Differential Killing of Mismatch Repair-Deficient and -Proficient Cells: Towards the Therapy of Tumors with Microsatellite Instability

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Abstract

DNA mismatch repair (MMR) defects bring about a strong mutator phenotype and microsatellite instability (MSI). In an attempt to exploit MSI in cancer therapy, we constructed expression vectors carrying a thymidine kinase/blastidin deaminase fusion gene downstream from a (C)₁₂ or an (A)₉ microsatellite and stably transfected these constructs into human cells in which the MMR status could be regulated by doxycycline. We now show that ganciclovir-resistant clones arising through frameshifts in the (C)₁₂ microsatellite were 20 times more frequent in cells in which MMR was inactivated. This difference may be exploited in gene therapy of tumors with MSI, which represent a substantial proportion of cancers of many different tissues.

Introduction

A substantial proportion of tumors of different organs displays MSI¹, a phenotypic trait characterized by a large increase in the frequency of frameshift mutations within repeated sequence elements, the so-called microsatellites. This anomaly is caused by inactivation of the postreplicative MMR system, which normally corrects strand misalignments arising in these repeats during DNA replication (1). In hereditary nonpolyposis colon cancer kindreds, which represents ~5% of colon cancer patients, the MMR defect and MSI are linked to inherited mutations in genes encoding MMR proteins. In ~10% of sporadic colon cancers, MSI arises as a result of epigenetic silencing of the MMR gene hMLH1 (2, 3), and an ever-increasing number of reports describes MSI also in cancers of head and neck, lung, prostate, breast, bladder, and other tissues (reviewed in Ref. 4). Past attempts to identify agents able to selectively kill MSI⁻ cells largely failed. Upon treatment with a range of DNA damaging agents, substantial differences in the response of MMR-deficient and -proficient cells were observed only for cisplatin, which kills MMR-proficient cells ~3-fold more efficiently than MMR-deficient ones (5) and for SN1-type methylating agents in the response of MMR-deficient and -proficient cells were ~3-fold more efficient than MMR-deficient ones (5) and for SN1-type methylating agents, where the difference is ~100-fold (6, 7). MMR-deficient cells were reported to be more sensitive to killing by CCNU than MMR-proficient controls (8), but this difference appears to be limited to only a subset of MMR-deficient cell lines. Thus, in an attempt to identify a more general approach toward the therapy of MMR-deficient tumors, we set out to exploit the MSI phenotype. In cultured human cells established from these tumors, MSI was reported to be two to three orders of magnitude higher than in control lines.

Materials and Methods

Cell Lines. 293T Lα cells were derived from the hMLH1-deficient human embryonic kidney 293T cells by stable transfection with a vector carrying the hMLH1 cDNA under the control of the inducible Tet-Off expression system (7). The cells were grown in DMEM with Eagle salts (Life Technologies, Inc., Gaithersburg, MD), supplemented with 10% Tet System Approved Fetal Bovine Serum (Clontech, Palo Alto, CA), 2 mM l-glutamine (Life Technologies, Inc.), 100 IU/ml penicillin, 100 μg/ml streptomycin (Life Technologies, Inc.), 100 μg/ml Zeocin (Invitrogen, San Diego, CA), and 300 μg/ml hygromycin B (Roche Molecular Biochemicals, Basel, Switzerland). To obtain cells completely free of the MMR protein hMLH1 (293T Lα⁻), the cells were transferred for at least 7 days to a medium containing 50 ng/ml DOX (Clontech). Fresh DOX was added every second day. To induce hMLH1 expression (293T Lα⁺), the cells were transferred to a medium without DOX, the medium was changed the following day, and the cells were cultivated for at least 6 more days. Expression of hMLH1 in these cells fully restored MMR proficiency (7).

Vector Construction. The pSBCTKBSD vector (13) containing the fusion gene encoding thymidine kinase and blastidin deaminase was used as a template for a PCR reaction in an assay consisting of 1X Cloned PFU buffer, 1 μM forward primer, 1 μM reverse primer, 200 ng of template DNA, 0.2 mM deoxynucleoside triphosphates and 2.5 units/50 μl reaction Pfu turbo DNA polymerase (Stratagene, La Jolla, CA). The primers (Micsynth, Balgach, Switzerland) were as follows: forward 5’-GTT CCA GGA TCC ACC ATG ATT GAA GAA TTC ATT GAA CAA GAT GGA TTG CAC GCA GG-3’.

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¹ The abbreviations used are: MSI, microsatellite instability; BSD, blastidin deaminase; DOX, doxycycline; GANC, ganciclovir; MMR, mismatch repair; ORF, open reading frame; TKBSD, thymidine kinase/blastidin deaminase fusion gene.
forward (C)$_{12}$, TGG CCA GGA TCC ACC ATG ATT GAA CCC CCC CCC ATT GAA CAA GAT GGA TTG CAC GCA GG; forward (A)$_{26}$, TGG CCA GGA TCC ACC ATG ATT GTC AAAA AAAAA AAAAA AAAAA AAAAA ATT GAA CAA GAT GGA TTG CAC GCA GG; and reverse, TAC TGG CTC GAG TCA ATG TAT CTT ATC ATG TCT GGA TCG.

The PCR cycle was as follows: 98°C for 3 min, (98°C for 1 min, 69°C for 1 min, and 72°C for 5 min)$_{30}$, 72°C for 10 min. The PCR products were digested with BstHI and XhoI (both New England Biolabs, Beverly, MA) and cloned into the corresponding sites of pcDNA3 (Invitrogen), creating pcDNA3-TKBSD, pcDNA3-(C)$_{12}$TKBSD, and pcDNA3-(A)$_{26}$TKBSD vectors.

**Isolation of Stable Transfectants.** pcDNA3-TKBSD, pcDNA3-(C)$_{12}$TKBSD, and pcDNA3-(A)$_{26}$TKBSD vectors were digested with BglII and DraIII (both New England Biolabs) and subjected to preparative gel electrophoresis. The fragments containing the TKBSD fusion gene were isolated and used for transfection of 293T Lc cells using the FuGENE reagent (Roche, Basel, Switzerland). Selection was initiated 2 days after transfection with 10 μg/ml blasticidin S (Invitrogen). After 2–3 weeks, stable clones were isolated and additionally propagated with blasticidin (100 μg/ml).

**Mutagenesis Assays.** The selected clone, carrying the microsatellite repeat/TKBSD fusion stably integrated in the genome, was grown without or with 50 ng/ml DOX in a 6-well plate in a medium containing 100 μg/ml blasticidin for 7 days. At this time point, the cells grown in the presence of DOX were completely free of hMLH1 and thus MMR-deficient, and cells grown without DOX remained MMR proficient. The high concentration of blasticidin in the medium ensured elimination of cells with frameshifted inserts. The blasticidin was then removed and the cells were additionally propagated without or with DOX in a 6-well plate. As the doubling time is −24 h, the cells were split every 2 days in a ratio 1:4 to maintain a constant cell number. In the absence of blasticidin, cells in which the TKBSD fusion gene was inactivated by frameshift mutagenesis (or otherwise) survived. Immediately upon blasticidin withdrawal and at the selected time points (4, 8, and 13 days, −4, 8, and 13 generations), 1 × 10$^5$ or 5 × 10$^5$ cells were plated into 10-cm dishes in 10 ml of medium containing 30 μM GANC (Sigma, St. Louis, MO) to score for mutant (GANC-resistant) cells. At the same time, 300 cells were plated in a medium without GANC to assess plating efficiency (control). After 2 weeks of incubation, the colonies were stained with Giemsa (Fluka, Buchs, Switzerland) and counted (see Fig. 1C for a schematic outline of the experiment).

**MSI Analysis.** Chromosomal DNA from the GANC-resistant colonies was extracted using the TRI reagent (Molecular Research Center, Cincinnati, OH). The vector DNA sequence containing the repeat was amplified by PCR under the following conditions: 1× Taq buffer, 1 μM forward primer, 1 μM reverse primer, 300 ng of template DNA, 0.2 mM deoxynucleoside triphosphates, and 2 units/50 μl reaction TaqDNA polymerase (New England Biolabs). The following primers (Microsynth, Balgach, Switzerland) were used: forward (GGC GTA GGC GTG TAC GGT G), reverse (CCA GTC CTC CCG CCA CGA CC). The PCR procedure was as follows: 95°C for 2 min (95°C for 1 min, 60°C for 1 min, and 72°C for 1 min 20 s)$_{30}$, 72°C for 10 min. The PCR products were purified, and the DNA regions containing the repeats were sequenced using the primer GTA CGT AGA CGA TAT CGT CG on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

**Results and Discussion**

The experimental system intended for use in gene therapy of tumors with MSI is based on transduction of the tumor cells with a vector carrying a toxin gene that is out-of-frame because of the insertion of a microsatellite immediately downstream from its AUG start codon (Fig. 1A). The inherent instability of the microsatellite in MMR cells should result in restoration of the correct reading frame in a given percentage of the transduced cells and thus in expression of the toxin and cell death. However, for the purposes of the present study, we decided to invert this strategy by making use of an in-frame reporter/toxin combination that allows for a more accurate estimation of mutation frequencies and is free of artifacts (Fig. 1, B and C). The reporter gene construct was a fusion of BSD from Aspergillus terreus and TK from Herpes simplex virus (13). This fusion gene was preceded by a microsatellite repeat, which was inserted immediately downstream from the start codon, but which maintained the correct reading frame of the fusion gene. For the initial experiments, we chose...
were adjusted, using plating efficiency, for 5 × 10^4 H11003 ods C

consisted initially exclusively of TK

we can calculate the mutation frequency in a cell population that

were grown for 8 days without blasticidin, treated

typical result of an assay described in Fig. 1 C

50 ng/ml DOX. The extracts of 293T L

5 frameshifts were observed in the (C) 12 repeat and

The (A) 26 and (C) 12 repeats, together with a control construct that
carried no repeat (Fig. 1B). The BSD protein confers resistance
against blasticidin, which permits the selection of clones carrying the
nonmutated construct stably integrated in the genome. Inducing MMR
deficiency in one-half of the cells by adding DOX to the culture
medium and propagating the cells independently in a MMR-deficient

frame (Fig. 2, C). The number of GANC-resistant colonies increased
with time in both MMR-proficient and -deficient backgrounds, but the fold-difference remained relatively stable (Fig. 2C).

In contrast to (C) 12 , the (A) 26 repeat was labile in both MMR-

TKBSD vectors, 293T L/H9251 selected clones carrying the pcDNA3-(A) 26 TKBSD and pcDNA3-

mutants), all carrying the vector integrated in the same genomic
sequence context and in a strictly isogenic genetic background.

Before initiating this study, we had to check the integrity of the stable clones carrying the reporter constructs, more specifically, the integrity of the Tet-Off system that controls the inducible expression of hMLH1. As shown in Fig. 2A, the 293T Lα cells and the clone stably transfected with the pcDNA3-(C) 12 TKBSD vector [denoted 293T Lα '(C) 12 TKBSD] expressed both hPMS2 and hMLH1 in similar amounts. In the presence of DOX, the transcription of hMLH1 was shut off, which resulted in the depletion of the hMLH1/hPMS2 heterodimer (7); these cells are denoted 293T Lα '(C) 12 TKBSD. The selected clones carrying the pcDNA3-(A) 26 TKBSD and pcDNA3-TKBSD vectors, 293T Lα(A) 26 TKBSD and 293T Lα(TKBSD, respectively, behaved similarly (data not shown).

Results of the mutagenesis assays ("Materials and Methods") indicated that the (C) 12 repeat remained stable in the MMR-proficient

in-frame, and upon addition of GANC, most of the cells were killed.
In contrast, frequent frameshift mutations within this repeat in a MMR-deficient background gave rise to a ~20-fold higher number of GANC-resistant clones in which the TKBSD gene was shifted out-of-frame (Fig. 2, B and C). The number of GANC-resistant colonies increased with time in both MMR-proficient and -deficient backgrounds, but the fold-difference remained relatively stable (Fig. 2C). In contrast to (C) 12 , the (A) 26 repeat was labile in both MMR-proficient and -deficient backgrounds, displaying only ~2 fold difference in stability (Fig. 2, B and C, and Table 1).

To confirm that the GANC-resistant phenotype resulted from a mutation in the reporter construct, we sequenced the DNA regions containing the repeat and its close proximity. Microsatellite frameshifts were detected in all of the 293T Lα samples sequenced (30 of 30). Only 1 frameshifts were observed in the (C) 12 repeat and ~1 or, less frequently, ~2 frameshifts in the (A) 26 repeat (Fig. 2D).

Table 1 Average fold-differences in the number of GANC-resistant colonies in MMR-deficient versus proficient background

<table>
<thead>
<tr>
<th>Days without selection</th>
<th>(C) 12 TKBSD</th>
<th>(A) 26 TKBSD</th>
<th>TKBSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>29.5 (±17)</td>
<td>1.6 (±0.1)</td>
<td>1.4 (±0.1)</td>
</tr>
<tr>
<td>8</td>
<td>17.1 (±3.9)</td>
<td>2.1 (±0.3)</td>
<td>1.2 (±0.1)</td>
</tr>
<tr>
<td>13</td>
<td>19.2 (±2.8)</td>
<td>2.6 (±0.2)</td>
<td>1.6 (±0.1)</td>
</tr>
</tbody>
</table>

* The values shown in the table were calculated by dividing the number of GANC-resistant colonies in the MMR-deficient background by the number of GANC-resistant colonies in the MMR-proficient background at each time point (4, 8, and 13 days) for each individual experiment. Shown are average values for each time point from five independent experiments.
Although most of the DNA samples isolated from the GANC-resistant MMR-proficient cells also contained −1 or −2 microsatellite frameshifts, other types of mutations (3 of 30) were also detected (data not shown) in agreement with previous studies (9). In control clones not containing a repeat within the reporter gene, the construct remained relatively stable and we did not detect significant differences in stability between MMR-proficient and -deficient backgrounds (Fig. 2C and Table 1). The above information is invaluable for the design of the therapeutic out-of-frame vector (Fig. 1A). In theory, only a fraction of frameshift mutations should lead to the restoration of the correct reading frame because of the possibility of both insertions and deletions. Our data demonstrate that deletions of a single repeat unit of a given microsatellite repeat predominate. Taking this evidence into account, it should be possible to design vectors with a high propensity toward shifting into the correct reading frame, i.e., by having the toxin gene insert in the vector in a +1 reading frame in cases where it is preceded by a mononucleotide repeat.

Microsatellite mutation frequencies measured in our MMR-deficient cells (Fig. 2C and Table 2) roughly corresponded to those described by others (9, 14, 15). However, the relative differences between MMR-proficient and -deficient cells were somewhat smaller: −20-fold in our system as compared with 16−30-fold as described by Hanford et al. (9) or 25−100-fold as described by Kahn et al. (12). However, the latter studies compared mutation frequencies of MMR-deficient colon carcinoma cells either with those of unrelated MMR-proficient colon carcinoma cells or even with those of MMR-proficient normal cells of different type. It has been well documented that cells acquire a plethora of mutations during transformation. Some of these mutations might inactivate cell cycle checkpoint pathways, which might allow DNA replication in the presence of DNA damage, and thus permit the accumulation of additional mutations even in a MMR-proficient background. Indeed, Boyer and Farber (16) found a 75-fold difference in the mutation frequency of the same microsatellite in MMR-proficient normal human fibroblasts and fibrosarcoma cells. Clearly, genetic differences between cells of different origin make a direct comparison of mutation frequencies very difficult.

A recent study (10) used the human MMR-deficient colon cancer cells HCT116 and their MMR-proficient counterparts (HCT116+Chr3) where the MMR defect was corrected by chromosome 3 transfer. These two cell lines, although not isogenic, are more closely related than those used in the studies cited above. Interestingly, the observed 30-fold difference in the stability of a (CA)12 microsatellite is quite close to that of the (C)12 repeat examined in our study.

The 20-fold difference in the stability of the (C)12 microsatellite between MMR-proficient and -deficient cells is lower than might have been anticipated from the results of earlier studies, however, given the extremely low mutation frequency in the MMR-proficient cells, it is likely to be therapeutically exploitable. Moreover, it is highly likely that analysis of a larger number of mononucleotide and dinucleotide repeats will identify a sequence with a substantially higher therapeutic index. Thus, exploitation of the MSI phenotype, which is currently estimated to segregate with −15% of colon cancers, may represent a valid approach toward combating these tumors and, more importantly, their metastases.

One of the major challenges of tumor therapy is acquired resistance to treatment. Because the MSI phenotype is linked with defective MMR, the only chance the transduced cell has to escape death is either to stop replicating, which would in itself lead to tumor regression, or to silence the transcription of the transgene. The latter scenario is unlikely because gene silencing requires as a rule many cell divisions and because the microsatellite repeat tested in our study was unstable after only four replication cycles. Even if this problem should arise, it could be overcome by repeated transductions. It is therefore likely that the problem of resistance will not pose a substantial threat to this approach.

The study described above represents an initial step toward this goal. As the environment of cells in tumors differs dramatically from that in cell culture, it will be necessary to carry out in vivo experiments using, in the first instance, human tumor xenografts in nude mice. Should these experiments meet with success, the transducing vector will be remodeled to carry the suicide gene out-of-frame, which would be moved into the correct reading frame through selective frameshifting in MSI cells, as shown in Fig. 1A. In a gene therapy setting, most cells in solid tumors are not transduced and it is likely that not each cell will mutate the microsatellite. But as tumor cells are in close contact, it is anticipated that the suicide gene will exert a bystander effect, which should bring about the death not only of the transduced cell that acquired the frameshift mutation but also of a number of surrounding cells (17). A complete eradication of a tumor expressing TK has been reported, although only 10% of the tumor cells expressed the enzyme (17). Cytosine deaminase, another suicide gene frequently used in gene therapy trials, has been reported to be effective even if only 2% of the tumor cells were transduced (18), and strategies causing even more effective bystander effects are being developed (19). The system described in this study should permit the identification of the most effective microsatellite/enzyme/prodrug combination that could then be further developed for therapeutic use.

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