Increased Sensitivity of Hydroxyurea-resistant Leukemic Cells to Gemcitabine

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ABSTRACT

Tumor cell resistance to certain chemotherapeutic agents may result in cross-resistance to related antineoplastic agents. To study cross-resistance among inhibitors of ribonucleotide reductase, we developed hydroxyurea-resistant (HU-R) CCRF-CEM cells. These cells were 6-fold more resistant to hydroxyurea than the parent hydroxyurea-sensitive (HU-S) CCRF-CEM cells and displayed an increase in the mRNA and protein of the R2 subunit of ribonucleotide reductase. We examined whether HU-R cells were cross-resistant to gemcitabine, a drug that blocks cell proliferation by inhibiting ribonucleotide reductase and incorporating itself into DNA. Contrary to our expectation, HU-R cells had an increased sensitivity to gemcitabine. The IC50 of gemcitabine for HU-R cells was 0.06 ± 0.03 μM versus 0.16 ± 0.02 μM for HU-S cells (P = 0.005). The cellular uptake of [3H]gemcitabine and its incorporation into DNA were increased in HU-R cells. Over an 18-h incubation with radiolabeled gemcitabine (0.25 μM), gemcitabine uptake was 286 ± 37.3 fmol/106 cells for HU-R cells and 128 ± 8.8 fmol/106 cells for HU-S cells (P = 0.03). The incorporation of gemcitabine into DNA was 75 ± 6.7 fmol/106 cells for HU-R cells versus 22 ± 0.6 fmol/106 cells for HU-S cells (P < 0.02). Our studies suggest that the increased sensitivity of HU-R cells to gemcitabine results from increased drug uptake by these cells. This, in turn, favors the incorporation of gemcitabine into DNA, resulting in enhanced cytotoxicity. The increased sensitivity of malignant cells to gemcitabine after the development of hydroxyurea resistance may be relevant to the design of chemotherapeutic trials with these drugs.

INTRODUCTION

Tumor cell resistance to chemotherapy is one of the major obstacles to the successful treatment of cancer. Hence, knowledge of cross-resistance among different antineoplastic agents is important because it provides direction for the design of clinical trials.

Because of its key role in the synthesis of deoxyribonucleotides, ribonucleotide reductase is an important target for antineoplastic drugs (1, 2). Mammalian ribonucleotide reductase is composed of two subunits termed R1 and R2 (or M1 and M2; Refs. 3–5). The R1 subunit has substrate and effector binding sites, whereas the R2 subunit contains a nonheme iron center and a tyrosyl free radical, both of which are essential for enzyme activity (3–6). Of the various ribonucleotide reductase inhibitors available, hydroxyurea has been in clinical use for over two decades, and its action on malignant cells in vitro and in vivo has been studied extensively (7). It is known that hydroxyurea inhibits ribonucleotide reductase activity by action on the R2 subunit (6, 8) and that resistance to cell growth inhibition by hydroxyurea results from overexpression of the R2 subunit (9–12).

Gemcitabine (2’,2’-difluorodeoxycytidine) is a new deoxynucleoside analogue that has shown clinical antitumor activity against a variety of malignancies (13, 14). This drug exerts its cytotoxicity by action on ribonucleotide reductase and incorporation into DNA, the latter of which results in a block in DNA replication (15).

In the present investigation, we have examined whether lymphoid leukemic cells that are resistant to hydroxyurea by virtue of overexpression of the R2 subunit of ribonucleotide reductase are cross-resistant to gemcitabine. Contrary to our expectation, we found that HU-R3 CCRF-CEM cells take up and incorporate a greater amount of gemcitabine into DNA than HU-S cells and display an increased sensitivity to growth inhibition by gemcitabine.

MATERIALS AND METHODS

Gemcitabine and [3H]gemcitabine (specific activity, 20 Ci/mmol) were obtained from Lilly Research Laboratories (Indianapolis, IN). Hydroxyurea was purchased from Calbiochem, whereas MTT was obtained from Sigma Chemical Co. (St. Louis, MO). [32P]dCTP was obtained from New England Nuclear Life Sciences (Boston, MA). The cDNA for the R2 subunit of human ribonucleotide reductase cloned into vector pCRII (Invitrogen, Carlsbad CA) was kindly provided by Dr. Yun Yen (City of Hope, Duarte, CA) and has been described previously (16). The R2 cDNA insert was excised from the plasmid using the restriction enzyme MluI.

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3 The abbreviations used are: HU-R, hydroxyurea-resistant; HU-S, hydroxyurea-sensitive; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCA, perchloric acid.
BamHI and labeled with $^{32}$P by the random primer method using the RadPrime DNA Labeling System (Life Technologies, Inc., Gaithersburg, MD). Mouse monoclonal antibody AD203 against human R1 was obtained from Accurate Chemical and Scientific Corp (Westbury, NY), whereas rabbit antiserum against human R2 was generously provided by Dr. Timothy Kinsella (Case Western Reserve University, Cleveland, OH) and has been described previously (17).

**Cells and Cell Growth Conditions.** Human T lymphoblastic leukemic CCRF-CEM cells were obtained from the American Type Culture Collection (Rockville, MD) and grown in RPMI 1640 supplemented with 10% FCS in an atmosphere of 6% CO$_2$. A subclone of these cells that was relatively resistant to growth inhibition by hydroxyurea (HU-R cells) was developed by incubation with incremental concentrations of hydroxyurea over several months. HU-R cells were routinely maintained in culture medium containing 90 μM hydroxyurea. The effects of hydroxyurea and gemcitabine on the proliferation of HU-S and HU-R CCRF-CEM cells were measured by MTT assay as described by Mosmann (18), with modifications as reported previously by us (19). In this assay, cells were plated at a density of 2 x 10$^5$ cells/ml in 96-well microwell plates in the presence of increasing concentrations of hydroxyurea or gemcitabine (shown in the figures) and analyzed for growth after 72 h of incubation.

**RNA Isolation and Northern Blotting.** Total cellular RNA was isolated from cells using RNAzol (Tel-Test, Inc., Friendswood, TX) as recommended by the manufacturer, and the integrity of the RNA was verified by agarose gel electrophoresis before use. RNA (20 μg) was electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde and transferred from the gel to a Nytran membrane (Schleicher & Schuell, Keene, NH) by capillary blotting. Equal loading of RNA on the gel was confirmed visually by ethidium bromide staining of the RNA bands. R2 mRNA was detected by hybridization of the membrane to $^{32}$P-labeled cDNA probe (1.2 x 10$^6$ cpm/ml) using QuickHyb Hybridization Solution (Stratagene, La Jolla, CA) as recommended by the manufacturer. The membrane was autoradiographed by exposing it to Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY) with intensifying screens at −70°C for 24–48 h.

**Western Blotting.** R1 and R2 protein levels in HU-S and HU-R cells were detected by Western blotting using an enhanced chemiluminescence Western blotting detection system (Amersham, Arlington Heights, IL). Cells in exponential growth phase were harvested and lysed in 200 μl of radioimmunoprecipitation assay buffer (1% Triton X-100, 1% sodium deoxycholate, 50 mM Tris, and 150 mM NaCl) containing 100 μg/ml phenylmethylsulfonyl fluoride, 75 μg/ml aprotinin, and 1 mM sodium orthovanadate. The protein content of the clarified lysate was measured by the BCA protein assay (Pierce, Rockford IL). SDS-PAGE of the samples was performed as described by Laemmli (20), and proteins were transferred from the gel onto a nitrocellulose membrane as described by Towbin et al. (21), using a Transblot system (Bio-Rad, Richmond, CA). Membranes were incubated with specific primary antibodies against R1 or R2, followed by secondary antibodies conjugated to horseradish peroxidase. For detection of protein bands, membranes were immersed in enhanced chemiluminescence detection solution and autoradiographed using Kodak XAR-5 film as recommended by the manufacturer.

**Cellular Uptake of $[^3]$H]Gemcitabine and Incorporation of $[^3]$H]Gemcitabine into DNA.** HU-S and HU-R cells were plated in fresh medium (10$^6$ cells/ml) containing 0.25 μM $[^3]$H]gemcitabine. After an 18-h incubation, cells were harvested and washed with ice-cold 0.4 N PCA and 0.2 m M hydroxyurea. HU-R cells were detected by Western blotting using an enhanced chemiluminescence Western blotting detection system (Amersham, Arlington Heights, IL). Cells in exponential growth phase were harvested and lysed in 200 μl of radioimmunoprecipitation assay buffer (1% Triton X-100, 1% sodium deoxycholate, 50 mM Tris, and 150 mM NaCl) containing 100 μg/ml phenylmethylsulfonyl fluoride, 75 μg/ml aprotinin, and 1 mM sodium orthovanadate. The protein content of the clarified lysate was measured by the BCA protein assay (Pierce, Rockford IL). SDS-PAGE of the samples was performed as described by Laemmli (20), and proteins were transferred from the gel onto a nitrocellulose membrane as described by Towbin et al. (21), using a Transblot system (Bio-Rad, Richmond, CA). Membranes were incubated with specific primary antibodies against R1 or R2, followed by secondary antibodies conjugated to horseradish peroxidase. For detection of protein bands, membranes were immersed in enhanced chemiluminescence detection solution and autoradiographed using Kodak XAR-5 film as recommended by the manufacturer.

**RESULTS**

**HU-R Cells Overproduce R2 mRNA and Protein.** The growth of HU-S and HU-R CCRF-CEM cells in the presence of hydroxyurea is shown in Fig. 1. HU-R cells were approximately 6–7-fold more resistant to growth inhibition by hydroxyurea than HU-S cells. To confirm that our HU-R cells had an increase in the expression of the R2 subunit of ribonucleotide reductase similar to that reported by others (9–12), steady-state R2 mRNA levels were measured by Northern blotting. These studies showed that R2 mRNA was indeed increased in HU-R cells.
Consistent with prior reports, the cDNA probe for R2 detected two transcripts of 3.4 and 1.6 kb (16). Western blot analysis revealed that HU-R cells had a corresponding increase in their content of R2 subunit protein but no increase in R1 subunit protein (Fig. 3).

**Effect of Gemcitabine on Cell Growth.** HU-S and HU-R cells were incubated with gemcitabine, and the effects on cell growth were measured by MTT assay. As shown in Fig. 4, HU-R cells were approximately 2.5-fold more sensitive to gemcitabine than HU-S cells. The IC₅₀ for gemcitabine was 0.16 ± 0.02 and 0.06 ± 0.03 μM for HU-S and HU-R cells, respectively (P = 0.005). The differences between all of the data points shown in Fig. 4 for HU-S and HU-R cells are highly significant (P < 0.0005).

**Gemcitabine Uptake and Incorporation into DNA.** To gain an insight into the mechanism responsible for the increased sensitivity of HU-R cells to gemcitabine, the cellular uptake of [³H]gemcitabine and its incorporation into DNA were measured after an 18-h incubation of cells with radiolabeled drug. As shown in Fig. 5A, the uptake of [³H]gemcitabine was significantly greater in HU-R cells than in HU-S cells (286 ± 37.3 fmol/10⁶ cells for HU-R cells versus 128 ± 8.8 fmol/10⁶ cells for HU-S cells (mean ± SE); P = 0.03). In addition, as shown in Fig. 5B, the incorporation of [³H]gemcitabine into DNA was approximately 3.4-fold greater in HU-R cells than in HU-S cells (75 ± 6.7 fmol/10⁶ cells for HU-R cells versus 22 ± 0.6 fmol/10⁶ cells for HU-S cells (mean ± SE); P < 0.02).

**DISCUSSION**

In the present investigation, we sought to determine whether cross-resistance exists between hydroxyurea and gemcitabine. We developed HU-R CCRF-CEM cells, and, consistent with other reports (9–12), these cells displayed an increase in the expression of mRNA and protein of the R2 subunit. However, our studies show that rather than being cross-resistant to gemcitabine, HU-R cells displayed a significant increase in sensitivity to gemcitabine. Further examination of the mechanism involved revealed that both the cellular uptake of gemcitabine and its incorporation into DNA were significantly increased in HU-R cells. It is known that in addition to inhibiting ribonucleotide reductase, gemcitabine exerts its cytotoxicity by incorporating into DNA and terminating DNA chain elongation (15). Our results strongly suggest that the increased sensitivity of HU-R cells to gemcitabine is likely to be due to the increased cellular uptake of gemcitabine, resulting in an expanded intra-
cellular uptake of \( ^{3} \text{H} \)gemcitabine was measured as described in “Materials and Methods.” Values shown represent the means ± SE (n = 3).

Fig. 5 A, cellular uptake of \( ^{3} \text{H} \)gemcitabine. HU-S and HU-R cells were incubated with 0.25 \( \mu \text{M} \) \( ^{3} \text{H} \)gemcitabine for 18 h, and the total cellular uptake of radiolabeled gemcitabine was measured as described in “Materials and Methods.” B, \( ^{3} \text{H} \)gemcitabine incorporation into DNA. Incubation conditions were similar to those described in A. Radiolabeled gemcitabine incorporation into DNA was determined as described in “Materials and Methods.” Values shown represent the means ± SE (n = 3).

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