Factors Affecting Growth and Drug Sensitivity of Mouse Mammary Tumor Lines in Collagen Gel Cultures¹

Bonnie E. Miller,² Fred R. Miller, and Gloria H. Heppner

Michigan Cancer Foundation, Detroit, Michigan 48201

ABSTRACT

A series of mouse mammary tumor subpopulation lines were compared for growth properties and sensitivity to chemotherapeutic drugs when grown as boluses in a collagen gel matrix versus in monolayer culture. Although the cell lines exhibited characteristic rates of bolus expansion in collagen, this growth was not paralleled by an exponential increase in cell number with time. Cell boluses contained a higher proportion of cells in G₀-G₁ phases of the cell cycle than did the same cell lines in monolayer cultures. Histological examination revealed areas of necrosis in boluses. Thus cells growing in collagen cultures resembled cells growing as solid tumors and cells from other three-dimensional culture systems.

The growth of cell boluses in collagen gel cultures was reduced nonexponentially by melphalan, methotrexate, and 5-fluorouracil in contrast to the exponential decrease in growth measured in cloning assays. The lowest concentration to which cells first responded to drug was in general similar for collagen gel assays and for cloning assays. The rank order of sensitivity of different cell lines in the two assays was identical for methotrexate (four cell lines), similar for melphalan (four of five lines), but quite different for 5-fluorouracil. In contrast to cloning assays cell boluses continued to grow, albeit at a reduced rate, in the presence of high drug concentrations. This was not due to either diminished drug availability in collagen gel or drug penetration into the bolus.

INTRODUCTION

Many animal and human tumors have been shown to contain multiple subpopulations which differ in a number of characteristics, including sensitivity to chemotherapeutic drugs (reviewed in Refs. 1 and 2). We have been interested in interactions between tumor cells of different subpopulations and between tumor cells and preneoplastic or normal cells, which can affect the growth and chemosensitivity of target tumor cells. We have described several such interactions, the mechanisms of which differ depending on the particular subpopulations and drugs tested (3–5).

Cloning assays for drug sensitivity by their very nature eliminate cellular interactions that may affect the drug sensitivity of heterogeneous tumors in vivo. We have designed an assay for drug sensitivity of tumor cells which allows such interactions to take place in vivo. The assay consists of embedding small pieces (<1 mm³) of tumor or a bolus of tumor cells from culture in collagen gel and measuring the outgrowth over 7 to 10 days by projecting the cross-sectional microscopic image of the expanding colony onto paper and measuring it with a planimeter (6). Growth in this system is three-dimensional and reminiscent of tissue density and morphology (7, 8).

In this report we describe characteristics of the growth and drug sensitivity of tumor subpopulation lines of a mouse mammary tumor in the collagen gel assay. Concentration-response curves for melphalan, methotrexate, and 5-fluorouracil obtained in this assay are compared to those obtained in a cloning assay. The lines used are internally homogeneous in sensitivity to these drugs, since our purpose was to study the contribution of three-dimensional growth to drug response without the added complication of cellular heterogeneity. Future studies will explore the influence of tumor subpopulation interactions on drug sensitivity in collagen gel and the relationship of that sensitivity to drug response in vivo.

MATERIALS AND METHODS

Tumor Lines. The cells used in this study are mouse mammary tumor subpopulations derived from a single, spontaneously arising mammary tumor of a BALB/cF3H mouse (9–11). These tumor lines, designated 66, 67, 168, 410, and 410.4, have been shown to be intrinsically different in sensitivity to several antineoplastic drugs, both in vivo and in vitro (4, 6, 8). The tumor cells are maintained in monolayer culture.

Media. Cell lines were grown in monolayer culture at 37°C in 5% CO₂ in air atmosphere in Waymouth’s Medium MB752/1 supplemented with 2 mM glutamine, penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum. Most experiments were carried out in DMEM (DME supplemented with penicillin, streptomycin, glutamine, 0.1 mM nonessential amino acids, and 10% calf serum). The calf serum used was low endotoxin, defined, iron-supplemented serum purchased from Hyclone-Sterile Systems, Logan, UT. Fetal bovine serum was low endotoxin, defined serum purchased from Grand Island Biological Co., Grand Island, NY. Other culture media and supplements were purchased from Grand Island Biological Co.

Drugs. Methotrexate (Lederle Laboratories, Pearl River, NY; 25-mg/ml solution) and 5-fluorouracil (Roche Laboratories, Nutley, NJ; 50-mg/ml solution), were diluted in 0.9% NaCl solution, sterilized by passage through a 0.2-μm filter, and diluted at least 20-fold further with medium before use. Methotrexate and 5-fluorouracil stock solutions were stored at 4°C for up to 3 months before use. Melphalan (Sigma Chemical Co., St. Louis, MO) was dissolved in acid alcohol (concentrated HCl:ethanol, 1:120), diluted 18-fold with acid saline (0.05 N HCl in 0.9% NaCl), filter sterilized, and further diluted with medium at least 20-fold (pH brought back to 7.4 with NaOH) for in vitro use. Melphalan solutions were prepared fresh daily.

Collagen Gel Assay. The system used is a modification of that of Yang et al. (7) and has been described previously (6). Collagen stock was prepared from rat tail fibers suspended in a 1:1000 dilution of glacial acetic acid. Complete collagen mixture was prepared from collagen

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²To whom requests for reprints should be addressed, at Michigan Cancer Foundation, 110 E. Warren Ave., Detroit, MI 48201.

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stock, concentrated DME, and NaOH and kept on ice before use. Complete collagen mixture (0.5 ml) was layered into each well of 24-well cluster plates and allowed to warm up to room temperature to gel. A bolus of cultured cells was placed on each surface and overlaid with 0.4 ml complete collagen mixture and then 0.9 ml DME-10 containing 2-fold concentrated DME, and NaOH and kept on ice before use.

Cell boluses were prepared by centrifuging tumor cell suspensions and then resuspending the pellet in complete collagen mixture at 1:5 × 10^6 cells/ml. One-µl boluses were embedded in each well with an Oxford ultramicropipet (Sherwood Medical Co., St. Louis, MO). Cell boluses were examined periodically with an inverted microscope equipped with a split image tracing device, using the x2.5 or x1 objective (E. Leitz, Wetzlar, West Germany). The projected image was drawn on a piece of paper and the area of the image was measured with a compensating polar planimeter (Kauffel and Esser, Morristown, NJ). Linear dimensions were converted to units of actual size of the cell bolus by projecting and measuring the image of a stage micrometer to determine the magnification (45-fold with the x2.5 objective).

Counting Nuclei from Cell Boluses. Cell boluses from collagen gel cultures were released by incubation in a shaking water bath at 37°C with collagenase type III (Worthington Biochemicals, Freehold, NJ), 2 mg/ml in Waymouth's medium without serum, added in equal volume to collagen gel cultures. After 1 h incubation, cells were collected by centrifugation, resuspended overnight in 0.1 M acetic acid to swell cells, and lysed with Zapon-Aceton detergent (Coulter Electronics, Hialeah, FL).

FACS Analysis of Cellular DNA Content. Cell boluses from collagen gel cultures were released with collagenase as above. From 10 to 12 individual cell boluses were pooled for each analysis. A single cell suspension was prepared from the released cell boluses with 3 ml/cell of protease type IX (Sigma), 10 mg/ml in Waymouth's medium without serum. Cells were collected by centrifugation after 15 to 20 min incubation, rinsed twice, resuspended in DME-10, pipetted up and down vigorously, and passed several times through a syringe with a 25-gauge needle to break up clumps and obtain a single cell suspension. Cells were recentrifuged and the pellet was fixed by slowly adding 2 ml of iced 70% ethanol while vortexing. Cells were kept on ice for 15 min and then pelleted and incubated for 30 min at 37°C in 0.1 ml RNase (Sigma), 1 mg/ml in phosphate-buffered saline, pH 7.6. Cells were collected by centrifugation and suspended with propidium iodide (50 µg/ml) in phosphate-buffered saline. Stained cells were stored in the dark at 4°C until analysis (within 24 h). Analysis was performed on a Becton Dickinson FACS 440 flow cytometer with a Consort 40 data acquisition package.

Cell suspensions prepared from normal mouse spleen, used as a control, were prepared as follows. Spleens were mechanically dispersed through fine mesh screen, cells were collected by centrifugation, red blood cells were lysed with Tris-buffered 0.14 M NaCl for 5 min, and the resulting cell suspension was filtered through 45-µm Nitex cloth (Tetko, Elmsford, NY). Spleen cells were then rinsed, suspended in DME-10, fixed, and stained by the same method used for tumor cells.

Cloning Assay. Cells were removed from monolayer cultures with trypsin:EDTA, rinsed with DME-10, passed through a syringe with a 25-gauge needle several times as necessary to obtain a single cell suspension, and plated at low density, in some experiments, in 60-mm tissue culture dishes (500 cells/dish) or, in other experiments, in 6-well tissue culture cluster plates (200 cells/well). Cells were incubated at 37°C in 10% CO₂ atmosphere for 2 to 4 h before an equal volume of DME-10 containing 2-fold concentrated drug was added. Plates were incubated for 7 to 10 days, then fixed with Carnoy's solution (methanol:acetic acid, 2:1), and stained with crystal violet. Colonies of greater than 32 cells were counted with a dissecting microscope.

Cloning in Collagen. Cells from monolayer were prepared as a single cell suspension as above; then 1 volume of cell suspension in DME-10 was diluted 20-fold with complete collagen mixture and added to 60-mm dishes (500 cells/dish) or 6-well cluster plates (200 cells/well), over an equal volume of gelled complete collagen mixture. Another equal volume of 3-fold concentrated drug solution in DME-15 (15% cell suspension) was added after 1 h incubation. Plates were incubated for 10 to 14 days, then cells were fixed, and gel was dehydrated with two 10-min treatments of methanol. Colonies were stained with Giemsa and counted with a dissecting microscope.

RESULTS

Growth Characteristics of Tumor Cells in the Collagen Gel Assay System. Within 1 or 2 days after embedding, cell boluses from cultured tumor cell lines begin to expand at a rate which is constant when the measured size of the cell bolus is expressed as the square root of area (6) (equal to a constant times mean diameter for a bolus of circular cross-section). This linear growth rate is illustrated for several cell lines in Chart 1A. Different cell lines have characteristic growth rates in collagen (6), and tumor pieces obtained from a tumor grown in vivo grow at about the same rate in collagen gel cultures as do cell boluses of the same tumor cell line from in vitro culture (6). Although the size of the cell bolus is correlated with the number of nuclei within it (6), the increase in the number of nuclei over a 7- to 10-day observation period is small and not exponential (Chart 1B).

In order to investigate whether many cells in the collagen gel cultures are arrested in G₂-M, or in other phases of the cell cycle, or whether many cells are dying, we used flow cytometry to compare propidium iodide-stained cells from monolayer and collagen gel cultures for DNA content. In Table 1 are summarized the results of flow cytometric analysis of five to six experiments with line 66 and line 168 and one experiment with line 67 cells. These three lines were chosen because metaphase cells from monolayer cultures of these cell lines were quite uniform in chromosome number, line 66 being diploid and lines 168 and 67 approximately tetraploid. As data from Table 1 indicate, for a given cell line collagen gel cultures contain a higher percentage of cells in G₂-M, and a lower percentage in other phases than do monolayer cultures, although there are some cells in cycle in the collagen gel cultures for all cell lines. From the monolayer cultures, 80 to 95% of the particles counted by FACS were analyzable, with DNA content in the correct range and reasonably uniform sizes as ascertained by forward scatter. On the other hand, in collagen gel cultures only 35 to 50% of the particles counted by the cell sorter were analyzable. There were many

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small particles with low DNA staining. These were probably cell fragments.

Cell boluses were fixed, sectioned, mounted on slides, and stained for histological examination. Over time, areas of necrosis developed in the central portions of boluses (not shown).

**Drug Sensitivity of Tumor Cells in the Collagen Gel Assay.**

Drug sensitivity as measured by the collagen gel assay differs from that measured by a cloning assay, both in the characteristic response of any individual cell line and in the sensitivity of several cell lines relative to each other (Chart 2A). All five lines demonstrated an exponential response to melphalan over the tested range of concentrations, with lines 168 and 410 being most sensitive and line 66 being least sensitive. In a few experiments higher concentrations of melphalan than shown here were used in the cloning assay. These values also fell on the same straight line.

Chart 2B shows melphalan sensitivity in the collagen gel assay. The measured response was not exponential but rather appeared to plateau at growth rates approximately 10 to 30% of control. Over most of the concentration range tested, all cell lines were less sensitive by the collagen gel assay than by the cloning assay. However, the concentration marking the threshold of sensitivity was similar for the two assays. On the basis of the IC_{50} for clonogenic survival and the IC_{50} for inhibition of the growth rate of boluses, we ranked the cell lines according to their sensitivity to melphalan. The rank order of sensitivity of four of the five cell lines was similar in the two assays. However, line 410 was the most sensitive line by the cloning assay but relatively insensitive by the collagen gel assay.

In Chart 3 the sensitivities of four cell lines to methotrexate are compared by the two assays, using the IC_{50}s for the two assays as before. The shapes of the two sets of concentration-response curves are similar to those seen for melphalan. Line 410.4 was most sensitive, lines 168 and 410 were intermediate, and line 66 was least sensitive in both assays.

In Chart 4 the sensitivities of four cell lines to 5-fluorouracil are compared by the two assays. The IC_{50} for 5-fluorouracil for these four cell lines were not significantly different in the cloning assays. The survival curves have a broad shoulder as shown.

However, there are differences in the response of each line to higher concentrations of the drug, as measured by the IC_{50}s. We used these values to compare the sensitivity of these lines by the two assays. In contrast to results with melphalan and methotrexate, the rank order of sensitivity to 5-fluorouracil measured by the two assays was not correlated. In cloning assays the cell lines were grouped into two sets: lines 66 and 168 were more sensitive; and lines 410 and 410.4 were less sensitive. On the other hand in collagen gel assays line 410.4 was more sensitive than other lines, lines 168 and 410 were intermediate, and line 66 was less sensitive.

**Analysis of Factors Affecting Drug Sensitivity in Collagen Gel Cultures.** In order to determine whether the lessened sensitivities toward high drug concentrations by cells in the collagen gel assay were due to the presence of collagen itself, we performed several cloning assays in collagen. These results are shown in Table 2. Sensitivities to 5-fluorouracil (three experiments) and melphalan (one experiment) of several cell lines as measured by cloning in collagen were greater than those obtained by cloning on plastic, and concentration-response curves had the typical exponential relationship in both assays.

As a further test of the effect of collagen per se on drug sensitivity, we plated line 410.4 cells for cloning on the plastic bottom of multwell dishes, covered them with collagen, and embedded boluses of line 410.4 cells in the same wells. Each well was continuously treated with one of several concentrations of methotrexate. The growth of the monolayer colonies was
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Chart 3. Effect of methotrexate on mammary tumor lines measured by cloning assay (A) and collagen gel assay (B). Methods as described for Chart 2. O, line 66; •, line 168; □, line 410; ■, line 410.4.

Chart 4. Effect of 5-fluorouracil on mammary tumor lines measured by cloning assay (A) and collagen gel assay (B). Methods as described for Chart 2. O, line 66; •, line 168; □, line 410; ■, line 410.4.

inhibited at concentrations of methotrexate at which cell bolus growth was unaffected (data not shown).

Evidence that the plateau in chemosensitivity in collagen gel cultures is not due to depletion of drug with time, allowing cells to escape from inhibition, is shown in Chart 5. When fresh medium containing the same drug concentration was added every other day to line 168 cultures, cell boluses treated with 5-fluorouracil or 30 μM melphalan were not inhibited more than when drug was added once at the start of the experiment, without a change of medium (our usual procedure). Only cell boluses treated with 10 μM melphalan were more inhibited when fresh drug was added.

To further examine whether differences between the assays are due to differences in drug delivery to cells, we assayed the response of line 168 cells by treating monolayer cultures of cells with melphalan for 4 h; we then trypsinized the cells and divided the treated cultures, plating some cells on plastic for the cloning assay and embedding some cells for the collagen gel assay. The results of this experiment are shown in Chart 6. The concentration-response curves obtained are characteristic of the assay type; the cloned cells were more sensitive to the drug than are the embedded boluses.

A similar experiment, in which line 410.4 cells were exposed to 5-fluorouracil for 4 h before cloning or embedding as a bolus in collagen, is shown in Chart 7. Although the response of these cells to high doses of 5-fluorouracil for a short time was nonexponential for either assay, the cloned cells were more sensitive to the drug than were the embedded boluses.

DISCUSSION

We have demonstrated that the collagen gel assay can be used to measure growth, through colony expansion, of tumor pieces or boluses of tumor cells from culture. These cultures have many characteristics in common with multicellular sphe-
We have compared the drug sensitivity of tumor subpopulation lines as assayed by the collagen gel assay and by cloning assays. Each of these lines was quite homogeneous in drug sensitivity as shown by exponential concentration-response curves obtained in cloning assays. Although the shape of the concentration-response curves for the collagen gel assays was nonexponential, the ranked sensitivities of the cell lines were the same for the two assays for four of four cell lines with methotrexate and for four of five cell lines with melphalan. With 5-fluorouracil, however, there was no correlation between the assays. We intend to test 5-fluorouracil with these four lines in vivo and melphalan with line 410 versus other lines in vivo, in order to determine whether one in vitro assay or the other better predicts in vivo response.

We have been puzzled by the fact that some cell boluses can continue to expand in the presence of drug concentrations which allow survival of less than 0.1% of cells in a cloning assay. We have shown that this "drug resistance" is not an artifact of lowered effective drug concentration due to the presence of drug inhibitors or drug-binding sites in the collagen preparation because cloning assays in collagen have concentration-response curves similar to those obtained from cloning on plastic (Table 2). Addition of fresh drug-containing medium did not further inhibit bolus expansion except with low doses of melphalan (Chart 5). This is further evidence that "drug resistance" is not due to depletion of drug in the collagen gel. Nor can this resistance be due entirely to differences in drug delivery, penetration of drug into the gel, or percentage of cells in cycle at the time of drug delivery, because similar differences between the assays appear when cells are treated with drug in monolayer culture before cloning or embedding in collagen. This is true when cells are treated with melphalan (Chart 6), a rapidly acting alkylating agent with a half-life of only 1 h under physiological conditions (24), and also when cells are treated with 5-fluorouracil, a metabolic inhibitor, high concentrations of which are required to inhibit cell replication or kill cells with only a short time of exposure (Chart 7). We have also seen these same characteristic differences in the assays when cells are treated for a short time with Adriamycin before embedding or cloning.4

Laboratories working with multicellular spheroid cultures have also tried to determine the cause for a shift toward lessened drug sensitivity in these cultures. Several laboratories have used sequential trypsinization of spheroids after drug exposure to demonstrate that inner cell layers of spheroids are less sensitive to drugs than are outer layers (21, 23, 25). Lack of drug penetration into the inner layers has been shown (20, 21, 25, 27). In addition a hypoxic radiosensitizer has been shown to enhance cell killing in the inner cell layers, indicating that hypoxic conditions may protect cells from potentially lethal damage (22). The conditions of limited drug access (28) and hypoxia (29) certainly exist in tumors, and insofar as they influence drug sensitivity any culture system which incorporates them must be a valuable addition to, information obtained by assays in which cells are cloned.

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**Chart 6.** Effect of 4-h melphalan pretreatment in monolayer on subsequent growth of line 168 in collagen gel cultures and as clones on plastic. Line 168 cells were plated at 2 x 10^4 cells in each of four T-25 flasks. After 24 h each flask was treated with a different concentration of melphalan in DME-10 for 4 h, then cells were suspended and counted, and portions of each cell suspension were grown in each assay system. Growth in each assay was measured and expressed as described for Chart 2. ●, collagen gel assay; ○, cloning assay. Bars, SD.

**Chart 7.** Effect of 4-h 5-fluorouracil pretreatment on subsequent growth of line 410.4 in collagen gel cultures and as clones on plastic. Methods as described for Chart 6, using line 410.4 cells and 5-fluorouracil.

roids, another three-dimensional culture system, including linear increase in colony diameter with time (12), presence of many dead cells (13, 14) and, in comparison with monolayer cultures, increased percentage of cells arrested in G2-M phases of the cell cycle (15, 16) and a decreased sensitivity to antineoplastic drugs (17-23). Many of these characteristics are also seen in tumors in vivo. Thus we believe that collagen gel assays may yield information on drug sensitivity of cells in tumors which is different than, and a valuable addition to, information obtained by assays in which cells are cloned.

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changes in the cell triggered by cell shape or degree of differentiation (30). This resistant subpopulation might be protected from potentially lethal damage and thus continue to divide at a relatively constant rate in the presence of very high drug concentrations. Wallen et al. (31), using some of the same cell lines as in our study, showed that nonreplicating, quiescent cells were able to reenter the proliferating state when the conditions that induce quiescence were relieved. One can imagine that the dynamics of growth in three-dimensional collagen gel, especially under drug treatment, could lead to shifts in those factors (pH, oxygenation, nutrition) that affect transition from quiescence to proliferation and thus allow for recruitment, over time, of cells into replication.

However, another possible explanation for continuing bolus expansion in the presence of high drug levels is that some proportion of the measured colony expansion is due to cellular migration (i.e., movement of nonreplicating cells through the collagen matrix) rather than cell division. The drugs used here are not energy poisons and thus would be unlikely to block any such migration at the concentration used.

A third possibility is that the collagen gel assay may measure, only or disproportionately, the replication rate of the fastest growing cells in the bolus instead of averaging the replication rate of all cells. If this is true the presence of a drug at a concentration which kills a large proportion of cells in the bolus may have little effect on the rate of bolus expansion, as long as some cells are still replicating at a normal rate.

We are currently investigating these possibilities in several ways: (a) by measuring cell bolus expansion in collagen of lethally irradiated cells alone (which may still be capable of migration) and of lethally irradiated cells mixed with various numbers of nonirradiated cells to determine how the growth rate varies with the number of replicating cells; and (b) by cloning cells from boluses treated with high drug concentrations in order to directly enumerate the number of clonogenic survivors at each drug concentration. Whatever the mechanisms involved they all are of potential importance in vivo and could contribute to reduced chemosensitivity of tumors whose cells are inherently sensitive to drug as assessed by cloning assays.

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