Correlation of in Vitro Clonogenic Assay Data with in Vivo Growth Delays and Cell Cycle Changes of a Human Melanoma Xenograft

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ABSTRACT

We have reported previously that, in a human melanoma xenograft line, plating efficiency and drug sensitivity in vitro were enhanced under hypoxic conditions. In the present study, we show that the in vitro chemosensitivity data using clonogenic assays at 5% oxygen also correlate better with the drug-induced in vivo growth delay. Tumors implanted in bilaterally symmetrical locations grew at different rates, and response to chemotherapy was also variable. Cell cycle changes in the xenograft, as monitored by flow cytometry, suggest that major changes in cell cycle traverse occur when a drug is active in vitro and also causes in vivo growth delay. These studies indicate the usefulness of the nude mouse human xenografts to study correlations between soft agar clonogenic assays, in vivo growth delay, and cell cycle traverse in response to chemotherapeutic manipulation.

INTRODUCTION

In vitro colony-forming assays are better indicators of drug-induced cytotoxicity and effects on tumor cell clonogenicity than are the conventional dye exclusion or cell viability methods (21). Recent studies have shown that, under certain well-defined conditions, it is possible to grow human solid tumor colonies in semisolid media (8, 12). In vitro and in vivo studies have shown that a 95% patient correlation is seen between drug resistance in vitro and a lack of tumor response in vivo. In contrast, drug sensitivity in vitro has only a 65% patient correlation with a positive clinical response (23, 32). A major factor limiting the widespread use of soft agar colony-forming assays for screening drug sensitivity of human solid tumors is their poor growth in semisolid media and the resulting low PE3 (22). A number of growth-stimulatory factors and procedures have been described to enhance the PE of the human solid tumor cells in the soft agar assays (7, 12).

We have compared earlier the growth of human melanoma xenograft cells cultured at 5 or 20% oxygen concentration. We have also reported that the drug sensitivity of these tumor cells in the soft agar colony-forming assay was dependent on the oxygen concentration in the incubating atmosphere (11). In view of the positive correlation between in vitro colony-forming assays and the response of human tumors and their xenografts to chemotherapy (5, 24, 25) and the clinical importance of these data, we have used a human melanotic melanoma xenograft to study parameters affecting the growth of human melanoma cells in soft agar assays. On the basis of the in vitro drug sensitivity data from soft agar assays, we have monitored the growth-inhibitory effects of drugs injected in vivo on the xenografts of this tumor in athymic nude mice. In addition, we have attempted to make correlations between the drug sensitivity in the soft agar assay and changes seen in cell cycle distribution of the treated population as measured by FCM.

MATERIALS AND METHODS

A human melanotic melanoma established initially in our laboratory as a xenograft was serially transplanted in athymic nude mice of BALB/c origin. The xenograft tumor line has retained its near diploid human karyotype through 18 serial passages in vivo and has a tumor doubling time of 3 to 4 days. Tumor biopsies from a number of animals were pooled, minced, and incubated in a mixture of 0.25% trypsin (Grand Island Biological Co., Grand Island, N. Y.) and 0.002% DNase I (Sigma Chemical Co., St. Louis, Mo.) in Hank’s balanced salt solution for 20 to 30 min at 37° in a flask with a magnetic stirrer. The resulting cell suspension was filtered through a 20-μm nylon monofilament cloth mesh (Small Parts, Inc., Miami, Fla.) and centrifuged, and the cell pellet was resuspended in tissue culture medium. This procedure resulted in recovery of single cells with greater than 95% viability as determined by the trypan blue dye exclusion method.

Athymic mice of the BALB/c strain were given injections s.c. of a single-cell suspension of 5 x 10^6 cells/0.1 ml of Hank’s balanced salt solution into the left and right anterolateral chest wall with a 21-gauge needle. After 19 days of growth, tumors were measured in 2 dimensions, and the tumor volume (V) was calculated according to the formula (28)

\[ V = \frac{(\text{length} \times \text{width})^2}{2} \]

Groups of 2 to 3 mice (4 to 6 tumors) were treated with the 10% lethal doses. A single i.p. injection was given and called Day 0, and tumor volume measurements were made every 3 to 4 days for a period of 21 days. For some of the drugs used, a second i.p. dose was given on Day 21. Drugs used were BLM (32 mg/kg), DDP (13.5 mg/kg), VLB (3.0 mg/kg), BCNU (39 mg/kg).

Serial tumor volume measurements were expressed as relative tumor volume (tumor volume at a given time divided by the tumor volume at the start of chemotherapy). Data were plotted on semilog scale of relative tumor volume versus time and analyzed for statistical significance using the Dunnet’s test (16).

Serial fine needle aspirates of xenografts were stained in propidium iodide-hypotonic citrate (18) and analyzed on a TPS-1 cell sorter (Coulter Electronics, Inc., Hialeah, Fla.). For DNA histogram analysis, software programs developed by Dr. B. Bagwell for Coulter Electronics were used.

RESULTS

In an earlier study (11), we have reported that 5% oxygen increased the PE and drug sensitivity of tumor cells from this xenograft over that of cultures incubated in an atmosphere of
20% oxygen. From this study, we have derived Table 1 to show the effect of clinically achievable plasma \( C \times t \) as tabulated by Alberts and Chen (2) on the clonogenicity of tumor cells in soft agar. In plates incubated under 5% oxygen, VLB and DDP would have been listed as active (causing greater than 70% reduction in the number of colonies), whereas in plates incubated under 20% oxygen, none of the drugs tested was active. In fact, both BLM and BCNU stimulated colony growth by 48 and 68%, respectively.

To study the effect of chemotherapy on tumor volume, we implanted the same number of tumor cells in symmetrically similar locations on the left and right sides of nude mice. In 13 untreated animals on Day 19, tumors implanted on the left side had a tumor volume range of 0.18 to 2.0 cu cm with a mean ± S.D. of 0.73 ± 0.47 cu cm, whereas the tumors on the right side had a tumor volume range of 0.35 to 2.30 cu cm and a mean ± S.D. of 1.36 ± 0.74 cu cm. These differences are statistically significant at a \( p \) value of 0.025 by \( t \) test. In addition, an analysis of variance at each time point measured showed that left- and right-sided tumors behaved as independent observations.

Chart 1 shows the growth curves expressed as relative tumor volumes of the control and treated groups of tumor-bearing mice given injections of a single dose of the various drugs. Each drug-treated time point was compared against the control value for statistical significance as shown in Table 2. On this basis, DDP would be classified as the most active agent, and VLB of intermediate activity, while BLM and BCNU would be considered inactive. The growth curve also shows that the time course for growth delay varies with the drug injected. Thus, significant early growth delays were seen for VLB on Day 3, while DDP caused significant growth delays between Days 3 and 9.

On Day 21, we reinjected a second dose of DDP, VLB, or BLM into the tumor-bearing mice. Of these tumors, only DDP produced growth delay similar to those caused by the first injection (data not shown here). No significant growth delays of tumor were seen in mice given injections of the other drugs.

The effects of VLB and DDP on the relative tumor volumes of different size and implanted on the right and the left side are shown in Chart 2A, B to D. In Chart 2A (VLB), the 2 tumors implanted on the right and the left side were of approximately similar initial tumor volumes (0.72 and 0.92 cu cm, respectively), yet the individual tumor response to VLB was significantly different as indicated by the shape of the growth curves. As shown in Chart 2B, another animal with different initial tumor volumes, 1.36 (left) and 0.54 (right) cu cm, had relatively similar regression curves in response to VLB. Chart 2C shows the growth curves of tumors, 0.7 and 2.3 cu cm, implanted on the left and right sides, respectively, of a mouse treated with DDP. In this instance, the large tumor shows more pronounced and longer lasting growth delays.

### Table 1

**Oxygen concentration and effect of drugs on colony formation**

<table>
<thead>
<tr>
<th>Drug</th>
<th>( C \times t ) (( \mu \text{g} \cdot \text{hr/ml} ))</th>
<th>5% Oxygen</th>
<th>20% Oxygen</th>
</tr>
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<tbody>
<tr>
<td>VLB</td>
<td>0.15</td>
<td>20</td>
<td>50</td>
</tr>
<tr>
<td>DDP</td>
<td>1.90</td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>BLM</td>
<td>5.00</td>
<td>40</td>
<td>148</td>
</tr>
<tr>
<td>BCNU</td>
<td>1.00</td>
<td>64</td>
<td>168</td>
</tr>
</tbody>
</table>

*These values represent clinically achievable plasma \( C \times t \) as tabulated by Alberts and Chen (2).

*Number of colonies in treated cultures expressed as percentage of control.
growth delay than does the tumor with the smaller volume. In another tumor treated with DDP, Chart 2D, the tumor with the smaller volume, left (0.2 cu cm), showed more growth delay but recovered faster from the effects of chemotherapy than did the larger tumor, right (2.3 cu cm).

Histograms in Chart 3 are from serial biopsies from an animal given an injection on Day 0 of DDP (13.5 mg/kg). To facilitate comparison of cell cycle changes with changes in tumor volume, the in vivo growth delay curve of this tumor is shown in Chart 2D (O). In the pretreatment biopsy (Chart 3A), the cell cycle distribution was G1, 69.7%; S, 12.2%; G2-M, 18%; CV, 9.77%. The CV of the G1 peak was broad, indicating the presence of cells with aneuploid DNA content. Human lymphoblasts of CCRF-CEM analyzed at the same time had a CV of 4% for the G1 peak. In the serial needle biopsy obtained after 24 hr of therapy (Chart 3B), a large increase in the S-phase cells (21.7%) was seen. In the Day 3 sample (Chart 3C), a large build-up of cells with DNA content of G1-M (57.6%) was seen. These changes occurred during a period of time when the tumor doubled its volume (Chart 2D). In the 7-day sample (Chart 3D), a decrease in the number of cells with G2-M DNA content was seen during a time period when the tumor volume was diminishing (Chart 2D). FCM of untreated tumor aspirates performed at the same time did not show changes in the DNA histograms as a function of increasing tumor volume. Chart 3E represents an untreated tumor histogram on Day 21. A second injection of DDP was given on Day 21, and in needle aspirates taken on Day 22 (Chart 3F), 24 hr posttreatment, there was no increase in the S-phase cells as seen previously. However, on Day 24, 3 days posttreatment (Chart 3G), a major build-up of cells with DNA content of G1-M was seen. By Day 34, 13 days posttherapy (Chart 3H), build-up of G1-M cells was still higher than that of the pretreatment samples.

In contrast to DDP, administration of other chemotherapeutic agents made minor changes in the DNA distribution histogram. In biopsies taken after 2 days of VLB injection, a 5% increase in the number of G1-M cells was seen. By Day 7, the histograms had DNA distribution similar to that of the pretreatment samples.

DISCUSSION

The role of drug pharmacokinetics in vitro and in vivo determines to a large extent the cytotoxic effects of drugs on tumor cell proliferation. The C x t is an important pharmacological parameter, the knowledge of which is necessary to determine correct drug exposure times which may correlate with in vivo pharmacokinetics. Although the model used in the present study is that of a human solid tumor growing as a xenograft, it is generally believed that the maximally tolerated drug dose in humans correlates closely with the 10% lethal dose in mice and that the drug C x t under these conditions is similar for a variety of drugs (20). At the present time, there is no general consensus as to what is the best and correct criterion for the selection of drug concentration for in vitro testing which may be used to predict drug activity in vivo. For example, Bateman et al. (5) used the average drug concentration over 1 hr at the peak of the plasma clearance curve, while Salmon et al. (23) used a sensitivity index based on the area under cell survival curve, and Van Hoff et al. (32) used one-tenth peak plasma drug concentration.

In the present study, we have used the tables generated by Alberts and Chen (2) to select the drug concentrations for comparison of in vitro and in vivo drug sensitivity testing. A similar confusion exists as to what should determine positive or negative drug results in the clonogenic assay. Using the criterion of greater than 70% colony kill, the data in our study would have indicated that both VLB and DDP were active under 5% oxygen concentration, whereas in plates incubated in 20% oxygen, none of the drugs tested would have been considered positive. In fact, both BLM and BCNU stimulated colony formation by 48 and 68%, respectively, under 20% oxygen. Since large differences in in vitro drug sensitivity of this xenograft are dependent on the oxygen level, it became imperative to find out which set of data correlated with the drug-caused in vivo growth delay. A comparison of our in vitro and in vivo data shows that drug cytotoxicity data at 5% oxygen correlate better with the in vivo growth delay in the xenografts. On the basis of 20% oxygen data, DDP would have been clearly classified as inactive, and BLM and BCNU would have been predicted to enhance melanoma xenograft growth.

The role of oxygen in tumor cell proliferation and chemotherapy has been studied by a number of workers. The effect of low oxygen concentration (N2 or <0.001% O2) on drug cytotoxicity in tissue culture cell lines has been studied by exposing cells to an N2 atmosphere for variable time periods, followed by drug exposure and incubation in 20% oxygen (26, 29, 30). Using this method, Tannock and Gutman (29) reported no major differences in cytotoxicity of drugs. Recently, Tveit et al. (31) have published data comparing the drug cytotoxicity seen with the Hamburger and Salmon (12) method (using 20% oxygen) and the Courtenay and Mills (7) method using a combination of RBC and 5% oxygen but without addition of heavily irradiated cells. In 3 of 4 drugs tested by this group, the published data are

![Chart 3](chart3.png)
These observations suggest that cell cycle traverse determines sensitive and -resistant P388 cell lines, significant cell cycle traverse disturbances are seen only in the sensitive cells (10). These observations suggest that cell cycle traverse determination by FCM may be useful in predicting drug sensitivity in clonogenic assays.

Present studies using the nude mice human xenografts indicate that this model is useful for testing the various modifications and rationale for the use of in vitro soft agar assays as predictors of response in vivo.

ACKNOWLEDGMENTS

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REFERENCES


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