Decreasing Sensitivity to Cytotoxic Agents Parallels Increasing Tumorigenicity in Human Fibroblasts

Anne R. Kinsella and M. Sally Haran

INTRODUCTION

Resistance to cytotoxic drugs is generally accepted to be a more common property of tumor cells than of normal cells. This has been attributed in part to the ability of tumor cells to more readily amplify their target genes than normal cells (Ref. 1 and references therein) and to progression-linked changes in the pathways of purine and pyrimidine biosynthesis (2).

The availability of a series of human fibroblast cell lines of common genetic origin that exhibit normal, immortalized, and tumor phenotypes (3, 4), provides the ideal system in which to address the following questions. Do tumor cells really have the potential to be more resistant to cytotoxic drugs than normal cells, and if so can this be correlated with the presence and expression of a specific oncogene, general chromosomal instability which might be a preliminary to gene amplification (5), a transformation-linked imbalance in the purine and pyrimidine salvage pathways, difference in drug uptake, or some as yet undefined property of the tumor phenotype?

To test these possibilities the human fibroblast cell lines were exposed to MTX, an inhibitor of purine biosynthesis used in the clinic, resistance to which in vitro and in vivo can be due to amplification of the gene for dihydrofolate reductase (6, 7). The same cells were also exposed to PALA, a specific inhibitor of de novo pyrimidine biosynthesis, in vitro resistance to which is due almost exclusively to amplification of the CAD gene (8-10). For both drugs the intrinsic sensitivities of the different cell lines were compared with the phenotype and the resistant clones were tested for gene amplification.

MATERIALS AND METHODS

Cell Lines. The cell lines chosen for study (Table 1) were the “normal” KMS-6 human embryo fibroblast cell line, its 60Co-immortalized derivative KMST-6 (3), the KN-NM cell lines established from the fibrosarcomas that resulted from activated N-ras infection of the immortalized KMST-6 cell line (4), the SUSM-1 immortalized human fetal liver cell line (3), and its N-ras-infected derivative (4). Normal diploid human skin fibroblasts (FF) and an activated N-ras-infected subclone were used as controls. All cell lines were routinely maintained in Dulbecco’s modified Eagle’s medium (GIBCO, Paisley, Scotland) supplemented with nondenized fetal bovine serum (GIBCO; batch 9805). The same serum batch was used for the entire series of experiments.

Measurement of Intrinsic Drug Sensitivities. To assay for drug resistance (sensitivity) 500 test cells were plated onto a feeder layer of 5 x 10^4 irradiated (5000 rads) EJ human bladder carcinoma cells (5 plates/point). The feeder cell line was really necessary only for the successful cloning of the normal fibroblast cell lines but was included for all cell lines to eliminate any complications that might arise from feeder cell metabolites with regard to the supply of “salvage pathway” substrates. MTX (Lederle, Pearl River, NY) and PALA (Drug Synthesis and Chemistry Branch, NIH, Bethesda, MD) were added 4 h later and the plates medium changed once a week, i.e., twice during an incubation period of 3 weeks. After 3 weeks resistant clones were isolated and maintained under selection for slot blot analysis (11). The remaining plates were fixed and stained. Identical experiments were performed using dialyzed fetal bovine serum, and where appropriate hypoxanthine (100 μM) or uridine (10 μM) were added; all were purchased from Sigma Chemical Co., St. Louis, Mo. All experiments were performed at least three times. All the illustrative data to be presented under “Results” will be of representative experiments. Experiments done within the same time frame were extremely reproducible, but generally the cells became more resistant with increasing time in culture, although the differential resistance was always maintained.

RESULTS

The chromosomally and phenotypically normal FF, cell line and the “normal” KMS embryonic fibroblast cell line were equally sensitive to MTX (Fig. 1). The chromosomally abnormal, immortalized derivative of KMS-6, KMST-6, showed increased resistance, which was increased still further in the activated N-ras-infected KMST cell pool (Fig. 1), to a level equal to that observed for the KN-NM tumor cell line derived from a fibrosarcoma produced on injection of the KMST-activated N-ras-infected cell pool into nude mice. The 50% lethal dose for the tumor KN-NM cells (Fig. 1) was 10 times that for the normal KMS cells.

The differences in sensitivity were even more marked when the same cell lines were tested for resistance to PALA, with the KN-NM tumor cell line exhibiting absolute resistance to PALA concentrations of 10 mM (Fig. 2). The resistance was unaffected by retroviral infection per se as seen for KMST PALA resistance (Fig. 3), but in the case of the two normal fibroblast cell lines KMS and FF, resistance was increased by infection with the activated N-ras-cDNA (Fig. 2, inset). N-ras injection of the SUSM-1 cell line, although it failed to induce tumorigenicity, resulted in increased resistance to MTX (Fig. 4). Similarly, all

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The abbreviations used are: MTX, methotrexate; PALA, N-(phosphonacetyl)-L-aspartate.
**Table 1** Growth characteristics and phenotypic properties of the cell lines used

<table>
<thead>
<tr>
<th>Cell strain</th>
<th>Origin</th>
<th>Morphology</th>
<th>Lifespan</th>
<th>Agar growth</th>
<th>Tumors in nude mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>FF&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Human foreskin 46,XY</td>
<td>N&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Finite</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>FF, N-ras</td>
<td>Activated N-ras infection of FF&lt;sub&gt;1&lt;/sub&gt;</td>
<td>T</td>
<td>Finite</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>KMS-6</td>
<td>Human fetus 46,XX</td>
<td>N</td>
<td>Finite</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>KMS-6 N-ras</td>
<td>Activated N-ras infection of KMS-6</td>
<td>N</td>
<td>Finite</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>KMST-6</td>
<td>&lt;sup&gt;*Co-ray treatment KMST-6&lt;/sup&gt;</td>
<td>T</td>
<td>Immortal</td>
<td>± (0.2%)</td>
<td>0</td>
</tr>
<tr>
<td>KN-NM</td>
<td>Fibrosarcoma; activated N-ras infection of KMST</td>
<td>T</td>
<td>Immortal</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SUSM-1</td>
<td>Human fetal liver</td>
<td>T</td>
<td>Immortal</td>
<td>± (0.06%)</td>
<td>0</td>
</tr>
<tr>
<td>SUSM-N-ras</td>
<td>Activated N-ras infection of SUSM-1</td>
<td>T</td>
<td>Immortal</td>
<td>+</td>
<td>0</td>
</tr>
</tbody>
</table>

* N, normal morphology; T, transformed morphology.

![Fig. 1](image1.png)

![Fig. 2](image2.png)

![Fig. 3](image3.png)

Anchorage-independent, nontumorigenic clones derived from activated N-ras infection of the KMST cell line exhibited the same resistance to PALA as the tumorigenic KN-NM cell line (data not shown).

Unfortunately, very few of the MTX- and PALA-resistant clones isolated have been analyzed for amplification of their target dihydrofolate reductase (MTXR) or CAD (PALA<sup>+</sup>) genes. This was due to their lack of stable resistance (particularly in the case of PALA<sup>+</sup> clones) to the drug concentration in which they were originally isolated. Consistent with this unstable resistance, none of the clones analyzed exhibited gene amplification.

When the experiments to measure the intrinsic sensitivities of the cell lines to MTX and PALA were repeated under conditions in which dialyzed fetal bovine serum was substituted for nondialyzed fetal bovine serum the sensitivities of the cell lines to both drugs increased and the differential resistance of the KMST and KN-NM cell lines was almost completely eliminated (Figs. 5 and 6). However, the resistance of the KN-NM cell line could be restored by supplementing the medium containing dialyzed serum with uridine (10 μM) (Fig. 7). In the case of MTX, the resistance of the KN-NM cell line could be restored to approximately 80% of that seen in nondialyzed serum by the addition of 100 μM hypoxanthine (data not shown). The presence of both thymidine (50 μM) and hypoxanthine (100 μM) made no difference to the survival seen following
the addition of hypoxanthine alone. Thymidine alone (50 μM) failed to restore the high level of PALA resistance of the KN-NM cell line (Fig. 7) suggesting salvage pathway specificity. Also, although the isolated MTX<sup>R</sup> and PALA<sup>R</sup> clones selected in dialyzed serum were stably resistant to the drug concentration in which they were originally isolated they again did not exhibit amplification of their target genes. The increased resistance did not correlate with an increase in growth rate (data not shown).

DISCUSSION

MTX and PALA are powerful inhibitors of de novo purine and pyrimidine biosynthesis. Resistance to both drugs is commonly due to amplification of the corresponding target gene. The relationship between gene amplification and tumorigenicity, as assessed by resistance to PALA and MTX, has recently been studied in mouse fibrosarcoma (12) and rat liver cell lines (5) exhibiting different degrees of tumorigenicity. In both studies a striking parallel was observed between the acquisition of drug resistance due to gene amplification and increasing tumorigenicity. Although gene amplification need not necessarily be distinct from “salvage pathway” effects which might facilitate the rescue of cells for long enough to facilitate gene amplification, it is not the mechanism operating in the present system.
This is not surprising for the normal cells (1) but perhaps is surprising for the highly chromosomally abnormal immortal and tumorigenic cell lines (5). However, the increased sensitivity of all the cell lines to both MTX and PALA when the experiments were performed in dialyzed fetal bovine serum suggests an important role for the “salvage pathways” of purine and pyrimidine biosynthesis in their original resistance. This is confirmed by the restoration of the resistance of the KN-NM cell line to PALA to the levels observed in nondialyzed serum by the addition of uridine (Fig. 7) and restoration of the MTX resistance by hypoxanthine. Interestingly, thymidine alone had no influence on MTX toxicity in dialyzed serum in contrast to some clinical reports (12) and this observation coupled with that for hypoxanthine and thymidine together might suggest that excessive thymidine in the serum is masking the effect. The elimination of the difference in resistance to MTX and PALA between the immortal KMST-6 cell line and KN-NM tumor cell line in dialyzed serum also suggests that the differences in resistance observed (Figs. 1 and 2) are due to differences in “salvage pathway” activity between the cell lines.

These data therefore, although preliminary, support the concept of a progression-linked change in the key metabolic pathways of purine and pyrimidine synthesis (2). Not surprisingly, inhibitors of de novo pyrimidine synthesis, such as PALA, produce a reduction in the intracellular pyrimidine ribonucleoside (UTP and CTP) and deoxynucleoboside (dCTP) triphosphate pools (14–17). Moreover, the antiproliferative, cytotoxic, and antitumor effects of PALA can in large part be reversed by the addition of exogenous uridine (18–22) and partially reversed by exogenous deoxycytidine (23). What all these exogenous agents have in common is that they are either substrates of “salvage pathway” enzymes or components of the de novo pathway distal to the inhibitory block.

Interestingly PALA in preclinical trials showed a unique spectrum of activity, being cytotoxic to several experimental tumors (Lewis lung and B16 melanoma) but inactive against several more rapidly spreading murine leukemias (24, 25). However, in clinical trials PALA failed to show any significant effect against a wide spectrum of tumor types in humans (26–33). One explanation of this might be that human tumor cells are able to use circulating uridine in the synthesis of pyrimidines, i.e., use their salvage pathways more efficiently than mouse cells. The data from the present study strongly support this interpretation. Certainly, Denton et al. (34) demonstrated a concurrent increase in the activities of the key enzymes of both the de novo and salvage pathways of pyrimidine synthesis in human colon tumors, while in studies of rat hepatomas (2), the activities of the salvage enzymes were higher than the rate-limiting enzymes of de novo pyrimidine synthesis. Moreover, it is well known that the relative rate of reduction of the purine nucleotide and thymidylate pools in response to MTX varies from cell type to cell type and is dependent on the partition of the reduced folate into those forms required for inosinate versus thymidylate synthesis (35) and the availability and ability of the cells to utilize exogenous hypoxanthine and thymidine. Comparison of the enzymic capacities of the de novo and salvage pathways for purine biosynthesis in normal and neoplastic animal tissues (36) showed the high activity and high affinity for substrate of a key salvage pathway enzyme (purine phosphoribosyltransferase) when compared with a key enzyme of the de novo synthesis pathway (amidophosphoribosyltransferase). All these data suggest an important role for salvage pathway enzymes in the circumscription of the action of inhibitors of de novo purine and pyrimidine biosynthesis, supported by studies using the transport inhibitor of nucleosides and bases, dipyridamole (37).

Thus, we have demonstrated for the first time in isogenic human cells, exhibiting a spectrum of phenotypes representative of the stages in the progression to malignancy, the importance of salvage pathways in the “rescue” of human cells from the cytotoxicity of inhibitors of the de novo pathways of purine and pyrimidine biosynthesis. The fact that the effect is in part N-ras specific may reflect the influence mutant N-ras has on the membrane and the transport of nucleosides and bases into the cell.

The data therefore support strongly the hypothesis that the concentrations of nucleosides in the plasma (38) and possibly from adjacent necrotic cells might protect tumor cells from the inhibitors of de novo synthesis. This confirms the need for inhibitors of the salvage enzymes to be administered together with inhibitors of the de novo pathway enzymes during cancer chemotherapy (2, 34, 36–38). In a recent study in rat hepatoma cells and human colon cells (39) azidodeoxythymidine inhibition of thymidine kinase acted synergistically with MTX and 5-fluorouracil (inhibitors of de novo synthesis) to enhance cytotoxicity.

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REFERENCES

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