Cytogenetic and Molecular Characterization of Tumors in Nude Mice Derived from a Multidrug-resistant Human Leukemia Cell Line

Anna B. Hill, William T. Beck, and Jeffrey M. Trent

ABSTRACT

We have evaluated whether selection of a human tumor leukemic line for resistance to vinblastine (Velban; VLB) alters its tumorigenicity. To address this question, CEM and CEM/VLB<sub>100</sub> cells [which express the multiple drug-resistant (MDR) phenotype via amplification of the P-glycoprotein gene] were characterized by several techniques including chromosome banding, in situ hybridization, Southern blotting, RNA dot blotting, in vitro drug sensitivity, and tumorigenicity in nude mice. Analysis of the chromosome banding patterns of both drug-sensitive CEM cells and the MDR CEM/VLB<sub>100</sub> cells revealed that the two lines differed primarily by the presence of a large metacentric marker chromosome associated with the acquisition of VLB resistance. In situ hybridization of a P-glycoprotein complementary DNA to metaphase chromosomes showed that the amplified P-glycoprotein genes in the CEM/VLB<sub>100</sub> cell line were localized to this large marker. Tumorigenicity of both the CEM and CEM/VLB<sub>100</sub> cell lines was measured after injection of 10<sup>7</sup> cells/nude mouse. The results showed that 4 of 4 drug-sensitive and 4 of 5 drug-resistant cell lines formed tumors in 5–10 wk. By comparison with the parental line, three of the four tumors arising from the CEM/VLB<sub>100</sub> line retained their drug-resistance properties as measured by vinblastine resistance in vitro and elevated P-glycoprotein mRNA expression associated with P-glycoprotein gene amplification. In addition, tumors retaining the MDR phenotype also retained the large metacentric marker chromosome. One tumor arising from CEM/VLB<sub>100</sub> reverted to the drug-sensitive phenotype, with a resultant decrease in P-glycoprotein mRNA expression and loss of P-glycoprotein gene amplification. This revertant was also missing the large metacentric marker present in all cells from the CEM/VLB<sub>100</sub> parent. Our experiments show that the acquisition of the MDR phenotype resulting from overexpression of P-glycoprotein in the plasma membrane does not effect the tumorigenicity of human CEM cells.

INTRODUCTION

Cultured mammalian cells exposed to a diverse group of amphiphilic heterocyclic cancer chemotherapeutic agents often become cross-resistant to other members of this group and are referred to as MDR. Some of the agents associated with MDR include colchicine, VLB, doxorubicin, and actinomycin D. The principal mechanism of resistance to all of these agents appears to be reduced net drug accumulation within the cell (1). Human, mouse, and hamster MDR cell lines overexpress a M, 170,000 plasma membrane glycoprotein, termed P-glycoprotein (2). Overexpression of P-glycoprotein is often correlated with amplification of the P-glycoprotein gene (3); however, Trent et al. (4) have shown that P-glycoprotein can be overexpressed in cell lines without gene amplification. Recently, P-glycoprotein was shown to be similar in amino acid sequence to the bacterial transport protein hemolysin-B and by analogy is thought to play an important role in exporting chemotherapeutic drugs from the cell (5).

Studies by Biedler and coworkers have shown that as Chinese hamster fibroblast cell lines developed MDR, their cellular morphology was markedly altered: the drug-sensitive lines appeared morphologically transformed while the drug-resistant variants developed a normal, apparently more differentiated, cellular morphology (6). When these MDR cell lines were tested for tumorigenicity they were uniformly nontumorigenic, in contrast to the drug-sensitive lines which readily induced tumors in hamster cheek pouches. In addition, MDR hamster cell lines had an increase in the number of EGF receptors compared to the drug-sensitive lines (7).

Related studies suggesting a loss of tumorigenicity following selection for drug resistance have been reported by others: drug-resistant murine leukemia cells were shown to be more immunogenic than their drug-sensitive parent and demonstrated a decreased ability to grow as tumors (8–10). Finally, a recent study using a rat brain cell line transformed by adenovirus showed that a variant resistant to methylglyoxal bis-(guanylhydrazone) had also lost its ability to form tumors in nude mice (11). Unlike the results from rodent systems described above, human tumors that become clinically “MDR” following noncurative chemotherapy continue to grow aggressively in vivo. Also, recent studies of biopsies from ovarian carcinomas have shown that at least a portion of these clinically progressive MDR tumors overexpress P-glycoprotein in vivo (12).

In this report, we examined whether a MDR subline of the human T-cell leukemia cell line CEM has lost its ability to form tumors concordant with its development of MDR. Our study suggests that in contrast to a loss of tumorigenic potential associated with the development of MDR in rodent-derived cell lines, selection for the MDR phenotype in human cells has no effect on tumorigenicity.

MATERIALS AND METHODS

Cell Culture and Chromosome Preparation. The development of the drug-sensitive CEM line (13), derived from a human T-cell leukemia, and a VLB-resistant CEM subline (CEM/VLB<sub>100</sub>) (14) have been previously described. Both cell lines were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum; medium for CEM/VLB<sub>100</sub> also contained 100 ng VLB/ml (Eli Lilly).

Chromosome preparation and G or Q banding was performed as previously described (15). A minimum of 25 banded cells were analyzed per specimen, with results expressed according to International System for Human Cytogenetic Nomenclature recommendations (16). Cells for chromosome analysis from nude mouse tumors were removed under sterile conditions, normal tissue was separated from tumors, and tumors were weighed in sterile Petri dishes, finely minced, and placed into M3 medium (17) that was modified by adding 20% bovine pituitary extract. This medium has been especially developed for growing primary tumor cells. Once cell growth from tumors was well established (~2 wk in vitro) chromosomes were prepared as above.

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3 The abbreviations used are: MDR, multidrug resistance; VLB, vinblastine; EGF, epidermal growth factor; SDS, sodium dodecyl sulfate; LD<sub>50</sub>, 50% lethal dose; CEM, CCRF-CEM, a T lymphoblastoid cell line.

4 A. Leibovitz, unpublished observations.

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The generation time of CEM and CEM/VLB100 parental lines was measured by plating 2,000 cells/60-mm dish in 0.4% agar and counting colonies on Days 1 and 3 as described by Leibovitz and Mazur (18).

In Situ Hybridization. Plasmid CHP1 (3) containing a hamster P-glycoprotein cDNA insert was nick translated with [3H]thymidine and hybridized in situ to CEM and CEM/VLB100 metaphases using methods previously described (19). In order to ensure the correct identification of all chromosomes, cells were Q banded and photographed prior to in situ hybridization.

Induction of Tumors in Nude Mice. CEM and CEM/VLB100 cells were washed in serum-free medium and injections of 10^7 cells/0.5 ml medium were given subcutaneously in the left groin of male BALB/c nude mice. Five mice per cell line were injected. Tumors which grew as solid masses at the site of injection were observed in 5 to 10 wk. Animals were sacrificed and tumors were placed into culture as described above. Since tumor cells were grown in suspension cultures, no contaminating fibroblasts from the nude mouse were present.

DNA and RNA Analysis. DNA was isolated by direct plating tumor cells in a buffer containing 0.2 M Tris, 0.1 M EDTA, pH 8.0, 50 µg/ml proteinase K/ml and 0.5% SDS. The lysate was incubated overnight and purified by phenol and chloroform extraction followed by ethanol precipitation. The amount of DNA was quantitated by the 4,6-diamino-2-phenyl indole dihydrochloride method (20). Ten µg of DNA from each CEM or CEM/VLB100 subline were digested with EcoRI and separated on a 1.0% agarose gel. DNA was transferred by the method of Southern (21) to Gene Screen Plus (New England Nuclear). Prehybridization and hybridization of the filter were done according to the manufacturer's instructions.

RNA was extracted by treating exponentially growing cultures of tumor cells with guanidinium isothiocyanate and 10 mM vanadylribonucleoside complexes (Bethesda Research Labs) as previously described (22). The lysate was placed over a cesium chloride cushion (5.7 M in 0.1 M EDTA) and RNA was pelleted by ultracentrifugation (150,000 x g for 18 h). The RNA pellet was resuspended in 10 mM Tris, 1 mM EDTA, pH 8.0, and 0.5% SDS and was ethanol precipitated. The amount of RNA was quantitated by spectrophotometry. RNA dot blots were prepared by applying 10, 5, 2.5, and 1.25 µg of RNA from each sample onto nitrocellulose using a minifold apparatus (Schleicher and Schuell). RNA filters were washed with 10× saline sodium citrate, baked at 80°C in a vacuum oven for 2 h, prehybridized, and hybridized according to the manufacturer's instructions. RNA filters were washed with 0.1% saline sodium citrate, 1.0% SDS, at 55°C, dried, and placed on X-ray film. Plasmids used for DNA and RNA hybridizations included a human P-glycoprotein cDNA clone HRP1 (23) (generously provided by V. Ling), human β-actin cDNA (24), and a cDNA clone (pE7) encoding a portion of the EGF receptor gene (25). Restriction fragments from plasmids were 32P-labeled by the random primer method of Feinberg and Vogelstein (26).

Drug Resistance Assays. Drug sensitivity was determined by cloning cells in soft agar as previously described (27). Underlayers of modified M3 medium containing 0.9% low melting point agar (Bethesda Research Labs) were placed in 60-mm dishes. Two hundred cells per sample (performed in duplicate) were placed into modified M3 medium containing 0.4% low-melting-point agar and plated over the underlayers. In plates containing VLB, the drug was present in both the upper and underlayer. Plates were incubated at 37°C and colonies counted manually using an inverted microscope on Day 10. Plating efficiency of the control cells was 15%; LI50 values were calculated by normalizing growth of colonies in drug to the control value.

RESULTS

Cytogenetic Characterization of CEM and CEM/VLB100 Cell Lines

Chromosome banding analysis of the CEM cell line has been presented elsewhere (28). Briefly, the line is characterized by several clonal numeric and structural chromosome alterations, including a modal chromosome number of 87 and the following identifiable structural chromosome alterations: dup(1)(p32 → 36); del(4)(q31); del(6)(q13); and t(9;?)(p22;?). Additionally, four unidentifiable marker chromosomes (Umars) were clonal features of the drug-sensitive CEM cell line (Fig. 1).

Chromosome banding analysis of CEM/VLB100 was similar to the CEM cell line in modal chromosome number (n = 89) and retained the majority of numeric and structural abnormalities of CEM. However, clonal chromosome changes unique to the resistant subline included a loss of the del(4)(q31) and del(6)(q13) chromosomes and replacement of two of the four Umars with two new Umars (Fig. 2A). The most striking change unique to the CEM/VLB100 subline was the presence of a large submetacentric marker chromosome (approximately the size of a chromosome 1). The long arm of this marker (termed M1) displayed a large area of intermediate banding suggestive of an ABR (Fig. 2, inset). ABRs (like homogeneously staining regions) have been demonstrated to represent sites of DNA sequence amplification (29).

In situ hybridization to metaphase chromosomes from the CEM/VLB100 subline was performed to identify whether the chromosomal locations of amplified P-glycoprotein sequences were associated with the putative ABR region. A final DNA concentration of 0.5 µg/ml of the probe was used, with slides incubated for 7 days at ~80°C. Distribution of all grains from 33 cells was examined with 32% (10 of 33) of all cells exhibiting one or more grains to the ABR of the metacentric marker (M1 ABR) (Fig. 2, B-E). Results revealed that ~10% of all chromosomal grains were localized to the M1 ABR, while 90% of grains along this marker were localized to the ABR.

Tumor Formation of CEM and CEM/VLB100 Cell Lines

We tested whether the tumorigenicity of CEM/VLB100 and CEM cells differed by giving injections of 10^6 cells s.c. into 5-wk-old nude mice. Tumors from both cell lines were observed within 5 to 10 wk (Table 1). Tumors grew as solid masses at the sites of injection. The frequency of tumor formation was

![Fig. 1. Q-banded cell from the parental (VLB-sensitive) CEM cell line. Detailed banding analysis of this line has been presented elsewhere.](cancerres.aacrjournals.org)
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Fig. 2. A, Q-banded karyotype of the CEM/VLB100 cell line. This cell line was characterized by a near-tetraploid modal chromosome number and relatively few clonal structural chromosome alterations. Arrow, dup(1)(p32–36) chromosome found in both the CEM (Fig. 1) and CEM/VLB100 cell lines. Closed box, two copies of a large metacentric marker chromosome (M1) which was unique to the CEM/VLB100 cell line (see text); M, marker. B–E, pictorial documentation in the CEM/VLB100 cell line of the localization of amplified P-glycoprotein genes. B and D, partial Q-banded metaphases from CEM/VLB100 with arrows denoting a large metacentric marker chromosome (M1) containing an ABR. C and E, same partial metaphases as shown in B and D demonstrating grain localization to the M1 marker following in situ hybridization (using radiolabeled P-glycoprotein cDNA probe).

Table 1 Tumorigenicity of CEM and CEM/VLB100 sublines in nude mice

<table>
<thead>
<tr>
<th>Tumor subline</th>
<th>Overall tumorigenicity</th>
<th>Time of appearance (wk)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEM-1</td>
<td>1.73</td>
<td>5</td>
</tr>
<tr>
<td>CEM-2</td>
<td>2.26</td>
<td>6</td>
</tr>
<tr>
<td>CEM-3</td>
<td>1.35</td>
<td>8</td>
</tr>
<tr>
<td>CEM-4</td>
<td>0.65</td>
<td>8</td>
</tr>
<tr>
<td>CEM/VLB-1</td>
<td>0.35</td>
<td>7</td>
</tr>
<tr>
<td>CEM/VLB-2</td>
<td>1.35</td>
<td>8</td>
</tr>
<tr>
<td>CEM/VLB-3</td>
<td>0.65</td>
<td>8</td>
</tr>
<tr>
<td>CEM/VLB-4</td>
<td>0.26</td>
<td>10</td>
</tr>
</tbody>
</table>

* CEM, 4 of 4 mice with tumors.
* Mouse died before tumor could be removed.
* CEM/VLB, 4 of 5 mice with tumors.

essentially identical for the CEM and the CEM/VLB100 cell lines, with 4 of 4 CEM-injected mice forming tumors and 4 of 5 CEM/VLB100-injected mice forming tumors. Although the tumors derived from the CEM/VLB100 line were smaller than CEM-derived tumors (mean tumor weight, 0.65 and 1.79 g, respectively), this is likely based upon a difference in cell doubling time for these two cell lines; using the method of Lelovitz and Mazur (18), we determined the generation time of CEM parental cells to be 32 h and the generation time of CEM/VLB100 parental cells to be 37.5 h.

Characterization of Nude Mouse Tumors Derived from CEM and CEM/VLB100 Cell Lines

In Vitro Drug Resistance. Our next series of experiments was performed in order to determine whether tumors from the CEM/VLB100 cell line maintained the MDR phenotype. Tumors were excised from the mice and tissue was divided for DNA extraction or tissue culture. Viable tumor cells which could be expanded in vitro were obtained from all tumor specimens.

CEM and CEM/VLB100 parental cell lines and seven tumor-derived sublines were tested for VLB resistance. Survival in VLB was measured by colony formation in soft agar. The CEM/VLB100 parent demonstrated the highest relative resistance (Fig. 3A), with an ID50 of 100 ng/ml. In comparison, the CEM parental cell line had an ID50 of 0.35 ng/ml with no surviving cells at 1.0 ng/ml. The tumors derived from CEM/VLB100 cells (designated as CEM/VLB-1, -2, -3, and -4) maintained a high degree of VLB resistance compared to the CEM parental cell line (CEM/VLB-2 ID50, 62 ng/ml; CEM/VLB-3 ID50, 80 ng/ml; CEM/VLB-4 ID50, 59 ng/ml). These results suggest that the VLB resistance is stable, since cells derived from these tumors were not exposed to VLB for 2–3 mo during the time necessary for in vivo growth and subsequent in vitro expansion.

Of particular interest to this study was the CEM/VLB-1 subline which reverted to a level of VLB resistance comparable to the drug-sensitive CEM cell line and its derived tumors (CEM-1, CEM-2, CEM-3, CEM/VLB-1: ID50, <1 ng/ml) (Fig. 3B).

The VLB sensitivities (Fig. 3A) of CEM/VLB100 and CEM/VLB100-derived tumors 2, 3, and 4 were compared by analysis of variance and covariance; the drug sensitivity of CEM/VLB100 is significantly lower than the sensitivities of CEM/VLB100-derived tumors 2, 3, and 4 (P < 0.01). The sensitivities of CEM/VLB100-derived tumors 2 and 4 were indistinguishable; however, the sensitivity of VLB-3 is significantly higher than that of VLB tumors 2 and 4 (P < 0.05), perhaps due to the higher levels of P-glycoprotein mRNA expression shown in Fig. 4.

P-Glycoprotein and EGF Receptor mRNA Expression. We next determined whether increased VLB resistance in the CEM/VLB100-resistant sublines was associated with overexpression of P-glycoprotein mRNA. Total cellular RNA was isolated from...
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Fig. 3. VLB resistance in vitro. Drug resistance was measured by plating cells into 0.4% soft agar containing increasing concentrations of VLB as described in "Materials and Methods." Surviving colonies were counted 10 days later. The data are expressed as percentage of survival compared to non-drug-treated controls. A, VLB resistance in CEM/VLB, cell-derived tumors. CEM/VLB, parent (*); CEM parent ( ); CEM/VLB tumor 2 (O); CEM/VLB tumor 3 (L); CEM/VLB tumor 4 ( ). B, VLB resistance in CEM-derived tumors. CEM parent ( ); CEM tumor 1 (O); CEM tumor 2 (O); CEM tumor 3 (O); CEM/VLB tumor 1 (L).

Fig. 4. P-glycoprotein RNA expression. Total cellular RNA was extracted from each subline and 10, 5, 2.5, and 1.25 µg of RNA were applied to nitrocellulose filters. Filters were hybridized with either 32P-labeled cDNA to P-glycoprotein (HRp1; A) or 32P-labeled human actin cDNA (B); actin hybridization is an internal control for the amount of RNA present in the filter.

Fig. 5. EGF mRNA expression. Total cellular RNA was extracted from each subline and 10, 5, 2.5, and 1.25 µg of RNA were applied to nitrocellulose filters. Filters were hybridized with either 32P-labeled cDNA to P-glycoprotein (HRp1; A) or 32P-labeled human actin cDNA (B); actin hybridization is an internal control for the amount of RNA present in the filter.

DISCUSSION

When drug-sensitive CEM cells and drug-resistant CEM/VLB, cells were tested for tumorigenicity in nude mice, both cell lines were shown to be equally tumorigenic. These results

Because of recent findings by Meyers et al. (7), we also probed our cells for mRNA expression of the EGF receptor in CEM/VLB, cells. Total cellular RNA from the CEM and CEM/VLB, parental cell lines were hybridized to either human EGF receptor cDNA or human β-actin cDNA (Fig. 5). In contrast to the results of Meyers et al., we found no significant amount of EGF receptor RNA expression in either the CEM or the CEM/VLB, subline.

P-Glycoprotein Gene Amplification. We next analyzed whether overexpression of P-glycoprotein in CEM/VLB, cell-derived tumors was due to the amplification of P-glycoprotein genes. DNA from parental cell lines and tumor sublines was digested with EcoRI, Southern transferred, and hybridized to human P-glycoprotein cDNA. Our results confirm that the CEM/VLB, has 15–20 copies of the P-glycoprotein gene compared to a single copy of this gene in the CEM line (3) (Fig. 6). CEM/VLB tumors 2, 3, and 4 all show a similar level of amplified copies of the P-glycoprotein gene, while CEM derived tumors 1, 2, 3 and CEM/VLB-1 revertant subline retained a single copy of the P-glycoprotein genes.

Karyotypic Analysis of CEM and CEM/VLB, cell-derived Tumors. Since the in situ hybridization data showed that amplified copies of P-glycoprotein are present on a large metacentric marker (M), it was important to show that the VLB-resistant tumors also retained this marker chromosome. Karyotypes were prepared from each of the VLB-resistant tumors which were expanded in culture. As expected, karyotypic analysis showed that the M, marker was present in all cells from the parental CEM/VLB, cell line and in all cells from CEM/VLB tumors 2, 3, and 4. In contrast, the CEM cell line and the CEM-derived tumors 1, 2, and 3 completely lacked the M, marker. In the CEM/VLB-1 revertant line, the vast majority of cells (>98%) lacked the M, marker. However, in less than ~2% of cells, the M, marker was observed. These data strongly suggest that there is a direct correlation between the presence of the M, marker chromosome, the presence of amplified copies of the P-glycoprotein gene, and the acquisition of the MDR phenotype.
suggest that in our model system overexpression of P-glycoprotein does not alter the ability of these cells to grow as tumors. While differences were observed in tumor size between CEM and CEM/VLB-induced tumors, this is best explained by the longer doubling time for CEM/VLB cells compared to CEM cells.

In addition, our analysis of individual tumors showed that, with one exception, tumors derived from the VLB-resistant parent maintain their MDR phenotype in the absence of drug selection for the duration of the experiment, 3 mo. The CEM/VLB-1 tumor that lost its drug-resistant phenotype also coordinately lost the ABR marker (which carries the amplified P-glycoprotein genes), as well as the increased copy number and associated overexpression of the P-glycoprotein gene. This revertant tumor clearly shows that the absence or presence of amplified P-glycoprotein genes does not influence tumorigenicity. Finally, we found that neither the CEM nor CEM/VLB100 parent lines (or any of the tumors derived from them) had any detectable alterations in the level of EGF receptor mRNA expression.

Our results differ significantly from those reported for similar studies in rodent systems (6–11). (a) We could not demonstrate any evidence for altered cellular maturation or differentiation associated with the acquisition of drug resistance (30). (b) The ability of the MDR subline to form tumors was unaltered; and (c) there was no evidence for altered expression of the EGF receptor gene. It is not clear why these changes are seen in some rodent fibroblasts and not in this human leukemic cell line. It is possible that these differences may be related to the degree of resistance of cell lines, the amount of P-glycoprotein in the membrane, or the presence or absence of other membrane components such as EGF receptor (7) or complex hematosides (31). The cell lineage may also be important in this phenomenon, as the murine MDR line, P388/ADR, forms ascites tumors in mice as well as its drug-sensitive parent (32). However, the recent finding that two Adriamycin-resistant human small cell lung cancer lines grow as well as their drug-sensitive parents in nude mice (33, 34) suggests that other factors may be involved. For example, although the drug-sensitive Chinese hamster lung cells grew as tumors in hamsters, whereas their drug-resistant derivatives did not (6), the immunological competence of the host may play some role in recognition (or lack of recognition) of the plasma membrane-altered MDR cells. Thus, the hamster cells (6) were placed in an immunologically privileged site but in a syngeneic animal with normal immune response; in contrast, the human cells in our study were placed in immunologically deficient animals, which may provide better conditions for tumor growth for the membrane-altered mutants. In addition, we speculate that amplification of the P-glycoprotein gene may involve "coamplification" of other genes in rodent systems that may influence cellular transformation and lead to loss of tumorigenicity (6). These same genes may be on different chromosomes in the human genome so that amplification of P-glycoprotein sequences does not lead to coordinate changes in expression of proteins which alter cellular morphology and tumorigenicity. In summary, our current experiments show that P-glycoprotein overexpression in the plasma membrane of human tumor cells is not associated with the loss of tumorigenicity.

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REFERENCES

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