Model for specific rescue of normal hepatocytes during methotrexate treatment of hepatic malignancy

[receptor-mediated endocytosis/(+)-amethopterin/folinic acid conjugate]

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ABSTRACT We utilized the presence of hepatic receptors for galactose-terminal glycoproteins to achieve specific rescue of differentiated hepatocytes from methotrexate toxicity. This was accomplished by administration of a conjugate formed by covalent coupling of the methotrexate antagonist, folinic acid (leucovorin), to galactose-terminating (desialylated) bovine fetuin. Two cultured hepatocyte-derived cell lines were chosen to test rescue from methotrexate toxicity: Hep G2 cells are capable of receptor-mediated endocytosis of galactose-terminating glycoproteins; PLC/PRF/5 cells are not. In the presence of methotrexate alone, both cell lines were reduced in number to 20% of control populations by day 5. Growth rates of both lines returned to normal when free folinic acid was added to the medium containing methotrexate. When asialofetuin-folinic acid conjugate was given to both cell lines in the presence of methotrexate, Hep G2 cells doubled their numbers by day 4 and reached control (without methotrexate) populations by day 8. However, PLC/PRF/5 cells failed to respond to administered asialofetuin-folinic acid conjugate under identical conditions, and the growth curve was the same as that for cells which received methotrexate alone. Conjugates of asialofetuin bound to folinic acid (folate derivative requiring dihydrofolate reductase activity for conversion to an active metabolite) and fetuin (non-galactose-terminal glycoprotein) linked to folinic acid also were made for control studies. These conjugates failed to rescue either cell line.

Methotrexate is a potent inhibitor of dihydrofolate reductase and has been shown to be effective in the treatment of various malignancies—e.g., choriocarcinoma (1), Burkitt lymphoma (2), and acute leukemias (3). Similar success has not been the case for primary hepatocellular carcinoma (4). A major limiting factor in the usefulness of methotrexate is the lack of specificity for neoplastic tissue, resulting in damage to normal as well as malignant cells. Hepatotoxicity is a common complication (5). This toxic effect can lead to irreversible liver damage as evidenced by the appearance of hepatic fibrosis (5). Our investigation was designed to study the possibility of specifically shielding normal hepatocytes from the toxicity of methotrexate. It is based on two principles. (i) Normal hepatocytes express a specific receptor as a constituent of their plasma membrane, which recognizes glycoproteins having exposed galactose residues. Such asialoglycoproteins are bound, internalized, and degraded in lysosomes (6). (ii) Administration of folinic acid enables cells to bypass the metabolic block induced by methotrexate. This agent has been used to reduce toxicity to normal cells but also rescues their malignant counterparts. Unlike normal hepatocytes, primary hepatocellular carcinoma cells have virtually no receptor activity for asialoglycoproteins (7). If folinic acid were attached to an asialoglycoprotein, transported,

internalized, and released, a selective basis for rescuing normal hepatocytes would be achieved.

MATERIALS AND METHODS

Materials. Bovine serum fetuin was obtained from GIBCO; methotrexate [(+)-amethopterin], folinic acid (N^5 -formyl-5,6,7,8-tetrahydropteroyl-L-glutamic acid), folic acid, and neuraminidase (*Clostridium perfringens*, Sigma type IV), from Sigma; 1-ethyl-3-(3-dimethylamino)propylcarbodiimide, from Pierce; Bio-Gel A-1.5m chromatography beads, from Bio-Rad; and $Na^{125}I$, from Amersham/Searle. All other reagents were of the highest quality available.

Cells and Cell Culture. Hep G2 (receptor-positive) cells were a generous gift from B. Knowles (Wistar Institute). PLC/PRF/5 (receptor-negative) cells were kindly provided by D. A. Shafritz (Albert Einstein College of Medicine). All cells were grown in plastic ware (Falcon) at 37°C under 5% CO_2 /95% air in minimal essential medium supplemented with 10% fetal calf serum (GIBCO).

The designation of receptor positive for Hep G2 is based on the recent asialoglycoprotein uptake data of Schwartz *et al.* (7), who found an uptake rate of 120 pg of asialoglycoprotein per μg of cell protein per hr. Using the same assay system, we have confirmed this figure for Hep G2 and found PLC/PRF/5 cells to take up <10 pg/ μg per hr, earning the designation receptor negative.

Amino Acid Analysis. After hydrolysis in 6 M HCl at 110°C for 20 hr under vacuum, samples were analyzed with a Beckman instrument, model 119BL, equipped with a single column 3 buffer system.

Desialylation of Fetuin. The desialylated derivative of bovine fetuin was prepared by treatment with neuraminidase (8). The asialofetuin was isolated by molecular sieve chromatography with a Bio-Gel A-1.5m column, and the protein was eluted with distilled water. The protein fraction was tested for residual sialic acid (9).

Iodination. Iodination of AsF was performed with carrier-free $Na^{125}I$ by a chloramine-T method (10). The ^{125}I -labeled protein was isolated by Bio-Gel A-1.5m chromatography, and the protein peak fractions corresponding to 45,000 daltons were collected and pooled. Protein concentration was estimated by the method of Lowry (11), and the final preparation was found to have a specific activity of 0.5 mCi/ μmol (1 Ci = 3.7×10^{10} Bq).

Coupling of Folinic Acid to Asialofetuin. ^{125}I -Labeled asialofetuin (10 mg) was dissolved in 0.01 M sodium phosphate buffer (pH 7.0). Folinic acid (185 mg) was dissolved in 3 ml of

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distilled water adjusted to pH 7.0 by the addition of dilute NaOH and then was mixed with asialofetuin. To this solution, 14 mg of 1-ethyl-3-(3-dimethylamino)propylcarbodiimide was added slowly with stirring. The pH was adjusted with dilute NaOH to between 6.5 and 7.0 over 4 hr, and the reaction was allowed to continue overnight. The reaction mixture was then dialyzed in tubing (molecular mass exclusion, 12,000 daltons) against distilled water. The retentate was lyophilized, then applied to the Bio-Gel A-1.5m column, eluted with distilled water, and analyzed for ^{125}I radioactivity. Absorption at 285 nm was determined for the pooled protein fractions, and a sample was hydrolyzed for amino acid analysis. Fetuin-folinic acid and asialofetuin-folic acid conjugates were made similarly. These preparations remained stable during storage at -70°C for at least 3 months as assessed by the analysis of protein-bound folinic acid after re-isolation of the 65,000-dalton peak. Uptake studies on ^{125}I -labeled asialofetuin-folinic acid by receptor-positive and receptor-negative cells demonstrated virtually identical uptake results compared to nonconjugated ^{125}I -labeled asialofetuin itself—i.e., conjugation of folinic acid to the asialoglycoprotein did not change the uptake characteristics of the glycoprotein.

Methotrexate Toxicity and Free Folinic Acid Rescue. PLC/PRF/5 and Hep G2 cells were seeded separately in triplicate in dishes of medium containing various concentrations of methotrexate. Cells were removed daily for 7 days by trypsinization and stained with trypan blue. Live cell populations were determined by manually counting cells capable of excluding trypan blue (12). The growth curves were compared with those for control cells receiving no methotrexate.

To examine the effect of free folinic acid on methotrexate-treated cells, we incubated both cell lines in medium alone, medium containing $0.5\ \mu\text{M}$ methotrexate, and medium containing $0.5\ \mu\text{M}$ methotrexate/ $5\ \mu\text{M}$ folinic acid.

Asialofetuin-Folinic Acid Conjugate-Induced Rescue from Methotrexate Toxicity. PLC/PRF/5 and Hep G2 cell cultures were incubated in medium alone, medium containing $0.5\ \mu\text{M}$ methotrexate and medium containing $0.5\ \mu\text{M}$ methotrexate/ $15\ \mu\text{M}$ asialofetuin-folinic acid conjugate.

Fetuin-folinic acid and asialofetuin-folinic acid conjugates containing the same molar amount of folate derivative as present in asialofetuin-folinic acid conjugate were tested in a similar manner.

To determine the effect of unconjugated asialofetuin on the ability of asialofetuin-folinic acid conjugate to mediate rescue, Hep G2 cells were incubated as before in $0.5\ \mu\text{M}$ methotrexate alone, $0.5\ \mu\text{M}$ methotrexate/ $15\ \mu\text{M}$ asialofetuin-folinic acid conjugate, and $0.5\ \mu\text{M}$ methotrexate/ $15\ \mu\text{M}$ conjugate in medium to which was added asialofetuin to compete in a 10-fold molar excess.

RESULTS

Coupling of Folinic Acid to Asialofetuin. After desialylation and iodination, the protein was purified to yield a single chromatographic peak with a molecular mass of $\approx 45,000$ daltons. When the protein was coupled with carbodiimide to folinic acid, the protein was eluted earlier on the molecular sieve column, corresponding to a mass of 66,000 daltons. From the UV absorption at 285 nm, it was estimated that 40 residues of folinic acid were bound to each protein molecule. This was confirmed by amino acid analysis of an acid hydrolyzate of the conjugate in which it was determined that 40 glutamic acid residues per molecule of protein were attributable to folinic acid.

Effect of Methotrexate and Free Folinic Acid on Cell Population Growth in Culture. Fig. 1 shows a plot of the viable cells (as a percentage of control) after 5 days of incubation as a function of methotrexate concentration. Cell counts on day 5

FIG. 1. Toxicity of increasing concentrations of methotrexate (MTX) on cell population. The viability of Hep G2 receptor-positive (●) and PLC/PRF/5 receptor-negative (○) cells was determined and expressed as the percentage of the number of control cells grown in the absence of methotrexate.

were chosen as reference points because control (non-methotrexate-treated) cell cultures reached confluence at this time. The population growth of receptor-positive and receptor-negative cells was inhibited to 20% or less by methotrexate concentrations of $0.1\ \mu\text{M}$ or greater. Concentrations less than this permitted both cell lines to approach that of controls. Thus, both cell lines showed similar sensitivity to methotrexate.

Fig. 2 shows the effect of administration of folinic acid to methotrexate-treated cells. In this plot of viable cells versus time, the population of both cell lines treated with methotrexate alone decreased to 20% by day 3. However, the growth pattern became identical to that of control cells after the administration of folinic acid, indicating that both cell lines have the potential of being rescued when free folinic acid is provided to methotrexate-treated cells.

Specific Rescue of Receptor-Positive Cells by Asialofetuin-Folinic Acid Conjugate. A plot of viable cell number versus time shows that receptor-negative control cells reached confluence by day 6 (Fig. 3A). Those cells that received methotrexate alone steadily decreased in population. When asialofetuin-folinic acid conjugate was added to methotrexate-treated cells, there was no change in the growth pattern—i.e., no rescue had occurred. However, when receptor-positive cells treated with metho-

FIG. 2. Reversal of toxicity in methotrexate (MTX)-treated cells by free folinic acid. Hep G2 cells were incubated in medium containing either $0.5\ \mu\text{M}$ MTX alone (●) or $0.5\ \mu\text{M}$ MTX/ $5\ \mu\text{M}$ folinic acid (+), and the viable cell number was determined. PLC/PRF/5 cells treated in an identical manner responded in the same fashion.

FIG. 3. Specific rescue of methotrexate (MTX)-treated cells by asialofetuin-folinic acid conjugate. (A) PLC/PRF/5 receptor-negative cells grown in the absence of MTX (control) (●), in 0.5 μ M MTX alone (+), or in 0.5 μ M MTX/15 μ M asialofetuin-folinic acid conjugate (Δ). (B) Hep G2 receptor-positive cells grown in the absence of MTX (control) (●), in 0.5 μ M MTX alone (+), or in 0.5 μ M MTX/15 μ M asialofetuin-folinic acid conjugate (Δ). Viable cell number was determined as described.

trexate were given the asialofetuin-folinic acid conjugate, the cells overcame the toxicity and increased their population, eventually reaching confluence by day 8 (Fig. 3B).

Other conjugates were tested to determine the specificity of the rescue. First, a conjugate of fetuin and folinic acid was made. In this case, because galactose residues are not exposed, receptor-mediated endocytosis by differentiated hepatocytes should not occur. A second conjugate was made in which folinic acid, a compound dependent on dihydrofolate reductase activity for conversion to folinic acid, was coupled to asialofetuin. This conjugate would be expected to be endocytosed. However, release of folinic acid will not overcome methotrexate-induced metabolic block. When tested in our cell culture system (Fig. 4) with 5 μ M methotrexate/15 μ M conjugate, neither of these conjugates had any effect on the methotrexate-treated populations of either cell line.

A 10-fold molar excess of unconjugated asialofetuin was added to compete with asialofetuin-folinic acid conjugate during rescue of Hep G2 cells. Addition of the asialofetuin resulted in an

FIG. 4. Specificity of rescue. The percentage of viable cells as a function of time is shown for Hep G2 cells grown in medium containing 0.5 μ M methotrexate (MTX) alone (●), 0.5 μ M MTX/5 μ M folinic acid (+), 0.5 μ M MTX/15 μ M asialofetuin-folic acid conjugate (○), or 15 μ M fetuin-folinic acid conjugate (Δ).

inhibition of rescue such that the cell population never exceeded 60% of the control population. This quantity of non-conjugated asialofetuin had no effect on the toxicity of methotrexate in either cell line.

DISCUSSION

Whereas other investigators have shown that toxins can be delivered to differentiated cells (13), the results presented here demonstrate *specific rescue* of differentiated cells by a biochemical agent based on the presence of receptor-mediated endocytosis. Specific delivery of cytotoxins to malignant cells has been limited by the inherent difficulty presented by the disappearance of cellular functions in the undifferentiated state, thus making specific rescue of differentiated cells a reasonable alternate approach.

We have chosen to use the Hep G2 cell line as a model of normal differentiated hepatocytes because primary cultures of hepatocytes cannot at present be maintained in a rapidly proliferating state. The fact that these cells can be rescued from methotrexate toxicity by administration of folinic acid conjugate makes it highly likely that normal hepatocytes will respond in a similar fashion because Hep G2 cells, although derived from a well-differentiated tumor (14), possess at most 30% of the receptor activity of normal hepatocytes as determined by uptake studies of primary hepatocyte cultures (7).

Besides the liver, several other tissues are highly subject to methotrexate toxicity—e.g., intestinal mucosa and bone marrow (5). There is a good deal of evidence that a variety of receptor-mediated endocytoses exist that are based on recognition of different non-galactose-bearing glycoproteins. Such activity has been found in both intestinal mucosa (15) and bone marrow (16). Therefore, it may be possible to develop other folinic acid conjugates in order to specifically rescue differentiated cells in nonhepatic tissues that are also highly sensitive to methotrexate and as such limit the drug's usefulness in chemotherapy.

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