3 patients during and after 12-day IdUrd infusion. Each symbol (A, O, C) represents a different patient.

Fig. 3. Relationship between percentage of dThd substitution by IdUrd in DNA of granulocytes (A) and hematological toxicity. Peripheral granulocyte counts (O) and platelet counts (D) were determined from a single patient during and after 12-day IdUrd infusion.

Fig. 4. Granulocyte count versus percentage of dThd substitution by IdUrd. Granulocyte count in peripheral blood and percentage of dThd substitution by IdUrd in DNA of granulocytes were determined in 12 patients at the end of a 9- to 14-day IdUrd infusion.
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end-infusion data (c)
All Other Values (e)

Platelet Count, 10^6/ml

% Substitution by IdUrd

0 4 8 12 16 20

Fig. 5. Platelet count versus percentage of dThd substitution by IdUrd. Platelet count in peripheral blood and percentage of dThd substitution by IdUrd in DNA of granulocytes were determined in 12 patients during (•) or at the end (○) of 9- to 14-day IdUrd infusions.

infusion, the recovery phases were relatively short, especially for platelets (< 7 days).

The data obtained from all patients exhibited an inverse relationship \( r = -0.54, P < 0.05 \) between the extent of IdUrd substitution in DNA of granulocytes and the granulocyte counts in the peripheral blood at the end of the infusion (Fig. 4). A similar correlation \( r = -0.7, P < 0.001 \) has been found between IdUrd substitution in DNA of granulocytes and the number of platelets in peripheral blood at any time during IdUrd infusion (Fig. 5).

DISCUSSION

Measurements of drug concentration in plasma provide an excellent indication of extracellular exposure. However, for drugs which must be actively transported into cells and/or undergo metabolic activation within cells, plasma concentrations may not adequately reflect events at the target site. Although it would be desirable to measure more direct determinants of drug action, there are several practical difficulties: (a) inaccessibility of target tissues in human studies; (b) unavailability of analytical methodology with adequate sensitivity and specificity; and (c) inadequate knowledge of the mechanism of drug action.

To evaluate the treatment of disorders of hematological cells, the therapeutic target can be readily harvested from circulating pools. Although more invasive, aspiration or biopsy of bone marrow is acceptable in these disease types. Since myelosuppression is the dose-limiting toxicity for IdUrd and many other antitumor drugs, hematological cells are more broadly appropriate targets for detailed investigation.

Radiolabeled drugs have often been used to probe biochemical effects of drug administration, especially changes in endogenous pool sizes. However, there are limits for the total amount of radioactivity which can be administered to human subjects. Monoclonal antibody techniques have also been used to estimate the proportion of cells which have incorporated some IdUrd in their DNA, but these techniques have not been applied to a quantitative determination of dThd replacement into DNA (21). In this study, we have found that the UV absorption and chromatographic properties of IdUrd and endogenous pyrimidines provide adequate sensitivity and selectivity without the use of radioactivity.

Since antimetabolites are designed to interfere with specific biochemical pathways, this class of compounds offers a variety of approaches to more direct monitoring of drug actions in vivo. For example, Spears et al. (18) studied the inhibition of thymidylate synthetase in tissue samples obtained from patients following a dose of 5-fluorouracil. When IdUrd is used as a radiosensitizer, the biochemical target is well-defined, namely, the incorporation of drug into DNA.

IdUrd became detectable in the DNA of circulating granulocytes only after 5 to 6 days of infusion, because the DNA in these cells was synthesized approximately 6 days earlier, when they were formed within bone marrow. The maximum substitution by IdUrd was observed at the end of the 9- to 14-day infusion. At this time, most of the granulocytes found in the circulating pool were exposed to the drug during their mitotic time. In essence, substitution by IdUrd measured in peripheral granulocytes reflects what occurred 6 to 8 days earlier in the bone marrow.

The relationship found in this study between the percentage of incorporation into DNA of granulocytes and the circulating granulocyte count is an example of the value of obtaining observations as close to the target as possible. Since circulating platelets do not have a nucleus, it is not possible to examine DNA in these cells. However, since platelets and granulocytes are formed in bone marrow under the same drug exposure conditions, it would seem reasonable that the DNA content of granulocytes would serve to represent the stem cell conditions for both platelets and granulocytes.

Several reports in the literature have demonstrated that the extent of IdUrd incorporation into DNA can vary from one cell line to another. Some cell lines can tolerate a relatively high level of dThd replacement without important effects on cell survival (8, 19, 20). In our protocol, thrombocytopenia was the most frequent dose-limiting systemic toxicity (21). For our group of patients, there was a clear overall relationship between the extent of substitution by IdUrd and the hematological toxicity. The number of platelets and granulocytes in the peripheral blood increased or decreased inversely with IdUrd substitution. On the other hand, some individual patients developed significant hematological toxicity at a relatively low level of substitution by IdUrd, while others had shown only a small decrease in cell counts despite a high level of substitution.

The analytical technology developed for granulocytes can be adapted to determinations in solid tumor specimens. The primary difficulty with solid tissues is usually a lack of accessibility. Further problems arise due to the heterogeneous nature of most tumors or host tissues.

With the use of IdUrd as a radiosensitizer, some encouraging clinical results have been obtained at our institution (21). Since IdUrd incorporation into DNA has reached a limit with the current protocol, we are exploring strategies for increasing IdUrd incorporation into DNA. These strategies can include regional drug delivery, modifications in the schedule of administration, or concomitant utilization of drugs that are able to modulate the metabolism of pyrimidines. To be effective, these new strategies must increase the IdUrd incorporation into DNA in vivo with selectivity for the tumor cells. The ability to monitor IdUrd incorporation into DNA provides a facile guide to evaluate the impact of these maneuvers. The extension of these techniques to analysis of solid tumor specimens will help to determine the selectivity of therapy.

REFERENCES

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