Excellent In vivo Bystander Activity of Fludarabine Phosphate against Human Glioma Xenografts that Express the Escherichia coli Purine Nucleoside Phosphorylase Gene

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ABSTRACT

Escherichia coli purine nucleoside phosphorylase (PNP) expressed in tumors converts relatively nontoxic prodrugs into membrane-permeant cytotoxic compounds with high bystander activity. In the present study, we examined tumor regressions resulting from treatment with E. coli PNP and fludarabine phosphate (F-araAMP), a clinically approved compound used in the treatment of hematologic malignancies. We tested bystander killing with an adenoviral construct expressing E. coli PNP and then more formally examined thresholds for the bystander effect, using both MuLV and lentiviral vectoring. Because of the importance of understanding the mechanism of bystander action and the limits to this anticancer strategy, we also evaluated in vivo variables related to the expression of E. coli PNP (level of E. coli PNP activity in tumors, ectopic expression in liver, percentage of tumor cells transduced in situ, and accumulation of active metabolites in tumors). Our results indicate that F-araAMP confers excellent in vivo dose-dependent inhibition of bystander tumor cells, including strong responses in subcutaneous human glioma xenografts when 95 to 97.5% of the tumor mass is composed of bystander cells. These findings define levels of E. coli PNP expression necessary for antitumor activity with F-araAMP and demonstrate new potential for a clinically approved compound in solid tumor therapy.

INTRODUCTION

The mechanisms by which Escherichia coli purine nucleoside phosphorylase (PNP) mediates prodrug cleavage and tumor regressions have become increasingly well understood (1–7). Although reversible phosphorylasis of (2’-deoxy) purine ribonucleosides to free base and (2’-deoxy) ribose-1-phosphate is a shared property of prokaryotic and eukaryotic PNPs, the mammalian enzymes differ fundamentally in sequence, structure, and function from their bacterial counterparts (3–5, 7). An important difference is the ability of E. coli PNP (but not the mammalian enzyme) to cleave adenosine analogs. Expression of E. coli PNP in cancer cells mediates the glycosidic cleavage of nucleoside prodrugs to highly toxic adenine analogs. The toxins are activated to nucleotides in the cytosol by adenine phosphoribosyltransferase and are incorporated into cellular RNA, disrupting both RNA and protein synthesis. Cell death results over a period of days, causing RNA degradation and release of PNP toxins from nucleic acid pools into the extra-cellular space (1, 6, 8–11). Tumor cell killing in vitro exhibits dose and time dependence on both of the level of E. coli PNP expressed and the amount of toxin generated (6, 8, 10, 11).

The present experiments focused on in vivo activity of a less well studied E. coli PNP substrate, 9-(β-β-arabinofuranosyl)-2-fluoroadenine (F-araA). E. coli PNP converts F-araA to 2-F-adenine (F-Ade) with a catalytic efficiency that is approximately 1,000-fold less than the prototypic E. coli PNP substrate, 9-(2-deoxy-β-β-ribofuranosyl)-6-methylpurine (MeP-dr; ref. 2, 11). The clinically used form of F-araA (i.e., fludarabine phosphate, F-araAMP) is rapidly converted in the blood to F-araA. F-araAMP is routinely used in the treatment of chronic lymphocytic leukemia but has no therapeutic role in the treatment of solid tumors such as gliomas.

Fludarabine was selected in these experiments for a number of reasons. First, the compound (without E. coli PNP) has been studied extensively, and pharmacokinetics of the agent in animal models are well defined. Second, a convenient source of fludarabine is available (the drug can be purchased from a hospital pharmacy). Because there is no readily available source, MeP-dr has required chemical synthesis in our laboratories in the past for in vivo studies. A commercial supply of GMP-grade E. coli PNP substrate would be expected to facilitate preclinical testing in animal models. Third, a safety profile already exists for fludarabine in human subjects, a fact that would be expected to promote testing of E. coli PNP in the clinic. Finally, early in vivo experiments suggested that fludarabine might be a useful compound for mediating tumor regressions with E. coli PNP (ref. 11, see also below).

Little information is available concerning the in vivo limits to bystander killing with E. coli PNP and F-araAMP or the safety of F-araAMP in this setting. Measurements of intratumoral PNP activity after in vivo delivery of E. coli PNP to tumors and studies of PNP transgene expression in non-target tissue such as liver after viral delivery have not been conducted previously. We therefore performed a series of experiments to address the following questions. First, how effectively can F-araAMP mediate antitumor effects in vivo after vector-based delivery of E. coli PNP, and how potent is bystander killing by this approach? We examined intratumoral levels of E. coli PNP, ectopic (intrahepatic) expression, and the dose dependence of these effects. Second, how well do intermediate end points (intratumoral levels of E. coli PNP enzyme, accumulation of radiolabeled F-araA metabolites, and intratumoral enzyme distribution) predict tumor regressions and cures with E. coli PNP/F-araAMP. Because in vivo bystander killing with herpes simplex virus thymidine kinase, cytosine deaminase, and other suicide gene products has been difficult to standardize with different vectoring strategies, we designed the present analysis to evaluate antitumor efficacy using three independent expression constructs (adenovirus, MuLV, and lentivirus). The findings indicate strong bystander killing and tumor regressions in a fashion that exhibits in vivo dose dependence on both intratumoral levels of E. coli PNP and the dose of F-araAMP administered.

MATERIALS AND METHODS

Construction of Recombinant Adenovirus Encoding E. coli PNP. The E. coli PNP gene was excised from pSV-PNP (12) with BamHI and inserted into the pACCMVpLpA adenoviral transfer vector (a gift of Dr. R. D. Gerard). Recombinant adenovirus was constructed by co-transfecting 293 cells in

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6-week plates with the plasmid pJM17 (Microbix, Ontario, Canada) and pAC-CMVpLpA-PNP (13). A kit from Qiagen Inc. (Valencia, CA) was used to purify adenoviral DNA from 200 μL of tissue culture supernatant. PCR-based screening (by viral DNA as template) with PNP-specific primers was used to identify recombinant adenovirus, and recombinants were plaque-purified by interactive infection of 293 cells with serially diluted viral stocks from single plaques. Functional expression of PNP from recombinant virus was confirmed by high performance liquid chromatography (HPLC) of infected cells (see below). Adenovirus encoding E. coli PNP (Ad-PNP) was titrated after CsCl gradient centrifugation (14), and E1-specific primers that produce products only in the presence of replication competent adenovirus (15) were used to exclude replication competent adenovirus by a PCR-based assay. The same techniques and an otherwise similar vector backbone were used to construct a control recombinant adenovirus expressing EGFP (enhanced green fluorescence protein, Ad-EGFP).

Measurement of E. coli PNP Activity. Crude cell extracts were prepared as described previously (9, 10) from D54MG (human glioma) cells transduced with the E. coli PNP. The extracts were incubated with various concentrations of MeP-dR, and the formation of product was measured by HPLC analysis of the reaction mixture (16). Activity was expressed as PNP units; one unit represents 1 nmol MeP-dR-converted/mg tumor cell extract/hour.

Intratumoral Inoculation of Recombinant Adenovirus and EGFP Detection. Ad-PNP or Ad-EGFP were inoculated directly into tumors [0.25–2 billion plaque forming units (PFU) in 50 μL 27-gauge needle] along four separate needle tracks. Tumors were approximately 250 mg at the time of inoculation. A subset of animals inoculated with Ad-EGFP were sacrificed, the tumors were embedded in OCT media (Fisher Scientific, Hampton, NH), frozen in liquid nitrogen, and sectioned. Tumor sections were studied by two independent protocols. In the first, a Leitz Epifluorescent microscope was used to monitor the expression of EGFP based on green fluorescence of the reporter gene. In the second, tumor sections were fixed in 4% buffered formaldehyde at room temperature for 20 minutes and washed with PBS (pH 7.4) three times. After blocking of the nonspecific protein-binding sites with goat serum (diluted 1:20 in PBS) for 30 minutes, a secondary antibody (antimouse AlexaFluor 586, Molecular Probes, Eugene, OR) was added at 1:400 dilution and incubated for 1 hour. The sections were then washed three times, and a Leitz Epifluorescent microscope with a Photometrics SenSys digital camera and IPLab Spectrum software (Scanalytics, Fairfax, VA) was used for visualization.

Measurement of Tumor Regressions. Human glioma (D54MG) tumor cells, with or without stably-transduced E. coli PNP were selected because (a) the tumor is quite slow growing in vivo (doubling time approximately 15 days), making it a convenient model for examining bystander killing in a naturally metastatic phase of tumor cells; (b) adenovirus, herpes virus, and other viruses have been used in the past to select human gliomas as a clinical target for suicide gene therapy; and (c) low level PNP expressing cells described previously were available for comparison (11). Tumor cells (2 × 106) were injected subcutaneously into the flanks of nude mice (nu/nu) purchased from Charles River Laboratories (Wilmington, MA). Tumors were measured with calipers, and an estimate of weight (mg) was calculated as described previously (11). Mice were evaluated for weight loss, tumor mass, and overall appearance twice weekly. MeP-dR was made in our laboratories according to the method described by Montgomery and Rosman (17). F-araAMP was obtained from Schering A.-G. (Berlin, Germany). All animal tumor regression studies were conducted according to NCI standard procedures. Each depicted curve represents the median tumor size from six animals, and each tumor regression study has been confirmed.

F-Ade Injection, Pre-established Tumors. Susceptibility of F-Ade to tumor was determined in tumors implanted subcutaneously in the flanks of mice as described above. Tumors of approximately 300 mg were injected intratumorally in a delivery volume of 100 μL.

Intratumoral Measurements of F-araAMP and Metabolites. D54MG parental or D54MG tumor cells expressing E. coli PNP were injected subcutaneously into the flanks of nu/nu mice, as described above. When the tumors were 300 to 400 mg, the mice received injection intraperitoneally with 100 μg/kg [1H]-F-araAMP (Moravek Biochemicals Inc., Brea, CA), and the radioactivity in tumors was determined 4 hours later as described previously (1, 10). We have shown previously (10) that total radioactivity 4 hours after injection is a reliable indicator of F-Ade metabolites in tumor tissues in vivo and that radioactive compounds recovered from tumors under these conditions represent F-araAMP. The labeled compounds in tumor extracts exhibit both specificity and dose dependence on the level of intratumoral E. coli PNP. Further efforts to analyze the tumor extracts did not reveal any more information than was indicated by the HPLC assay as originally described (10), because of degradation of relevant compounds during the extraction process.

For experiments in which adenovirus was used, labeled compound was injected 48 hours after viral administration. The tumors were dissolved in 1 mL soluene 350 (Packard Instrument Company, Meriden, CT) by incubating at 55°C for 4 hours and then at room temperature until dissolved. A portion of each extract was mixed with scintillation fluid, and the radioactivity was determined. Four tumors were removed for each measurement.

Production of Recombinant Lentivirus Expressing PNP. Plasmids generously provided by the Trono laboratory (Geneva, Switzerland) were used to perform lentivirus construction according to the method of Trono et al. (18). To establish a lentivirus capable of PNP expression, the gene was PCR amplified by primers 5’-ggatccacagtctcagccatatag-3′ (BamHI site and ATG underlined) and 5’-ctcgcaacctttatgegccagc-3′ (Xhol site underlined). The resulting product was subcloned into Zero-Blunt vector (Invitrogen, Carlsbad, CA). After digestion with BamHI and Xhol, the luciferase gene in the pHR/CMVLuc was Sin-18 lentivirus vector was replaced with E. coli PNP. Correct insertion was verified (a) by sequencing the entire PNP gene and the ligation sites; (b) by transfecting the resulting plasmid, which encodes a CMV promoter-driven PNP gene, into 293T cells; and (c) by verifying E. coli PNP enzymatic activity by HPLC in vitro (see above). A total of 40 μg of plasmid DNA was used for calcium-phosphate transfection of one 10-cm dish. The DNA mixture contained 5 μg of envelope-coding plasmid pMD.G, 15 μg of the packaging plasmid pCMVΔR8.91, which expresses Gag, Pol, Rev, and VSV-G; and 20 μg of transfer vector plasmid. Replication-deficient viral particles encoding E. coli PNP were collected from tissue culture supernatant after transfection, and a Beckman SW-28 rotor sucrose cushion centrifugation was used to concentrate lentivirus stock 1,000-fold with one round of centrifugation at 26,000 RPM for 90 minutes at 8°C. After 2 hours of incubation on ice, the virus was resuspended into serum-free medium. Titer of lentivirus reaches approximately 1 × 109 infectious particles/ml following concentration by this method, and replication-deficient viral particles encoding E. coli PNP were collected from tissue culture supernatant after transfection.

Recombinant lentivirus encoding PNP was used to generate clonal D54MG human glioma cells with high level transgene expression. Briefly, 5 × 105 D54MG cells were seeded into individual wells of 6-well plates and infected with recombinant lentivirus. Clonal cells were obtained by limit-diluting the population of infected cells into 96-well plates. Cell clones were selected, and confirmed for E. coli PNP activity both by HPLC and a conventional cell killing assay in the presence of MeP-dR in vitro.

RESULTS

Delivery to Glioma Tumors by Adenovirus. In vivo activity following PNP gene transfer is shown in Fig. 1A. Ad-PNP conferred dose-dependent PNP expression in human glioma tumors after direct intratumoral inoculation of the virus. Injection of adenovirus through needle tracks also delivered PNP to other (extratumoral) tissues, as indicated by E. coli PNP measurements in liver. For experiments in which adenovirus was used, labeled compound was injected 48 hours after viral administration. The tumors were dissolved in 1 mL soluene 350 (Packard Instrument Company, Meriden, CT) by incubating at 55°C for 4 hours and then at room temperature until dissolved. A portion of each extract was mixed with scintillation fluid, and the radioactivity was determined. Four tumors were removed for each measurement.

An adenovirus in which E. coli PNP was substituted to express EGFP in an otherwise identical virus was used to visually confirm tumor transduction. EGFP expression was examined by both fluorescence of the protein and anti-EGFP monoclonal antibody binding. Excellent concordance between the two methods for detecting EGFP was observed. Because the transgene was administered by intratumoral inoculation, EGFP expression concentrated in regions sur-
rounding needle tracks. In areas where EGFP expression was highest, >50% of the cells expressed the protein as shown by both EGFP fluorescence and anti-EGFP antibody detection (Fig. 2). Negligible evidence of expression was observed outside areas of direct inoculation. Overall, <10% of the tumor cells expressed the reporter gene.

Overall, 10% of the tumor cells expressed the reporter gene.

Tumors treated with control (no EGFP) adenovirus demonstrated no fluorescence signal (data not shown).

The data in Fig. 1 and 2 indicate F-araAMP-dependent killing of tumor cells not expressing E. coli PNP (bystander killing). To further delineate limits of bystander killing with F-araA and to test specificity of these results, we studied stably-transduced D54MG tumors. Figure 3 describes substantial activity of fludarabine against MuLv-transduced tumors, in which E. coli PNP is expressed from an SV40-driven promoter (11). We next expressed E. coli PNP, using a third, independent (lentiviral) vector system. Lentivirus confers a very high level expression in epithelia compared with first-generation adenoviral constructs, as well as those derived from MuLv. In Fig. 4A, the lentiviral expression system is used to depict a study in which 100% of cells express E. coli PNP at high levels (126,000 units). Complete regressions and cures of tumors (12 of 12) were obtained with F-araAMP under these conditions. Tumors established from lower proportions of

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PNP-expressing cells (10%, 5%, 2.5%) exhibited dose dependence on both the amount of prodrug added (Fig. 4B) and the intratumoral PNP activity (Fig. 4C). Antitumor effects were observed with F-araAMP when as few as 2.5% of tumor cells in vivo expressed PNP (average PNP activity = 3600 units). Administration of F-araAMP at either dose did not alter growth of D54MG tumors expressing a control transgene [green fluorescent protein expressed from a lentivirus (not shown)]. In B, tumors were established from an inoculum in which 10% of cells expressed E. coli PNP. Expression at the time of drug treatment was confirmed by measurement of enzymatic activity within the tumor, indicating a level of 3100 PNP units. Antitumor efficacy was greater at 100 mg/kg F-araAMP (●) than at 50 mg/kg (▲). In C, D54MG glioma tumors were established with decreasing proportions of PNP expressing cells [and increasing nonexpressing (parental) cells]. Delays in tumor growth exhibited dependence on the percentage of PNP-transduced cells. Open symbols, F-araAMP therapy; closed symbols, no prodrug therapy; circles, 2.5%; triangles, 5%, and squares, 10% PNP-transduced cells. In D, F-Ade was administered intratumorally through four needle tracks in a delivery volume of 100 µL. The MTD of F-Ade given by this route is approximately 90 mg/kg.

Discussion

In contrast to other suicide genes, bystander killing by E. coli PNP does not use gap junctions or related cell-cell communication systems and therefore does not require structures that may be poorly expressed in many tumor types (6, 8, 9, 11). Moreover, because the plasma

Table 1 Accumulation of F-araA metabolites in tumors following E. coli PNP expression

<table>
<thead>
<tr>
<th>Tumor</th>
<th>% Wild-type cells</th>
<th>PNP units</th>
<th>F-araA metabolites</th>
</tr>
</thead>
<tbody>
<tr>
<td>D54</td>
<td>100</td>
<td>0</td>
<td>31 ± 3</td>
</tr>
<tr>
<td>D54/PNP</td>
<td>0</td>
<td>300</td>
<td>65 ± 12</td>
</tr>
<tr>
<td>D54/Adenovirus</td>
<td>&gt;90</td>
<td>3,000</td>
<td>75 ± 42</td>
</tr>
<tr>
<td>D54/PNP-lenti</td>
<td>90</td>
<td>15,000</td>
<td>390 ± 140</td>
</tr>
<tr>
<td>D54/PNP-lenti</td>
<td>0</td>
<td>120,000</td>
<td>1,100 ± 230</td>
</tr>
</tbody>
</table>

NOTE. Mice bearing D54MG tumors, D54MG tumors stably expressing PNP from an SV40 promoter in 100% of cells (D54/PNP; ref. 11), from lentivirus in 100% or 10% of cells; or adenovirally transduced D54MG tumors (D54/Ad) were injected (intraperitoneal) with 100 mg/kg [3H]F-araAMP (1.4 Ci/mol). For D54/Ad tumors, compound was injected 48 hours after Ad-PNP (2 × 10⁹ PFU). The mice were sacrificed 4 hours after injection of compound, and total radioactivity in the tumors was determined. Mean ± SD of four samples is provided for each experiment. Cell extracts were also obtained from tumors and livers of three mice bearing each tumor type 48 hours after adenovirus injection (2 × 10⁸ PFU), and the amount of PNP activity was determined (as in Fig. 1A). The PNP activity in the livers of these three mice was 1, 24, and 1,040 units at 48 hours after viral inoculation. There was no activity in the livers of mice bearing D54MG or D54/PNP tumors. PNP units = nanomole MeP-dR converted per milligram tumor tissue per hour. F-araA metabolites are expressed as nanomole per gram tissue.
half-life of fludarabine in experiments such as those shown above is <1 hour, and the tumor doubling time is approximately 15 days, our results indicate that E. coli PNP/fludarabine is capable of destroying the quiescent (non-cycling) compartment of tumor cells in vivo. The toxin generated by E. coli PNP works quite differently from ganciclovir monophosphate or 5-flourouracil (toxins generated by herpes simplex virus thymidine kinase and cytosine deaminase suicide gene products, respectively), in that F-Ade impairs one or more enzymes unrelated to DNA synthesis and kills cells that are not actively proliferating. The mechanism of action is also quite different from all of the compounds currently used in the treatment of cancer (1, 8, 19–22).

One goal of the present experiments was to evaluate a recombinant virus and examine the efficiency of E. coli PNP transduction. The results demonstrate that adenovirus confers dose-dependent expression of functional enzyme in vivo (Fig. 1). The data depicted in Figs. 1 and 2 indicates that adenovirus encoding E. coli PNP can elicit substantial tumor cell killing and slow tumor growth after F-araAMP administration, despite the expected tendency to express transgene primarily along inoculation sites. Ectopic expression of E. coli PNP (e.g., adenoviral infection of liver) was well tolerated after systemic dosing of F-araAMP in this setting. The data in Fig. 1 therefore points to both safety and substantial bystander killing by E. coli PNP/F-araAMP.

Antitumor effects with E. coli PNP exhibit dependence on both the level of prodrug administered (Fig. 4B) and the total amount of PNP activity present within solid tumors (Fig. 4C). As few as 2.5% of tumor cells expressing the PNP gene at high levels led to strong tumor responses. On the basis of adenov- and lentiviral studies in D54MG tumors, a transfection rate in which 2–5% of cells express PNP at sufficient levels of activity (e.g., 2,000–3,000 units) can be considered a benchmark for impairing tumor growth. The lentiviral protocol used at higher levels of expression led to tumor cures (for example, when 5–10% of cells express the transgene, Fig. 4C). Intratumoral F-araA metabolites above ~400 nmol/g tumor tissue were also associated with potent tumor regressions and cures (Table 1). The data in Table 1 demonstrates specificity and dose dependence on intratumoral levels of E. coli PNP expression, and describes a useful end point for monitoring PNP therapy in vivo.

Transduction of D54MG cells with a first-generation retrovirus (MuLV) led to E. coli PNP expression levels approximately 500-fold less than the levels observed with lentivirus. In tumors established from MuLV and comprised of 100% PNP expressing cells (i.e., no bystander killing required), substantially lower PNP levels than those described above (and correspondingly less toxin accumulation) effectively impaired tumor growth (Fig. 3). This is likely because of more potent killing of PNP-transduced (compared with bystander) cells, as we have reported earlier for other E. coli PNP substrates (ref. 6).7 Adenovirus and lentivirus (3,000–8,000 PNP units, 2–10% transduced cells) were used to compare tumors with similar levels of PNP activity and percentages of cells transduced and similar delays in tumor growth were observed (Figs. 1 and 4C).

Fludarabine has been reported to confer antitumor activity in an earlier study of E. coli PNP expression in hepatic tumors (23) and in glioma tumors transduced ex vivo (11). The results (Fig. 1, 3, and 4) establish that toxic compounds such as F-Ade can be liberated in tumor tissues by the action of PNP and cure malignancies without untoward toxicity to the host. Tumor regressions with F-Ade cannot be attained simply by intratumoral injection (Fig. 4D), presumably because of escape of the compound to the systemic circulation.

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