Establishment of Mutant Murine Mammary Carcinoma FM3A Cell Strains Transformed with the Herpes Simplex Virus Type 2 Thymidine Kinase Gene

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ABSTRACT. To establish cell systems appropriate for investigating the mode of action of antitherpetic nucleoside analogues, mutant cell strains were constructed from murine mammary carcinoma FM3A cells, which were deficient in TK, but were transformed with a recombinant plasmid DNA containing the HSV-2 TK gene. The transformed cells incorporated the viral DNA, expressed viral TK activity and showed unusually high sensitivity to the cytostatic action of the antitherpetic nucleoside analogues ACV and IVDU, both of which were only weakly inhibitory to the growth of the parent cells. Curiously, the FM3A cell strains transformed with HSV-2 TK gene showed a higher sensitivity to ACV and IVDU than the previously established cell line transformed with HSV-1 TK gene. This contrasts with the inhibitory effects of ACV and IVDU on acute HSV infection, since HSV-2 infection is slightly or considerably less susceptible than HSV-1 infection to inhibition by ACV or IVDU, respectively.

HSV encodes for a TK (EC 2,7,1,21) that differs from the cellular cytosol and mitochondrial TKs in a number of physical, immunological and kinetic properties (5). The viral TK is endowed with a broader substrate specificity, and hence is able to phosphorylate certain nucleosides and nucleoside analogues which are not detectably phosphorylated by the cellular TK. Several potent and selective antitherpetic agents have been developed; for example, ACV, IVDU, BVDU and other related compounds (4, 7–10).

In our previous study (2), we have established transformants from a TK-deficient mutant of murine mammary carcinoma FM3A cells to which a cloned HSV-1 TK gene was stably introduced to produce viral TK activity. Taking advantage of the differential properties as mentioned above for the viral and cellular TKs, we have
successfully demonstrated that the transformant cell line designated FStk-10/aprt-3/HSV-1 tk+ is a useful cell strain to track down nucleoside analogues that are preferentially activated by the HSV-1 encoded TK. Such nucleoside analogues exert a marked cytostatic effect on the transformant cells (2, 4). HSV-2 also encodes for a specific TK, which differs from the TK specified by HSV-1 in several aspects including substrate affinity. Such differences in substrate affinity may at least in part account for the differential susceptibility of HSV-1 and HSV-2 to the inhibitory effects of IVDU and BVDU (8, 11, 12). Therefore, it would be useful to establish a transformant cell line which is deficient in cellular TK activity but expresses HSV-2 TK activity. In conjunction with the previously established FStk-10/aprt-3/HSV-1 tk+ cell line (2), the HSV-2 tk+ transformant may constitute an attractive probe for nucleoside analogues which are activated differentially by the HSV-1 TK and HSV-2 TK and which would therefore differ in their cytostatic action on the two transformant lines. We have now established such HSV-2 tk+ transformant line by introducing a cloned DNA for the HSV-2 TK gene into murine FM3A cells that are deficient in TK. The biological and biochemical characteristics of these cell strains are described in the present report.

MATERIALS AND METHODS

Cell cultures. F28-7 is a wild-type subclone of FM3A, which was originally derived from a spontaneous mammary carcinoma in a C3H/He mouse. A mutant cell line deficient in TK and APRT (FS tk− 10/aprt−3) isolated from F28-7 and its transformant (FS tk−10/aprt−3/HSV-1 tk+) were described previously (2). The cells were grown in suspension culture at 37°C in ES medium (Nissui Seiyaku Co., Tokyo) (13) supplemented with 2 % fetal calf serum (Gibco, N.Y.)

Transformation of TK-deficient cells with HSV-2 TK gene. The HSV-2 TK gene was introduced into FStk−10/aprt−3 cells by the calcium phosphate coprecipitation method as calf described previously (2, 3), using pBR322 recombinant plasmid, pGR18 (14). Briefly, 5 ml- aliquots of cell suspension at 5×10⁶ cells/ml in ES medium containing 0.5 % fetal serum were inoculated into 6-cm glass Petri dishes and the cells were allowed to adhere to the bottom for 30 min. Then, plasmid DNA (20 μg), circular or linearized with restriction endonuclease Hind III, coprecipitated with calcium phosphate without carrier DNA, was added to each dish. As described previously (3), the cells were allowed to take up the plasmid DNA for 5 h, with 10% dimethylsulfoxide present during the last 30 min. Then the medium was replaced by 20 ml normal growth medium and the cells were cultured for 20 h to allow expression of the transformed phenotypes. The resulting transformants were selected on the surface of HAT- agarose medium [ES medium plus 5% dialyzed fetal calf serum and 0.35% agarose (Seakem agarose ME, Marine Colloids Inc., Mich.) containing 100 μM hypoxanthine, 1 μM amethopterin (Sigma Chemical Co., St. Louis, Missouri) and 10 μM thymidine]. The transformant clones isolated were then cultured in medium containing 100 μM hypoxanthine and 10 μM thymidine until the cells recovered from the toxicity of amethopterin. The transformant was designated FStk−10/aprt−3/HSV-2 tk+.

Enzyme assay. The cells were disrupted by repeating 3 x freeze-thawing and centrifuged at 105,000 x g for 1 h. The supernatant of the crude cell extracts was then assayed for TK activity as described previously using [methyl-³H]thymidine (42 Ci/mmol; Amersham, U.K.) as substrate (2). Protein content was measured using Biorad Protein Assay Kit (Biorad, U.S.A.).
Assay of drug activity. As described previously (1, 2), cell suspensions each containing 10^4 cells/ml were prepared, and aliquots of 2 ml were transferred to 35-mm plastic dishes. Cells were cultured in the presence of various concentrations of ACV or IVDU for 3 days at 37°C, and the cell numbers were counted with a microcell counter (Sysmex, model cc-150A, Toa Iyodenshi, Co., Kobe, Japan). The cell number counted in the presence of drugs was expressed as a percentage of that without drug. Values obtained in this way were almost equal to those calculated from the cell doubling time as described previously (1).

Southern blot hybridization. High-molecular weight DNA was prepared as described previously (3), and digested with restriction endonuclease EcoR1 (New England Biolabs, U.S.A.) under the conditions recommended by the suppliers. 15 μg of the DNA was subjected to electrophoresis in a 1.0% agarose gel, as described (3). The DNA was transferred onto a membrane filter (Gene Screening) (New England Nuclear U.S.A.) and the resulting blots were hybridized with a nick-translated DNA prepared from recombinant plasmid, pGR18. Nick-translation, hybridization, and washing conditions were as described previously (3). All other chemicals used in this study were of the highest grade commercially available.

RESULTS AND DISCUSSION

Transformant cells were obtained by introducing the HSV-2 TK gene into the TK-deficient FStk-10/aprt-3 cells by a gene-transfer technique described in MATERIALS.

Fig. 1. Detection of HSV-2 TK gene sequences in cells transformed by the HSV-2 TK gene. DNAs digested with endonuclease EcoRI were subjected to Southern blot analysis. The probe used was a whole DNA of plasmid clone pGR18 containing the viral TK gene. For details, see MATERIALS AND METHODS. Lanes: 1, F28-7 (wild-type); 2, FStk-10/aprt-3; 3, FStk-10/aprt-3/HSV-2 tk+ c2; 4, FStk-10/aprt-3/HSV-2 tk+ c5.
AND METHODS. Using circular plasmid DNA, 39 transformants were isolated at a frequency of $8 \times 10^{-6}$. Using Hind III digested linearized plasmid DNA, 5 transformants were obtained at a frequency of $1 \times 10^{-6}$. The resulting transformants FStk-10/aprt-3/HSV-2 tk+ c2 (c denotes a cloned transformant obtained with circular plasmid DNA) and FStk-10/aprt-3/HSV-2 tk+ c5 incorporated the HSV-2 TK genes into high molecular weight DNAs and stably maintained these genes upon passages for more than 3 months (Fig. 1). They produced TK activity (Table 1) and grew in HAT medium (see MATERIALS AND METHODS) to the same extent as in normal medium. The Southern blot hybridization patterns for the 2 transformants differed from each other, and this may reflect different patterns of DNA integration. Since digestion of clone pGR18 DNA by EcoRI gives rise to two fragments of 4.75 kilobase (kb) and 3.85 kb, respectively (14), one of the two bands of about 10 kb seen in Fig. 1 lane 4 should originate from the viral TK gene integrated in the nuclear genome. The size of the two bands seen in lane 3 is close to those of the above mentioned Eco RI digestion products of clone pGR18, but the significance of this coincidence is not clear. Fig. 1 shows that there is no sequence homology between mouse cellular DNA and HSV-2 TK gene. In the two transformants, the number of incorporated HSV-2 TK genes could be estimated to be at most 10 if the faintest band corresponding to about 4 kb in lane 4 in Fig. 1 was taken as 1. However, the number of TK gene copies does not necessarily coincide with the TK activity (Table 1 and Ref. 2). The TK activity of FStk-10/aprt-3 cells was about 1% of that of the wild-type cells. The TK activities of the two transformant cells were respectively 61.4% and 9.6%, of the TK activity of the wild-type cells (Table 1). A TK activity as low as 10% sufficed for the cells to grow in HAT medium, as the FStk-10/aprt-3/HSV-2 tk+ c5 cells grew in HAT medium whereas the FStk-10/aprt-3 cells did not.

The viral TK is known to have a broader substrate specificity than the cellular TK (5), and, consequently, is able to phosphorylate a wide variety of nucleosides and nucleoside analogues (see Ref. 4). Two of the antierpetic compounds, namely ACV and IVDU were used as test reagents to show that the two transformant clones, c2 and c5 of FStk-10/aprt-3/HSV-2 tk+ cells, because of the presence of viral TK activity, became sensitive to the inhibitory action of the antierpetic compounds. As control, a transformant FStk-10/aprt-3/HSV-1 tk+ cell line which produces HSV-1 TK activity and has been established previously (2) was used. As shown in Fig. 2A, FStk-10/aprt-3/HSV-1 tk+ cells were 20-fold more sensitive to ACV than were the wild-type F28-7 cells, when the 50% inhibitory dose (ID$_{50}$) values of ACV were compared. More importantly, the two transformants producing HSV-2 TK activity, i.e., FStk-10/aprt-3/HSV-2 tk+ c2 and FStk-10/aprt-3/HSV-2 tk+ c5 cells were

<table>
<thead>
<tr>
<th>Cell strain</th>
<th>TK activity</th>
<th>dTMP formed /50 μg protein/30 min (cpm)</th>
<th>Specific activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F28-7 (wild-type)</td>
<td></td>
<td>515,400</td>
<td>100</td>
</tr>
<tr>
<td>FStk-10/aprt-3</td>
<td></td>
<td>5,000</td>
<td>0.97</td>
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<tr>
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<td>61.4</td>
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<tr>
<td>FStk-10/aprt-3/HSV-2 tk+ c5</td>
<td></td>
<td>49,400</td>
<td>9.6</td>
</tr>
</tbody>
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Values are averages for duplicate assays.
Mouse Cell Mutant Producing HSV-2 TK

respective 90- and 160-fold more sensitive to ACV than were the wild-type F28-7 cells. That the HSV-2 tk+ cells were more sensitive to the cytostatic action of ACV than were the HSV-1 tk+ transformants contrasts with previous findings that an acute HSV-1 infection is equally (12), if not more susceptible (6), to inhibition by ACV than HSV-2 infection. For IVDU, again, a higher sensitivity was noted with the HSV-2 tk+ than with the HSV-1 tk+ cell lines (Fig. 2B). The ID50 of IVDU for the HSV-2 tk+ transformant cell lines was exceptionally low (50 pg/ml), that is almost 5 orders of magnitude lower than that for the wild-type F28-7. That the HSV-2 tk+ transformant cell lines were more sensitive to the cytostatic action of IVDU than were the HSV-1 tk+ transformants is, again, a finding that contrasts with the differential susceptibility of acute HSV-1 and HSV-2 infections, the latter being about 100-fold less susceptible to inhibition by IVDU than the former (8, 11, 12). The
reason(s) for this apparent anomaly in the differential effects of IVDU against HSV-infected and HSV TK gene-transformed cells remain to be elucidated.

The differential susceptibility of HSV-1 and HSV-2 infection to inhibition by IVDU and BVDU has been attributed to the fact that, while in HSV-1-infected cells IVDU and BVDU are phosphorylated onto the 5'-mono-, 5'-di- and 5'-triphosphates, they are stuck at the 5'-monophosphate stage in HSV-2-infected cells (7-11). This may, in turn, be ascribed to the particular behavior of the viral TK: HSV-1 TK would be able to phosphorylate BVDU (and IVDU) successively to the 5'-mono- and 5'-diphosphate, whereas the HSV-2 TK would phosphorylate BVDU (and IVDU) to the 5'-monophosphate but not further onto BVDU (or IVDU) 5'-diphosphate. The present findings indicate that the HSV-2 tk+ transformant cell lines are at least as, if not more, susceptible to the cytostatic action of IVDU than is the HSV-1 tk+ transformant cell line. This may be interpreted to mean that either (i), assuming that the active form of IVDU is its 5'-triphosphate, HSV-2 TK of the transformed cells, unlike its counterpart of the infected cells, is capable of phosphorylating IVDU onto IVDU 5'-diphosphate, or (ii), if IVDU is phosphorylated only onto the 5'-monophosphate, its cytostatic action on HSV-2 TK+ transformant cells may be mediated by IVDU 5'-monophosphate possibly at the level of thymidylate synthase. Additional experiments have been planned to investigate the nature of the phosphorylated products of IVDU formed in HSV-2 TK+ cells.

It should also be mentioned, that the extent of sensitivity to a given antiviral nucleoside varied from one experiment to another. This fluctuation correlated with the stability of the integrated viral TK gene in the transformant cells: the cells which had lost the viral TK gene seemed to gain growth advantage during subsequent passages. Thus, the cytostatic action of the antiviral nucleoside analogues may be strongly influenced by the retention of integrated viral TK gene in the transformant cell population. To eliminate any untransformed cells, the transformant cells should, as described in MATERIALS AND METHODS, be passed in HAT medium in which only tk+ cells grow.

Our results thus indicate that the transformant cell line FStk-10/aprt-3/HSV-2 tk+ is a useful cell strain to detect nucleoside analogues that are specifically activated by the viral TK. Growth of the HSV-2 TK+ transformant cell line is inhibited by IVDU at a concentration as low as 50 pg/ml. While the mechanism of action of IVDU, ACV and other nucleoside analogues in the HSV-2 tk+ cell lines remains to be elucidated, especially with regard to the reversed order of sensitivity of the HSV-1 and HSV-2 tk+ transformants towards IVDU, the present findings may have far-reaching therapeutic implications in that they signal an extremely potent cytostatic action of nucleoside analogues such as IVDU on tumor cells which have been transformed by the HSV-2 TK gene.

Acknowledgements. We thank Dr. Gary S. Hayward (Johns Hopkins University, U.S.A.) for providing us with the recombinant plasmid pGR18. This work was supported in part by Grants-in-Aid for a comprehensive 10-Year Strategy of Cancer Control from the Ministry of Health and Welfare of Japan, and for Cancer Research and for Special Project Research from the Ministry of Education, Science and Culture of Japan.
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(Received for publication, July 1, 1986)