Tumor Chemosensitivity Conferred by Inserted Herpes Thymidine Kinase Genes: Paradigm for a Prospective Cancer Control Strategy

Frederick L. Moolten

Veterans Administration Medical Center, Bedford, Massachusetts 01730, and the Department of Microbiology, Boston University School of Medicine, Boston, Massachusetts 02118

ABSTRACT

The lack of highly exploitable biochemical differences between normal tissues and some tumors can theoretically be circumvented by a strategy utilizing gene insertion prophylactically to create tissue mosaicism for drug sensitivity, thereby ensuring that any tumor arising clonally will differ from part of the normal cell population. Elements of the strategy were tested with neoplastic BALB/c murine cell lines bearing the herpes thymidine kinase gene. Exposure to the herpes thymidine kinase-specific substrate 9-||2-hydroxy-1-(hydroxymethyl)ethoxy|methyl|guanine ablated the clonogenic potential of the cells in vitro, and administration of this drug to BALB/c mice bearing tumors produced by the cell lines uniformly induced complete regression of the tumors. The observed responses to therapy imply that the strategy may prove valuable when the genetic technology needed for its human implementation becomes available.

INTRODUCTION

The limited ability of antineoplastic therapy to distinguish neoplastic from normal cells on the basis of proliferative behavior has inspired a search for biochemical characteristics of neoplastic cells that are tumor specific rather than proliferation specific. Unfortunately, current molecular genetic studies (reviewed in Refs. 1 and 2) fail to support the expectation that such characteristics must be a consistent feature of neoplastic cells. Rather, these studies suggest that the neoplastic state can be explained without postulating tumor-specific functions, but merely the operation of normal proliferation-specific functions at abnormal levels, as a result of changes (sometimes minimal) in the structure of growth-regulatory genes or changes in their number or chromosomal environment. If confirmable, this conclusion implies that a continued search for highly specific attributes of neoplastic cells cannot be depended on for a general solution to the problems of cancer therapy and that major reductions in the lethality of cancer may require alternative approaches that do not depend on the natural occurrence of such attributes. An alternative strategy entailing the artificial creation of differences between normal and neoplastic cells through the prophylactic use of gene insertion techniques is described below, together with data suggesting the potential feasibility of the strategy when a gene insertion technology safe and efficient enough for its human implementation becomes available.

The strategy, which extends earlier concepts (3, 4), emerges from two principles: (a) as radiation studies have demonstrated, only a small minority of normal stem cells in a tissue subjected to cytotoxic therapy need survive in order to ensure host survival, in some cases fewer than 1% (5, 6); (b) most tumors are clonal, and when a clone of cells arises in a tissue composed of a mosaic of genetically diverse cells, the clone must inevitably differ from some of the other cells in the mosaic (7). This difference is what provides the basis for therapeutic specificity. The approach is the following. Genes that alter cellular sensitivity to various chemotherapeutic agents are introduced prophylactically into tissues in a scattered fashion, so that some cells acquire a given gene while others do not. The result is a mosaic pattern in which cells differ in terms of what drugs they are sensitive to. If a tumor later arises, therapy is directed at whatever sensitivity the tumor clone exhibits, while the host is protected by those cells in the mosaic normal population that do not share that sensitivity. Of the many ways in which the strategy might be implemented, the simplest entails the use of a single gene in a single tissue and is illustrated in Fig. 1.

Tests of the mosaicism strategy are likely to be feasible soon on the basis of emerging technology for inserting genes into mice (8–11). To justify the substantial time and resources these tests may require, it will be important to provide evidence that the strategy, once testable, can operate as predicted. The present study has gathered this evidence by simulating the two critical events that would be expected during therapy of a tumor that arose in a mosaic host. The first is an attack directed against the tumor on the basis of its drug susceptibility characteristics. Without mosaic mice, this was simulated by utilizing normal mice with transplanted tumors bearing a drug sensitivity gene that had previously been inserted into the tumor cells in vitro. The second event is an attack directed against a significant fraction of normal cells; this was simulated with irradiation. The gene chosen for insertion encodes the TK3 enzyme of HSV type 1. HSV-TK can catalyze the phosphorylation of a number of nucleoside analogues that are poor substrates for the TK of mammalian cells. The best known is the antiherpes drug acyclovir (12), which exhibits minimal toxicity to cells lacking HSV-TK activity, but is activated in cells expressing HSV-TK to a toxic form capable of inhibiting DNA synthesis, and which has been reported to exhibit selective cytotoxicity to cells possessing inserted HSV-TK genes (13–15). More recently, a related drug, HHEMG (16–18), has been reported to be a better substrate for phosphorylation by HSV-TK. Preliminary experiments with the neoplastic cell lines created for the present study indicated the cytotoxicity of HHEMG to be more specific for HSV-TK-positive cells than that of acyclovir; therefore HHEMG was chosen for the study. HHEMG has also been reported recently to be more specific than acyclovir in inhibition of the growth of HSV-TK-positive sublines of murine L-cells (19).

MATERIALS AND METHODS

Cell Lines. Cells were cultured in medium containing 10% fetal bovine serum and antibiotics. HSV-TK-positive cells were maintained

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2 To whom requests for reprints should be addressed, at Veterans Administration Medical Center (151), 200 Springs Road, Bedford, MA 01730. This is Publication 131 of the Hubert H. Humphrey Cancer Research Center at Boston University.
Gene A is introduced into about 90% of bone marrow stem cells (identified by dark nuclei)
A leukemia arises, clonally, from one of the 90% A-positive cells

After tests identify the A-positive nature of the normal cells, Drug A is given. What survive are 10% of the normal cells (i.e., the A-negative fraction)

Fig. 1. Paradigm for induced mosaicism in bone marrow. Gene A, DNA coding for exquisite sensitivity to Drug A. Cells bearing this gene are easily killed by doses of Drug A harmless to other cells. The results of the depicted sequence can be thought of as mimicking the effects of subjecting the neoplastic cells to lethal irradiation and of the normal cells to sublethal doses.

in minimal essential medium containing HAT (20, 21) (aminopterin concentration, 1 µg/ml), which is lethal to TK-negative cells. DMEM was used for TK-negative cells.

Derivation of HSV-TK-positive Sublines. Lineages are diagrammed in Fig. 2A. The TK-negative BALB/c fibroblast line 10E2(TK−) and the 10E2(HSV-TK) line (which contains an HSV-TK gene inserted as a result of exposure of 10E2(TK−) cells to HSV attenuated by UV irradiation (22)) were obtained from B. Hampar and S. Showalter. Insertion of the HSV-TK gene into a neoplastic subline of 10E2(TK−) cells, termed TK−, was accomplished by extracting DNA (23) from Escherichia coli harboring the plasmid PKOS17B2, which contains the HSV-TK gene in a 3.6-kilobase BamHI restriction fragment (24); purifying the plasmid DNA by cesium chloride density gradient ultracentrifugation; and applying it to cultures of TK− cells by means of a calcium phosphate coprecipitation method (25) in the presence of TK− DNA as a carrier. HAT selection was used to identify TK-positive transfectants. No HAT-resistant colonies arose from cultures exposed to TK− DNA alone. Of the multiple colonies obtained from transfections with HSV-TK DNA, two, randomly selected from different culture dishes, were cultured further to permit 5 × 10⁶ cells to be injected s.c. into BALB/c mice, yielding the tumor lines PK, and P, KM₂. For in vitro therapy studies, these and other tumor lines were serially passaged by s.c. injection of tumor fragments suspended in DMEM. In vitro studies were performed with cells explanted from the tumors. The TK-negative revertant line PK, R was derived by passage of PK, cells into increasing concentrations of 5-iododeoxyuridine to 100 µg/ml.

Neoplastic Transformation. K3T3 is an established sarcoma line originally derived by transformation of BALB/3T3 cells with Kirsten sarcoma virus (26). Tumorigenic sublines of 10E2(TK−) or 10E2(HSV-TK) cells were derived by exposing these cells to 7,12-dimethyl-benz(a)anthracene (0.02 µg/ml) for 2 days, plating surviving cells in 0.3% agar to select for anchorage-independent colonies, passaging cells from these colonies repeatedly in culture, and testing for tumorigenicity by injecting 1–5 × 10⁶ cells from various passages s.c. into BALB/c mice previously exposed to a sublethal dose of irradiation (425 rads).

The TK− tumor line arose at post-7,12-dimethyl-benz(a)anthracene passage 7 and the TK−22 line at post-7,12-dimethyl-benz(a)anthracene passage 9.

TK Phenotypes. The putative TK status of the cell lines used was confirmed by three independent methods. (a) TK enzyme activity was assayed as described (27). Cell extracts were screened for their ability to phosphorylate [³H]thymidine, with results concordant with the putative phenotypes of each line. HSV-specific TK activity of cellular extracts was then quantified by using [³H]iododeoxyuridine as a substrate in the presence of tetrahydouridine (28). (b) All cell lines were plated sparsely and allowed to grow to form a confluent mosaic of HSV-TK-positive and -negative patches, subsequent HHEMG treatment destroyed the HSV-TK-positive cells, leaving the HSV-TK-negative patches to survive. The TK−22 line. HSV-TK-negative lines were strikingly more resistant to HHEMG, surviving until exposed to drug concentrations 200 to >1000 times those lethal to HSV-TK-positive cells.

When 9/1 mixtures of HSV-TK-positive and -negative cells were plated sparsely and allowed to grow to form a confluent mosaic of HSV-TK-positive and -negative patches, subsequent HHEMG treatment destroyed the HSV-TK-positive cells, leaving the HSV-TK-negative patches to survive (Fig. 3); the TK-negative phenotypes of these patches were confirmed in two repetitions of the experiment by demonstrating their complete destruction upon exposure to HAT-containing medium. In contrast to this patchy mosaicism, when 9/1 mixtures were plated at high density, so that each TK-negative cell was surrounded by HSV-TK-positive neighbors, only rare TK-negative

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RESULTS

HSV-TK DNA Sensitizes Neoplastic Cells to HHEMG in Vitro. Six BALB/c murine sarcoma cell lines were utilized, three HSV-TK-negative and three positive for expression of an inserted HSV-TK gene (Fig. 2A). DNA hybridization analysis of the cells (Fig. 2B) demonstrated in the putatively HSV-TK-positive lines the expected 3.6-kilobase HSV-TK BamHI fragment (plus fragments of inserted plasmid DNA in the two plasmid-transfected lines). Interestingly, an absence of these DNAs was demonstrable not only in the TK− line but also in the PK, R revertant, suggesting that the TK-negative status of the latter reflected a loss of the inserted DNA rather than merely its failure to be expressed.

The in vitro sensitivity to HHEMG was measured by a colony inhibition assay, designed to simulate the effects of treating a tumor for several days in vivo to eliminate the clonogenic potential of the tumor cells. As seen in Fig. 2C, each of the HSV-TK-positive lines was highly sensitive to HHEMG (concentration inhibiting colony formation by 50% in the neighborhood of 10⁻⁴ M). Consistent with their greater HSV-TK activity, the PK, and P, KM₂ lines were slightly more sensitive than the TK+22 line. HSV-TK-negative lines were strikingly more resistant to HHEMG, surviving until exposed to drug concentrations 200 to >1000 times those lethal to HSV-TK-positive cells.

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Fig. 2. Correlation of HSV-TK status and in vitro HHEMG sensitivity. A, lineage of HSV-TK-positive and -negative tumor cell lines. Nonneoplastic lines (described in Ref. 22) are shown in circles, their tumorigenic derivatives in rectangles. The level of HSV-specific TK activity is cited in parentheses as the mean value obtained from triplicate samples after subtraction of background radioactivity found in enzyme-free blanks and is expressed as pmoles iododeoxycytidine phosphorylated/h/mg protein ± SE. DMBA, 7,12-dimethylbenz(a)anthracene; IUdR, iododeoxyuridine. B, Southern blots of genomic DNA from HSV-TK-expressing tumor lines and their TK-negative counterparts. The procedure utilized 2 µg DNA digest per sample and was repeated with 10 µg for samples which failed to manifest any hybridizing band at 2 µg; the 2-µg P2KM2 sample, which may have been underloaded on the gel as judged by ethidium bromide staining and yielded only a faint hybridization pattern (not shown), was also rerun at 10 µg. Arrows, position of the 3.6-kilobase BamH1 HSV fragment containing the TK gene. The higher molecular weight bands seen with the plasmid-transfected PK1 and P2KM1 cells represent plasmid sequences. C, sensitivity of HSV-TK-positive and -negative cells to colony inhibition by HHEMG. Duplicate or triplicate cell cultures, exponentially growing in nonselective medium (DMEM), were exposed to HHEMG for 3 days; the cells were resuspended by trypsinization, measured fractions of the suspension were replated in medium without HHEMG, and after an additional 7 days of incubation colonies that developed were stained with Wright's stain for enumeration. As controls, identically grown untreated cultures were always assayed concurrently with the HHEMG-treated cultures. Mean values from the HHEMG-treated cultures were expressed as a percentage of control values; the range of variation did not exceed ± 14%.

cells survived HHEMG treatment. This latter result is presumed to reflect transfer of phosphorylated HHEMG by “metabolic cooperation” (32) between contiguous HSV-TK-positive and -negative cells. Since any similar transfer of toxicity in vivo would jeopardize therapeutic specificity, the potential utility of the HSV-TK gene may require mosaicism that is patchy rather than diffuse. A report (33) that in normal mouse bone marrow only a limited number of clones are active at any one time is consistent with this type of patchiness, and evidence for patchiness has also been demonstrated in human epidermis (7). The extent to which any of these naturally occurring mosaics would be duplicated by artificially induced mosaicism remains to be determined.

HSV-TK-positive Tumors Respond to HHEMG Therapy. The six tumor lines that had been studied in vitro were used to induce tumors in BALB/c mice, and the response of these tumors to HHEMG therapy was measured, to determine whether the HSV-TK gene also sensitized tumor cells to the drug in vivo. An additional feature of some experiments was the exposure of the mice to sublethal irradiation immediately prior to injection of the tumor inoculum, at a dose (425 rads) sufficient to ensure that 6–14 days later, when tumors appeared and HHEMG therapy was started, significant depression of stem cell numbers in bone marrow was still present. This deliberate destruction of normal stem cells was performed to create experiments in which both of the two major events in the induced mosaicism therapeutic paradigm were simulated during overlapping intervals, the first being an attack by the therapeutic agent directed against the tumor and the second an attack directed against a major (but not intolerably large) frac-
tion of normal cells. An additional consequence of irradiation (not explicitly investigated) may have been to reduce immune function and thereby increase the degree to which the efficacy of therapy reflected only the efficacy of the administered drug.

The results of therapy are shown in Table 1. Untreated mice it was only occasionally effective. In neither case did tumors were invariably fatal. In contrast, HHEMG treatment of 55 mice bearing HSV-TK-positive tumors induced complete tumor regressions in all mice. Three of these mice (all irradiated) later developed tumor recurrences, whereas the remaining 52 remained tumor free over a 6-month observation period. In the case of HSV-TK-negative tumors, none of 57 HHEMG-treated mice experienced a tumor regression, and all died. Cyclophosphamide (a DNA-alkylating agent) was administered as a representative of agents that act independently of the HSV-TK pathways. It was variably effective against all the tumor lines except K3T3 when tested in unirradiated mice; in irradiated mice it was only occasionally effective. In neither case did its efficacy depend on the HSV-TK status of the tumor. More recently, mice bearing moderately large (1-cm) tumors have been treated with HHEMG at the same dose, but for a total duration of 8 days. All HSV-TK-positive tumors treated to date (4 P2KM2 and 3 PK1) have regressed completely, whereas no similar tumors treated with cyclophosphamide have regressed (0 of 7 P2KM2 tumors and 0 of 8 PK1 tumors). Also ineffective was HHEMG therapy of large HSV-TK-negative tumors. An example is shown in Fig. 4, which illustrates the difference between the consequences of possessing and lacking an HSV-TK gene.

Notwithstanding the therapeutic efficacy exhibited by HHEMG against HSV-TK-positive tumors in vivo, populations of these cells in vitro frequently harbored some cells that had reverted to a HHEMG-resistant phenotype. Although not analyzed biochemically, this phenomenon is consistent with reports of phenotypic loss of HSV-TK as a result of either gene loss (as seen in the PK,R line) or more often gene methylation (34, 35). Reversion was assayed by plating 5–6 replicate suspensions of about 5000 cells that had been grown in HAT-containing medium into nonselective medium (DMEM), allowing the cells to undergo 5–8 doublings, and replating a measured fraction of them at low density in DMEM containing 4 x 10^{-6} M HHEMG. After 10 days, the number of surviving colonies was determined, and this value was corrected for the effects of cocultivation with HSV-TK-positive cells (as determined by reconstruction exper-

<table>
<thead>
<tr>
<th>HSV-TK status</th>
<th>Tumor line</th>
<th>Irradiation status of recipients</th>
<th>Fraction of mice exhibiting lasting tumor regressions*</th>
<th>Cyclophosphamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ P2KM2</td>
<td>0/10/10</td>
<td>0/14 (43)</td>
<td>1/10 (58)</td>
<td></td>
</tr>
<tr>
<td>+ TK*22</td>
<td>0/10/10</td>
<td>0/17 (61)</td>
<td>3/10 (73)</td>
<td></td>
</tr>
<tr>
<td>+ PK1</td>
<td>0/10/10</td>
<td>0/12 (81)</td>
<td>7/10 (112)</td>
<td></td>
</tr>
<tr>
<td>- TK(-)</td>
<td>0/10/10</td>
<td>0/38 (53)</td>
<td>2/13 (76)</td>
<td></td>
</tr>
<tr>
<td>- K3T3</td>
<td>0/10/10</td>
<td>0/10 (47)</td>
<td>0/10 (59)</td>
<td></td>
</tr>
<tr>
<td>- PK,R</td>
<td>0/10/10</td>
<td>0/12 (55)</td>
<td>0/12 (46)</td>
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* These were defined as complete regressions lasting at least 6 months; in studies with other fast-growing murine neoplasms, this interval has been sufficient for tumors to recur if they have not been eradicated permanently (36, 44).

Numbers in parentheses, median survival times (in days) of mice that died.

All tumors regressed completely in these mice, but one P2KM2 and 2 TK*22 tumors later recurred, all at the original sites. No recurrences were detected in regrow mice at distant sites, by either inspection or by necropy performed on 8 randomly selected mice killed after 6 months of observation.
capable (even in irradiated mice) of eradicating a small tumor enlarged. D, Day 37. The PK, tumor has regressed completely; the TK(—) tumor flank and TK(—) into the left flank. Small tumors are visible at each site. >.Day 13 after I'K, tumor inoculation into the right tumors in the same mouse. I. Day 13 after I'K, tumor inoculation into the right system. One can conclude, however, that the presence of a subpopulation of drug-resistant cells in a tumor does not invar-

DISCUSSION
The present results demonstrate that tumors can be eradicated in vivo on the basis of acquired drug susceptibilities while their hosts survive significant depletion of normal stem cells. The demonstration that this critical principle of the mosaicism strategy operates as predicted strengthens the justification for proceeding with technologically more difficult attempts to create mosaic hosts for testing the strategy in its entirety. Recent reports suggest that gene insertion efficient enough to create a testable model of mosaicism in the hematopoietic system of mice will be feasible as the result of the development of efficient retroviral vector (8–11). In addition to a need for efficient gene delivery, other concerns that may need to be addressed include the following. (a) Mutational loss of activity of inserted genes. One potential solution is to induce mosaicism by inserting drug resistance genes into a minority of cells in a tissue rather than drug sensitivity genes into a majority; particularly effective would be genes that encoded resistance too great for tumor cells to emulate by spontaneous mutation, e.g., a dihydrofolate reductase enzyme activity displaying almost absolute resistance to methotrexate (37). A disadvantage is the need to insert the resistance genes into all tissues vulnerable to therapy; thus, the strategy cannot be confined to a single tissue, such as bone marrow, selected for convenience of gene insertion. An alternative solution would be to introduce into cells multiple independent chemosensitivity genes. (b) Transfer of toxicity by metabolic cooperation. This problem might be averted by developing therapies with agents too large to traverse intercellular channels or by using as sensitivity genes DNA segments with specific binding affinity for potentially lethal agents (38, 39). Finally, (c) a need for safety may conflict with the proposed use of retroviral vectors, mentioned above, since their random insertion patterns may bring them to areas of the genome where their presence is oncogenic. Attempts may be warranted to develop alternative vectors with recombination functions that are more site specific, as has been suggested for some inserted histocompatibility genes (40).

While the above list emphasizes how premature it would be to anticipate early general clinical applicability of induced mosaicism, use in specific high risk situations might be contemplated if the strategy is successful in animal model systems. Retroviral vectors of HSV-TK are currently being utilized in this laboratory in hopes of being able to test the curative potential of HHEMG in mosaic mice that develop hematopoietic neoplasms. When technology permits, any favorable results achieved in these tests may serve as a model for initial human trials of induced mosaicism in individuals at exceptionally high risk for fatal leukemias. These include adults with some preleukemia syndromes whose risk of developing acute nonlymphocytic leukemia may exceed 80% (41). In such individuals, the need for medical intervention would appear to be as urgent as that of candidates currently being considered for gene therapy trials to correct genetic disorders (42).

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