Intratumoral Conversion of 5-Fluorocytosine to 5-Fluorouracil by Monoclonal Antibody-Cytosine Deaminase Conjugates: Noninvasive Detection of Prodrug Activation by Magnetic Resonance Spectroscopy and Spectroscopic Imaging

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Abstract

The monitoring of antibody-directed enzyme-prodrug therapies requires evaluation of drug activation within the tissues of interest. We have demonstrated the feasibility of noninvasive magnetic resonance spectroscopy and spectroscopic imaging (chemical shift imaging) to detect activation of the prodrug 5-fluorocytosine (5-FCyt) to the cytotoxic species 5-fluorouracil (5-FU) by monoclonal antibody-cytosine deaminase (CD) conjugates. In vitro, L6-CD but not 1F5-CD selectively metabolized 5-FU to 5-FU on H2981 human lung adenocarcinoma cells because of the presence and absence of cell surface L6 and CD20 antigens, respectively. After pretreatment of H2981 tumor-bearing mice with L6-CD, in vivo metabolic activation of 5-FCyt to 5-FU within the tumors was detected by 19F magnetic resonance spectroscopy; the chemical shift separation between 5-FCyt and 5-FU resonances was ~ 1.2 ppm. 5-FU levels were 50–100% of the level of conjugates. In vitro, L6-CD but not 1F5-CD selectively metabolized 5-fluorouracil (5-FU) by monoclonal antibody-cytosine deaminase (CD) conjugates. In vivo, tumor-bearing mice demonstrated the highest signal intensity of 5-FU within the tumor region. This study supports further development of noninvasive magnetic resonance methods for preclinical and clinical monitoring of CD enzyme-prodrug therapies.

Introduction

Present therapeutic modalities for treating cancer are frequently ineffective because of the lack of specificity of the agents for cancer cells. The ensuing toxicity to normal tissues can severely limit the dose of drug administered. A promising new approach for increasing therapeutic index of anticancer therapy, ADEPT,3 is based on the delivery to cancer cells of a nontoxic antibody-enzyme conjugate. After a suitable time to allow for clearance of the unbound conjugate, a nontoxic prodrug is administered that is selectively converted to a toxic compound. This paradigm is exemplified by systemic administration of the monoclonal antibody-CD conjugate, L6-CD, which renders tumor cells susceptible to the nontoxic prodrug 5-FCyt (1, 2). The L6-CD contains a yeast enzyme, CD, which converts 5-FCyt to a potent cytotoxic agent, 5-FU. The specificity of this therapeutic approach to cancer cells is based on selective binding of the L6 monoclonal antibody portion of the conjugate to a cell surface antigen strongly expressed on most human carcinomas (3, 4). A similar approach that involves delivery of a gene that codes for bacterial CD has also been reported by other investigators (5–9).

Unlike traditional chemotherapeutic approaches for cancer that can be monitored by analysis of the drug and metabolite levels in blood, clinical monitoring of enzyme-prodrug therapies requires evaluation of drug activation within the tissue of interest, e.g., tumor. A clinically applicable noninvasive method for monitoring drug activation in target tissue will allow evaluation of drug activation profiles and selectivity for tumors versus normal tissues. Such methods may be more acceptable to patients than multiple biopsies, leading to increased compliance to the therapeutic protocols. In this study, we have demonstrated the feasibility of noninvasive MRS and spectroscopic imaging, CSI, for monitoring L6-CD/5-FCyt therapy in vitro and in mice bearing human lung cancer (H2981) xenografts. To our knowledge this constitutes the first report of its kind and is also applicable to the alternative approach involving delivery of a “suicide” gene that codes for CD (5–9). MRS of natural abundant fluorine, 19F MRS, has been used successfully to monitor the pharmacokinetics and metabolism of 5-FU in both experimental animals and patients (10–14). 19F MRS offers several advantages such as a spin of 1/2, 100% natural abundance, high detection sensitivity (0.83% that of protons), and low background signal. The technique is, therefore, suitable for monitoring in vivo levels of fluorinated compounds and their metabolites, particularly when such compounds are administered at high doses (>100 mg/kg body weight) as is the case for 5-FCyt (1, 5). In this study, we have shown that 19F MRS and CSI can be used to monitor signal intensities of 5-FCyt and 5-FU in tumors as well as their whole body biodistribution in mice. Issues relating to the use of these techniques to evaluate patient tumors are also discussed.

Materials and Methods

Materials. Antibody conjugates L6-CD and 1F5-CD and 13B anti-idiotypic antibody were obtained from Bristol-Myers Squibb (Seattle, WA). L6 (IgG2a) binds to a ganglioside antigen expressed on the surface of human carcinomas (4); 1F5 (IgG2a) binds to the CD20 antigen expressed on both normal and neoplastic B cells but not on carcinomas (2, 15); 13B anti-idiotypic antibody recognizes and binds to the L6 portion of circulating L6-CD but not on carcinomas (4); 1F5 (IgG2a) binds to the CD20 antigen expressed on both normal and neoplastic B cells but not on carcinomas (2, 15); 13B anti-idiotypic antibody recognizes and binds to the L6 portion of circulating L6-CD but not on bound L6-CD and enhances the clearance of unbound conjugate (1, 2). The L6-CD contains a yeast enzyme, CD, which converts 5-FCyt to a potent cytotoxic agent, 5-FU. The specificity of this therapeutic approach to cancer cells is based on selective binding of the L6 monoclonal antibody portion of the conjugate to a cell surface antigen strongly expressed on most human carcinomas (3, 4). A similar approach that involves delivery of a gene that codes for bacterial CD has also been reported by other investigators (5–9).

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In Vitro Studies. H2981 cells were cultured in Iscove’s modified Dulbecco’s medium (Sigma) supplemented with 10% fetal bovine serum, 200 units/ml penicillin, and 0.1 mg/ml streptomycin. To demonstrate the ability of MRS to detect selective drug activation in vitro, subconfluent H2981 cells (~ 2 × 10^7 cells; 4 repeats) were incubated for 30 min with growth medium alone or with medium containing 10 μg/ml L6-CD or 1F5-CD. Cells were then washed three times with PBS and incubated for 1 h with medium containing 5-FCyt. Cells
MR DETECTION OF 5-FCYT ACTIVATION

**Fig. 1.** Metabolism of 5-FCyt by H2981 cells pretreated with either L6-CD (a and b) or 1F5-CD (c and d). The vertical scale is proportional to the signal intensity, which was normalized to display comparable signal-to-noise levels for cell extracts (a and c) or media (b and d). Cells were incubated with conjugate for 30 min followed by three cycles of washing and, finally, were incubated with 5-FCyt for 1 h. Extraction and analysis of samples were performed as reported in "Materials and Methods."

**Fig. 2.** Typical in vivo 19F spectrum obtained from a H2981 tumor 60 min after 5-FCyt injection (400 mg/kg i.p.). Mice were pretreated with L6-CD and 13B anti-idiotypic antibody at 48 and 24 h, respectively, before the MRS study. Data were processed with a line broadening of 25 Hz.

Incubated with medium alone were treated with either 5-FCyt or 5-FU. Media containing 5-FCyt and 5-FU were collected after incubation and frozen in liquid nitrogen. Cells were washed three times, trypsinized, and frozen. The cells were homogenized, centrifuged to remove cell debris (15,000 rpm; 4°C; 15 min), and lyophilized. Both incubation media (0.5 ml) and lyophilized cell extracts dissolved in 0.5 ml of distilled water were analyzed for fluorine-related compounds on a Bruker 500-MHz NMR spectrometer. Spectra were acquired using a one-pulse sequence with a flip angle of 45°, 5 s repetition time, 16,384-block size, 22,000-Hz sweep width, and 16/64 scans.

**Tumors.** H2981 tumor sections of approximately 32 mm³ were implanted s.c. in the right flank of female BALB/cAnNCr-nu nude mice (National Cancer Institute, Frederick, MD). Mice were given laboratory chow and water ad libitum. Tumor sizes of 500-600 mm³ were used for the MR experiments. The experimental protocol was approved by the Institutional Animal Care and Use Committee. Mice were anesthetized by i.p. injection of a mixture of ketamine (25 mg/kg; Aveco Ltd., Fort Dodge, IA), acepromazine (2.5 mg/kg; Aveco), and 0.9% NaCl solution (1:1:2 by volume).

**In Vivo Studies.** In vivo experiments were performed to demonstrate: (a) activation of 5-FCyt within tumors by MRS; and (b) whole body distribution of 5-FCyt and 5-FU by CSI. H2981 tumor-bearing mice were treated i.v. with L6-CD (300 μg/25 g mouse) 48 h before the experiment. To eliminate unbound circulating conjugate, 13B anti-idiotypic antibody (200 μg/25 g mouse) was injected i.p. in the same mice 24 h before the experiment. Control animals did not receive antibody treatment. MRS experiments were performed on GE CSI 4.7T and 9.4T NMR spectrometers with a two-turn solenoidal coil tunable to 1H or 19F frequency. Mice were anesthetized and a catheter to deliver 5-FCyt was placed i.p. before commencing the MRS studies. Body temperature was maintained within the magnet by a thermostat-regulated heating pad. For MRS studies, 5-FCyt was injected as a single bolus dose of 400 mg/kg after acquisition of baseline spectra. To evaluate drug activation in tumors, serial 19F spectra were acquired every 5 min for 1 h using a one-pulse sequence (60° flip angle, 3-s repetition time, 1,024-block size, 10,000-Hz sweep width and 32/64 scans).

**Fig. 3.** Profile of 5-FCyt and 5-FU in H2981 tumors following treatment with 400 mg/kg 5-FCyt (i.p.). a, control mice not receiving conjugate (n = 3), in which the conversion of 5-FCyt (O) to 5-FU was not detected by MRS. b, L6-CD and 13B anti-idiotypic antibody-pretreated mice (n = 5), in which the conversion of 5-FCyt (●) to 5-FU (●) was detected by MRS. Mice were pretreated with L6-CD and 13B anti-idiotypic antibody at 48 and 24 h, respectively, before 5-FCyt injection. Data are means ± SE (error bars).
Both 5-FCyt and 5-FU resonances were detected noninvasively in tumors of mice treated with L6-CD/13B/5-FCyt with adequate spectral resolution (Fig. 2). Only the 5-FCyt peak was detected in control mice. For each mouse, resonance intensities of 5-FCyt or 5-FU were normalized to the highest 5-FCyt intensity. The mean intensities are illustrated in Fig. 3, a and b. In control mice, maximum tumor levels of 5-FCyt occurred early at 10 min after 5-FCyt injection and decreased to 50% of maximum levels at 60 min (n = 3). In L6-CD/13B-treated mice, maximum 5-FCyt tumor levels occurred at 25 min and were 60% of maximum levels at 60 min (n = 5). Maximum 5-FU tumor levels occurred early at 10 min, and the 5-FU/5-FCyt ratio (an index of CD activity) ranged between 0.5 and 1.0 within the study time (60 min). On the basis of previous studies using the L6-CD/13B/5-FCyt approach (1), these results imply that tumor concentrations of 5-FU will be 0.5-1.0 molar equivalent of intratumoral 5-FCyt when 5-FCyt rapidly to 5-FU. Levels of 5-FU in cells and media were 0.3 ± 0.03 µmol/2 × 10^7 cells and 1.62 ± 0.03 mM, respectively. 5-FCyt was only observed in the medium at this time point (0.4 ± 0.05 mM). In contrast, conversion of 5-FCyt to 5-FU did not occur in cells treated with 15F-CD. Levels of 5-FCyt in cells and media after 1-h incubation with 3 mM 5-FCyt were 0.42 ± 0.02 µmol/2 × 10^7 cells and 2.50 ± 0.08 mM, respectively. When incubated directly with 5-FCyt, 15F-CD was equally capable of activating 5-FCyt to 5-FU (data not shown). This means that 15F-CD did not bind to antigens on H2981 cells and, therefore, eliminated during the washing cycles. In control experiments in which cells were not incubated with conjugate, the levels of 5-FCyt in cells and medium after incubation with 3 mM 5-FCyt for 1 h were 0.43 ± 0.02 µmol/2 × 10^7 cells and 2.95 ± 0.42 mM, respectively. Corresponding control experiments with 3 mM 5-FU gave values of 0.36 ± 0.02 µmol/2 × 10^7 cells and 1.96 ± 0.16 mM for cells and media, respectively. Of interest was the finding that no other signals from 5-FU catabolites or anabolites were observed in these experiments within the experimental time used.

**Discussion**

5-FU is frequently used in combination therapy for the treatment of adenocarcinomas of the colon, breast, and lung. Both antibody-CD conjugates and genes coding for CD have been previously used to generate 5-FU from 5-FCyt (1, 2, 5-9). These novel approaches are capable of generating high and sustained levels of 5-FU selectively.
within mouse tumors and are, therefore, associated with dramatic cytotoxicity, tumor regression, and even cures (2, 5). A critical issue that may influence the clinical applicability of these approaches is the ability to detect drug activation and target tissue selectivity. To facilitate this, we have applied MR methods to detect selective prodrug activation in vitro as well as to define the magnitude and location of CD enzyme activity in mouse tissues after systemic administration of an antibody-enzyme conjugate.

In this study, we showed that MRS and CSI methods are capable of detecting conversion of 5-FCyt to 5-FU in vitro and in vivo. 5-FCyt and 5-FU resonances were adequately resolved (~1.2 ppm). This implies that one can monitor the levels of these compounds directly without resorting to methods that measure total signal after washout of the original compound. This is particularly important since the metabolism of 5-FCyt by CD was rapid, and both prodrug and metabolite were rapidly metabolized in H2981 cells, but despite a reduction in 5-FU levels of >30% after 1-h incubation of cells with 5-FU, no metabolites were detected within 20 ppm up- or down-field of the 5-FU resonance. This may be due to the low levels of these multiple putative 5-FU metabolites.

In vivo studies were performed by pretreating tumor-bearing mice with L6-CD followed by 13B anti-idiotypic antibody 24 h after having received L6-CD. 13B binds to and clears circulating L6-CD but not the conjugate that is tumor-associated. This has been shown previously (1) to lead to high tumor:blood conjugate ratios (42:1) and has enabled large doses of 5-FCyt to be subsequently administered. In H2981 tumors pretreated with L6-CD/13B, metabolic activation of 5-FCyt to 5-FU was detected and monitored for 60 min. Metabolic activation was rapid and 5-FU levels were between 50 and 100% of 5-FCyt tumor levels. No activation was observed in control mice. CSI experiments demonstrated that 5-FCyt localized mainly in the abdominal and lower thoracic regions of the mice after i.p. injection. The highest 5-FU levels were observed in the tumor region, supporting the selectivity of this ADEPT approach for tumors. However, other regions showed signal from 5-FU. This may be caused by the diffusion of 5-FU out of the tumor region and/or partial volume averaging, although we cannot completely rule out contribution from nonspecific activation. The absence of the hepatic catabolite of 5-FU, fluoro-ß-alanine, in in vivo spectra suggests that there were low levels of circulating 5-FU in the blood. The absence of anabolites of 5-FU, however, may be caused by the short observation time of 1 h and/or by low levels of such metabolites. The results obtained in these experiments are in general agreement with those obtained by removing the tumors from the animals and analyzing the drug concentrations by high-performance liquid chromatography (1).

These MR methods for detecting conversion of 5-FCyt to 5-FU will aid in the clinical testing of CD-based prodrug therapies. Clinical applicability at the present widely available magnetic field strength of 1.5 tesla may be hampered by low spectral resolution. This problem will be solved by employing high-field magnets such as 3, 4, and 9 tesla presently available in some institutions. MR methods are inherently insensitive. However, at the 5-FCyt dose (400 mg/kg) and the magnetic field strength (4.7 tesla) used, the compounds are easily detected. Lower doses of 5-FCyt (100 mg/kg) can be detected at this field strength, and sensitivity/spatial resolution can be further enhanced by increasing the number of scans or imaging time. In conclusion, we have demonstrated feasibility for using MRS and CSI methods to noninvasively detect the activation of 5-FCyt to 5-FU by ADEPT. These MR methods can be applied to the preclinical and early clinical testing of CD/5-FU therapy. In addition MRS and CSI may have applications in a variety of other enzyme-prodrug combinations of clinical interest.

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References

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