Extracellular Expression of Cytosine Deaminase Results in Increased 5-FU Production for Enhanced Enzyme/Prodrug Therapy

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Abstract. Background: The cytosine deaminase/5-fluorocytosine (CD/5-FC), strategy for cancer gene therapy shows considerable promise in experimental models but, because CD is a cytosolic enzyme, intracellular production of 5-fluorouracil (5-FU) causes the demise of the transduced cells before cytotoxic concentrations of 5-FU can be achieved within the extracellular milieu. Materials and Methods: A soluble secreted form of CD was constructed and evaluated compared to intracellular CD in vitro and in vivo. Results: The secreted form of CD temporarily spared transduced cells and enhanced accumulation of extracellular 5-FU. Cytosolic CD produced rapid inhibition of thymidylate synthase and cell death before significant extracellular concentrations of 5-FU developed. Finally, tumors expressing the secreted form of CD had an improved response to 5-FC treatment compared to tumors expressing intracellular CD. Conclusion: Further evaluation of extracellular expression of CD for enzyme/prodrug therapy may provide improvements in this commonly studied gene therapy strategy.

The low efficiency of gene delivery to target tumor tissues is a major impediment to the clinical use of cancer gene therapy. Current viral or non-viral delivery systems can transduce only a small percentage of cells within a tumor. Cytosine deaminase, an enzyme not present in mammalian cells, physiologically deaminates cytosine to uracil; however, it also has the capacity to deaminate the antifungal agent 5-fluorocytosine (5-FC) to the potent cytotoxic agent 5-fluorouracil (5-FU). The CD/5-FC enzyme/pro-drug strategy delivers the cytosine deaminase gene to tumor tissue thus resulting in tumor-specific generation of 5-FU. This approach has shown promise in preclinical studies (1, 2) with a major advantage of this strategy being that the 5-FU produced by a CD-expressing cell may cause toxicity to surrounding non-transduced cells resulting in so-called bystander killing, independent of gap junctional intercellular communication (1, 3, 4). In addition to direct cytotoxicity, 5-FU is also a potent radiosensitizer even at concentrations below that required for cytotoxicity (5).

A potential disadvantage of the CD/5-FC approach is the cytosolic location of CD and the resultant high intracellular concentrations of 5-FU attained in CD-expressing cells. This can lead to premature killing of CD-expressing cells (the "factory" for gene therapy) resulting in a smaller bystander effect as cytotoxic concentrations of extracellular 5-FU may not be achieved. Indeed, Mullen et al. (6) took advantage of this concept of premature killing of CD-transduced cells to demonstrate that the CD/5-FC strategy could be used as a negative selection system to isolate CD-nonexpressing cells. We have also observed that CD-transduced cells are preferentially killed and this death occurs before cytotoxic concentrations of extracellular 5-FU can be achieved (4). In an effort to overcome this limitation of the CD/5-FC strategy, we developed a soluble secreted form of E. coli CD (CD2) that results in extracellular production of 5-FU. Extracellular production of 5-FU spares CD-transduced cells from high intracellular levels of 5-FU and allows for more exposure to non-transduced cells. We demonstrate that addition of 5-FC to cells expressing the intracellular form of E. coli CD (CD1) results in a rapid inhibition of thymidylate synthase (TS), coincident with early cell death. In contrast, CD2-expressing cells show a more gradual inhibition of TS and are spared from immediate 5-FU toxicity. CD2-expressing cells, therefore, live longer to produce higher extracellular concentrations of 5-FU.

Materials and Methods

Construction of CD1 and CD2 expression plasmids. Isolation of the E.coli cytosine deaminase gene (CD1) has been described previously (4). The CD1 gene was subcloned into a mammalian expression plasmid (pZ, kindly provided by Genetics Institute, MA,
which encodes the CD gene and the neomycin-resistance gene from a single mRNA due to the presence of an encephelomyocarditis virus internal ribosome entry site (IRES). To direct the translated CD protein into the secretory pathway we used the pre-pro segment from human tissue-plasminogen activator (7, 8). Briefly, the pre-pro- segment of tPA was amplified using oligonucleotides tPA 5-prime (CCTCTAGAGTCGACG AAGCAATCAT GGATGCA) and tPA 3-prime (GTTATTCGACATTCTTCTGAATCGGGC). The tPA 5-prime primer contains XbaI and SalI restriction sites, and a consensus Kozak followed by the first four residues of human tPA. The tPA 3-prime primer is homologous to the first 12 bases of E. coli CD at its 5-prime end and homologous to tPA at its 3-prime end. The CD1 coding sequence was also amplified using the oligonucleotides CD2 5-prime (CATGCCCGATTCAGAATGTCGAA TAACGCTTTACAA) and CD2 3-prime (GCTCTAGAATTCCAGTCGTTCAACGTTT). The two resulting fragments were subsequently purified and joined in a PCR reaction which was subcloned into the expression vector pZ.

**Western blot analysis.** Expression of cytosine deaminase in transfected cells was determined by resolving the samples by SDS-PAGE transferring to nitrocellulose membrane using a semi-dry blottedtter (Bio-Rad, CA, USA) and then probing the membrane with a CD specific antisera (kindly provided by Brian Huber, Glaxo-Wellcome) to detect the presence of CD using enhanced chemiluminescence as recommended by the manufacturer (Amersham, CA, USA).

**Figure 1.** CD2 but not CD1 is secreted from WB cells. Western blot analysis of cell extracts and conditioned media prepared from WB cells expressing CD1 (WB-CD1) and CD2 (WB-CD2). Log phase cells were grown in the presence of serum-free media for 24 h after which the conditioned media (CM) and cell extracts (CE) were analyzed by Western blot analysis as described in Materials and Methods.

**Figure 2.** Extracellular expression of CD results in temporary sparing from 5-FC toxicity. Log phase WB-CD1 and WB-CD2 cells were treated with 5-FC (0.3 mM) for 10 days in identical dishes. One plate of each cell line was harvested each day to determine the cell number and conditioned media was stored for 5-FU determination (Figure 3). ■ WB-CD1 untreated controls, □ WB-CD1 5-FC treated, △ WB-CD2 untreated controls and ◆ WB-CD2 5-FC treated. The results presented here represent the means of three experiments with s.e.m.

**Figure 3.** Extracellular expression of CD results in accumulation of extracellular 5-FU. Log phase WB-CD1 (■) and WB-CD2 (●) cells were treated with 5-FC as described in Figure 2. Conditioned media were collected daily and analyzed for the presence of 5-FU using gas chromatography/mass spectroscopy as described in Materials and Methods. The results presented here represent the means of three experiments with s.e.m.

**Cell lines and cell culture.** Rat liver hepatoma cells (WB) and rat colon carcinoma cells (K12) were cultured in DMEM containing 10% calf serum, penicillin (50 units/ml), streptomycin (50 µg/ml) at 37°C in 5% CO₂. WB-CD1 and WB-CD2 as well as K12-CD1 and K12-CD2 cells were constructed by transfecting the cytosine deaminase expression construct (pZ-CD1 or pZ-CD2) using Lipofectamine (Life Technologies, MD, USA). Clones that stably integrated the appropriate CD expression vector were obtained...
upon G418 selection (600 Ìg/ml). Using Western blot analysis, CD1 and CD2 clones showing the greatest expression were identified and utilized for further experiments.

Measurement of 5-FU concentrations. Conversion from 5-FC to 5-FU was measured using a gas chromatographic, mass spectrometric assay as described previously (9). Briefly, conditioned medium was collected, derivatized and quantification of the derivatized products was performed using a Hewlett-Packard 5987A gas chromatograph/mass spectrograph in selected ion-monitoring mode.

Thymidylate synthase activity measurement. TS activity in cell extracts was determined using the method of Maley et al. (10) by measuring the conversion of $^3$H dUMP to dTMP with the release of $^3$H$_2$O. Cells were washed with cold PBS, scraped and sonicated. An aliquot of the extract was then incubated at 37°C for 30 min with $^3$H dUMP in the presence of 50 mM Tris, 5 mM MgCl$_2$, 50 mM NaF, 10μM dUMP and 1 mM reduced folate. The reaction mixture was then treated with activated charcoal suspended in 4% perchloric acid and centrifuged. The supernatant, containing $^3$H$_2$O produced by TS, was processed for liquid scintillation counting.

Animal model. Nude female mice (Nu/Nu CD-1, Charles River Laboratories, Wilmington, MA, USA) of 7-8 weeks were injected subcutaneously in the flank with 5 x 10$^6$ viable K12 rat colon carcinoma cells transduced with either CD1 or CD2. Non-transduced K12 cells served as control cells. Tumors were measured biweekly with calipers in 2 dimensions. Tumor volumes were calculated in mm$^3$ using the formula: $3.14/6 (L \times W^2)$. When tumors measured an average volume of 100-150 mm$^3$ treatment was started. Mice were injected daily i.p. with 500 mg/kg 5-FC for 3 weeks. Non-transduced animals were handled according to the established procedures of the University of Michigan Laboratory Animal Maintenance Manual.

Results

Construction of CD2-expressing WB and K12 cells. Soluble secreted cytosine deaminase (CD2) was constructed by the introduction of the tPA pre-pro sequence five prime to the initiator ATG of E. coli cytosine deaminase. The CD2 gene
was cloned into a plasmid allowing concurrent expression of the CD2 gene, an IRES signal and the neomycin resistance gene from a single bicistronic mRNA. The expression plasmid was confirmed by direct sequencing and transient expression in COS-1 cells followed by Western blotting of cell extracts and conditioned media (data not shown). The plasmids were then used to construct stable cell lines in WB, rat hepatoma cells, or K12, rat colon carcinoma cells. The highest expressing CD1 and CD2 clones were identified by Western blot analysis and utilized for all subsequent experiments (data not shown).

Western blot analysis of conditioned media and cell extracts from WB-CD1 and WB-CD2 cells was performed using a CD-specific antiserum (Figure 1) which demonstrated that inclusion of the tPA pre-pro sequence results in targeting of CD2 to the extracellular space. As previously reported CD1 was only expressed as an intracellular enzyme. The intracellular form of CD2 migrated with a higher apparent molecular weight on SDS-PAGE compared to CD1, as expected for intracellular CD2 should contain the 32 amino acid tPA pre-pro segment. In contrast, the extracellular form of CD2 was similar in size to intracellular CD1 due to the removal of the tPA pre-pro segment by endogenous signal propeptidases prior to secretion. Secretion of CD2 was efficient as witnessed by the significant accumulation of the CD2 peptide in the media. In addition, processing of the tPA pre-pro segment was also efficient since there was no evidence for the larger molecular weight peptide evident in the conditioned media.

Extracellular expression of CD results in temporary sparing of the "factory". To test whether extracellular expression of CD protects transduced cells from premature killing we cultured WB-CD1 and WB-CD2 cells in the presence and absence of 5-FC and monitored cell growth over a 10-day period. As shown in Figure 2, WB-CD1 and WB-CD2 cells had a similar growth rate in the absence of 5-FC. In contrast, when the cultures were treated with 1 mM 5-FC, WB-CD1 cells growth arrested immediately while WB-CD2 cells continued to propagate to a similar extent as untreated cells for approximately 6 days after which a growth arrest and eventual cell death were observed. To examine if continued growth of CD2-expressing cells for the first 6 days was due to a failure to produce 5-FU, the amount of 5-FU in the media was determined. As shown in Figure 3, the concentration of 5-FU in WB-CD2 cultures was significantly higher than in WB-CD1 cultures after as little as two days of 5-FC treatment and continued to accumulate with a peak of almost 5 μM 5-FU observed on day 9. The 5-FU concentration in WB-CD1 cultures did not increase significantly for the entire period of the study. To demonstrate that the above observations were not unique to WB cells, we constructed CD1- and CD2-expressing K12 cells (a rat colon carcinoma derived line). In the case of K12-CD1 cells, no cell growth was observed when cells were treated with 1 mM 5-FC, whereas K12-CD2 cells grew like untreated cells for 4 days, whereafter growth arrest was observed (data not shown).

Our hypothesis that intracellular expression and activity of CD1 results in preferential killing of transduced cells is supported by the inability of WB-CD1 and K12-CD1 cells to generate extracellular 5-FU and the ability of both WB-CD2 and K12-CD2 cells to temporarily proliferate in the presence of 5-FC and to accumulate extracellular 5-FU. To confirm that the rapid death of WB-CD1 cells was due to intracellular production of 5-FU, we examined intracellular levels of thymidylate synthase (TS) activity, since TS inhibition is a primary mechanism of 5-FU toxicity. In WB-CD2 cells TS activity remained essentially unchanged for at least 6 hours after treatment with 5-FC and decreased to about 40% of the level in untreated cells for at least the next 16 hours. In contrast, treatment of WB-CD1 cells resulted in a drop in TS activity levels to 40% of control as early as 30 minutes after the addition of 5-FC and decreased to approximately 10% of control within 8 hours (Figure 4). This is consistent with the hypothesis that premature killing of CD1-expressing cells is due to the production of high intracellular concentrations of 5-FU.

Efficacy of CD1 and CD2 in a co-culture system. To further demonstrate the ability of CD2-expressing cells to generate higher concentrations of extracellular 5-FU without killing the "factory" cell we designed a co-culture experiment. WB-CD1 or WB-CD2 cells (which are both G418-resistant) were cultured in the presence of parental WB (G418 sensitive) cells for 1-4 days in the presence of 1mM 5-FC. CD-expressing cells were plated as 5% of the population. The surviving fraction of WB-CD1 or WB-CD2 cells was determined by a colony formation assay using media containing G418. The concentration of 5-FU in the medium during the co-culture was also determined. These results were used to determine the surviving fraction of WB-CD1 or WB-CD2 cells as a function of extracellular 5-FU generated. As shown in Figure 5, for a given concentration of 5FC WB-CD2 cells resulted in a significant increase in the amount of 5-FU released into the media, but despite the increased concentration of 5FU a much greater fraction (almost 10-fold) of WB-CD2 cells survived compared to WB-CD1 cells. Because the two cell lines are equally sensitive to 5-FU (data not shown), the increased cytotoxicity observed in WB-CD1 cultures must be due to rapid production of intracellular 5-FU thus limiting the ability to produce significant 5FU to be cytotoxic to adjacent cells.

Efficacy of CD1 and CD2 in tumor xenografts. To test if expression of CD2 resulted in an improved therapeutic
response in vivo, we utilized CD1- and CD2-expressing K12 rat colon carcinoma cells. The stable cell lines were implanted into nude mice and the rate of tumor growth was monitored. K12-CD1 and K12-CD2 tumors had similar rates of growth when implanted into the flanks of nude mice. In response to 5-FC treatment (500 mg/kg/day), K12 tumors had no cytotoxicity and maintained a doubling time of 8.5 days. CD1-expressing tumors had a trend toward an increase in tumor doubling time of 35% (8.5 days vs. 11.5 days). In contrast, CD2-expressing tumors had a tumor doubling time that was 110% that of K12 tumors (8.5 days vs. 18 days). This was a 70% increase in tumor doubling time when compared to CD1-expressing tumors (11.5 days vs. 18 days). When treatment was stopped, the growth rate of K12-CD1 and K12-CD2 tumors quickly resumed a rate which was comparable to that of K12 tumors.

**Discussion**

Enzyme/prodrug strategies for the treatment of cancer provide a developing alternative to traditional chemotherapy. A major advantage of these strategies is the high intratumoral and low systemic levels of chemotherapy generated by enzymatic conversion of the prodrug. A number of enzyme/prodrug strategies have been described, each with its advantages and disadvantages. The HSV-TK/Ganciclovir strategy has the advantage of producing a highly toxic drug, ganciclovir triphosphate, but a significant disadvantage to this strategy is that it produces only modest bystander killing in tumor cells that lack gap junction mediated intercellular communication (3, 11). Because the cytosine deaminase/5-fluorocytosine strategy produces a membrane diffusable drug (5-fluorouracil) (1), it has a bystander effect that is independent of gap junction communication (4).

Although 5-FU is membrane permeable, substantial evidence suggests that the bystander effect produced by cells expressing intracellular CD is not yet optimized. The use of CD expression as an effective negative selection system suggests that 5-FU concentrations are higher in the CD1- and CD2-expressing cells than in surrounding cells (6). Our results are consistent with this observation. We found that TS activity was inhibited more rapidly and more profoundly after 5-FC treatment in CD1(non-secreted)- compared to CD2 (secreted)-expressing cells, consistent with greater intracellular accumulation of 5-FU in CD1 cells. Furthermore, 5-FC treatment of CD1-expressing cultures generated little or no 5-FU while in CD2-expressing cultures 5-FU could be detected on day 3 and continued to accumulate to a peak value of 3.5 μM on day nine. Interestingly, on day 6 when the extracellular 5-FU concentration reached 1 μM, CD2-expressing cells stopped proliferating. Previous studies with WB cells had suggested that 1 μM 5-FU was the minimal dose at which cytotoxicity was observed (data not shown). Therefore, the "factory" in this case was truly protected and able to continue to generate CD2 until fully cytotoxic concentrations of 5-FU were developed in the media. In contrast, CD1-expressing cells produced little extra cellular 5-FU before their growth was arrested due to intracellular 5-FU accumulation and TS inhibition.

Our results show a modest growth delay for K12-CD1 tumors compared to K12 tumors in 5-FC-treated mice. Tumors composed of K12-CD2 revealed a trend toward enhanced growth inhibition compared to K12-CD1 tumors, although this did not achieve statistical significance. There are a number of factors which may have contributed to this outcome. While early reports suggested a significant response of CD1-expressing tumors to 5-FC (1,12), the modest growth delay of K12-CD1 tumors described here is consistent with our previous data (13, 14) and others (15) where there was often little growth inhibition with CD1, and the witnessed growth inhibition only persisted during the course of 5-FC treatment. In addition, several investigators treating established animal tumors with various CD1-containing adenovirus vectors have also seen a limited response to CD/5-FC treatment (16-18). In addition, we have previously shown that the level of 5-FC safely attainable in experimental animals is low compared to the Km of CD1 for 5-FC. While the Km of CD1 is 17.9 mM-1 (14), the serum concentration of 5-FC following a single intraperitoneal injection of 5-FC of 1000 mg/kg (twice the daily dose used in this study) is 7.2 mM (19). This substantial difference between serum levels and the Km of CD1 places strict limits on how much benefit one may be able to achieve utilizing the E. coli cytosine deaminase, whether intracellular or extracellular.

Given the limited success of CD1 in GDEPT, several variations and combinations of treatments have been attempted to improve the efficacy of the CD/5-FC system. These include using the more efficient CD enzyme from S. cerevisiae (14,20), combining CD/5-FC with tk/ganciclovir (15,18), combining CD with uracil ribosyl phosphotransferase (UPRT) (17, 18) and adding radiation treatments to these genetic manipulations (13, 19). The combination with ionizing radiation is especially promising since even low, sub-cytotoxic, concentrations of 5-FC can be significantly radiosensitizing (5). Each of these approaches has shown promise, with significant responses in some settings. One intriguing, although yet untested, possibility may arise from the recent elucidation of the three- dimensional crystal structure of CD1 (22). With this information regarding the binding site for 5-FC, it may be possible to generate a variant of CD1 which deaminates 5-FC as well or better than cytosine. We have previously shown the Km for 5-fluorocytosine is significantly greater than the Km for cytosine (17.9 vs. 2.2 mM) (14). Alternatively, production of a soluble secreted form of the
more catalytically efficient yeast enzyme might also increase the observed bystander effect. The one exception may be combining a secreted extracellular CD with UPRT. This combination would lead to the extracellular production of fluorodeoxyuridine monophosphate (FdUMP) which is not membrane permeable.

Our results indicate that expression of a prodrug converting enzyme intracellularly may be disadvantageous due to high concentrations of the cytotoxic product resulting in death of the producer cell before sufficient concentrations of the drug have been achieved in the extracellular milieu. Other examples of this modification include secreted human beta-glucuronidase and the adriamycin prodrug HMR 1826 (23, 24). The glucuronidase system uses both secreted and membrane tethered forms of beta-glucuronidase in combination with glucuronidated prodrugs. This approach has led to significant bystander toxicity as well (24). Recent studies have also used an extracellular, membrane tethered form of carboxypeptidase G2 (CPG2). The extracellular, membrane tethered location of CPG2 allows it to act on its prodrug 4-[(2-chloroethyl) {2-mesyloxyethyl} amino] benzoyl-L-glutamic acid (CDMA) which is not accessible to intracellular enzymes (25). Additional prodrug substrates for this enzyme may further enhance the utility of the CPG2 system (26). Along these lines, we have developed soluble secreted and membrane tethered forms of endogenously activated mutants of carboxypeptidase A which convert methotrexate-α-peptide prodrugs to the active agent, methotrexate (27). Analogous to the membrane tethered CPG2 the cell-surface enzyme may allow for efficient bystander killing without diffusion of the active enzyme from the tumor. While these other enzyme/prodrug systems underscore the utility of extracellular prodrug conversion, the well known safety of 5-fluorocytosine and the well characterized cytotoxic and radiosensitizing activities of 5-FU make optimizing the CD/5-FC system especially relevant.

Acknowledgements

This research was supported by NIH Grant CA80145 (TSL) and NIH Grant PO1 CA85878 (AR), U.S.A.

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Received October 31, 2003
Accepted February 25, 2004