In: Human Tumor Cloning, S.E. Salmon and J.M. Trent (Eds.), Grune and Stratton, Inc., New York, 1984, 205-214.

DRUG APPLICATION TO THE SURFACE OF SOFT-AGAROSE CELL CULTURES

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INTRODUCTION

Evaluation of human tumor cell chemosensitivity using soft-agar colony formation assays generally have utilized one of two standard methods of drug application: 1) "One-hour exposure" followed by washing and resuspension of cells prior to inoculation in culture or 2) "continuous exposure" by incorporation of drug into soft-agar cell suspensions prior to inoculation (Alberts et al, 1980; Soehnlen et al, 1980). The present study was designed to assess possible advantages of an alternate method of drug exposure: that is, drug application to the surfaces of soft-agarose cultures following inoculation. In contrast to other techniques, surface application was viewed as a means to eliminate mechanical manipulation of cells in the presence of drug and to permit all cultures to be set up from a single, "bulk" cell suspension with minimal handling.

MATERIALS AND METHODS

Detailed descriptions of tumor acquisition, digestion, soft-agarose culture, and chemosensitivity testing by the drug incorporation technique have been reported previously (Agrez et al, 1982a,b; Alley et al, 1982; Alley and Lieber, 1984). The same procedures were utilized in the present study with the exception of the following modifications permitted by the culture surface drug application technique: After digestion and washing, cells from each specimen were suspended in "bulk" at a density of 5×10^5 cells/ml; 1 ml

aliquots of cell suspension containing 0.3% Seaplaque agarose were then applied to the base layer of each culture dish with a constant-volume step-syringe (Eppendorf Repeator 4780), the barrel of which was fitted with a large-bore fibrometer tip (Becton Dickinson and Co.). Following agarose gel formation, aliquots (100 µl) of each drug solution (20x final concentration) were applied to surfaces of 3 culture dishes and aliquots of each drug vehicle (water, 0.9% saline, 10% ethanol) were applied to 6 culture dishes. Note that culture dishes set up by the "drug incorporation" and "surface drug application" methodologies contained identical formulation: Base layers (1 ml) contained McCoy's medium supplemented with 9.8% fetal bovine serum, 140 µg sodium pyruvate, 28 µg L-serine, 1.3 µmol L-glutamine, 65 units penicillin, 65 µg streptomycin, 65 µg asparagine, 246 µg DEAE dextran, 0.5% tryptic soy broth, and 0.5% agar; cell layers (1 ml) contained 5 x 10^5 cells in CMRL 1066 medium supplemented with 10.5% fetal bovine serum, 1.4 units insulin, 0.21 µmol vitamin C, 140 units penicillin, 140 µg streptomycin, 14 µmol L-glutamine, 68 µg asparagine, 35 µmol 2-mercaptoethanol, and 0.3% agarose plus 100 µl drug solution.

For a given specimen culture, chemosensitivity was assessed shortly following exhibition of significant growth in "proliferation control" culture dishes (Alley and Lieber, 1984). Cultures were stained with a metabolizable dye, INT (Alley et al, 1982), and colonies were counted by a computerized image analyzer, the Omnicon Feature Analysis System, Model II (Bausch and Lomb, Inc., Rochester, NY). The evaluable region of each culture dish (35 contiguous fields [each 4.44 x 3.22 mm^2] equivalent to 500 mm^2 area) was assessed on the basis of a standard colony count program (Kressner et al, 1980). Selective scoring of viable cell groups was achieved in the gray-manual mode with the aid of a scintered glass filter placed between the light source and culture dish. The maximum optical density detection level (lower threshold value) was set to 456; the minimum optical density detection level (upper threshold value) was adjusted to exclude features of non-stained cell groups and debris from analysis (levels ranged from 520-640 depending upon specimen culture opacity). The mean colony count ($\geq$ 60 µ diameter) and standard error of the mean for each group of cultures (6 dishes/control group and 3 dishes/drug-treated group) were computed and tabulated by the analyzer. Colony formation in drug-treated cultures was expressed relative to that in vehicle-treated cultures as percent of control growth.

RESULTS

Preliminary Evaluation of Culture Surface Drug Application

A preliminary assessment of the drug overlay technique was performed with soft-agarose cultures of a human rhabdomyosarcoma continuous cell line (A204). As shown in Table 1, surface application of most clinically useful agents resulted in greater than 70% inhibition of colony formation; in fact, at therapeutically relevant concentrations most agents inhibited colony formation more than 90%. It was not unexpected that cyclophosphamide and procarbazine lacked activity in this in vitro system since each of these agents requires metabolic bioactivation (e.g., Lieber et al, 1982; Alley et al, 1984). While melphalan at 0.05 μg/ml was inactive (data not shown), subsequent evaluations revealed that significant inhibition of colony formation required the presence of 1.0 μg/ml or higher concentrations whether melphalan was introduced by culture incorporation or culture surface application. Thus, soft-agarose culture matrix appeared to provide no significant barrier to activity of standard chemotherapeutic agents.

In a subsequent experiment, drug activity following culture incorporation was compared with that following culture surface application for 7 agents in 9 primary human tumor cell cultures (2 colon, 4 kidney, and 3 ovary). Linear regression analysis of paired data is depicted in Figure 1. All entries except 3 (circled) fall within 95% confidence limits of the line, $Y = 0.914\ X + 22.1$, where X represents the percent survival resulting from the drug overlay technique and Y represents the percent survival measured by the drug incorporation technique ($r = 0.780$, $n = 42$, $p < 0.001$). A slope factor of 0.914 ($\pm$ 0.234, 95% CI), coupled with a Y intercept of 22.1 ($\pm$ 13.0, 95% CI) suggests that the culture surface application technique provides a somewhat more sensitive index of drug effect than the culture incorporation technique.

Use of Culture Surface Drug Application in the Chemosensitivity Testing of Primary Tumor Cell Cultures

Culture surface drug application was employed in the assessment of 145 consecutive evaluable human solid tumor specimens. Significant proliferation was observed in 73 specimen cultures, 55 of which were sensitive to one or more chemotherapeutic agents. As shown in Table II, use of surface application in this series of tumor cultures resulted

TABLE I
Sensitivity of Human Rhabdomyosarcoma Cells (A-204)
to Chemotherapeutic Agents Applied to
Soft-Agarose Culture Surfaces

Agent	Culture Concentration[1] (μg/ml)	Colony Formation[2] (% of Control Growth)
Vehicle Controls	—	100 ± 5.4
Actinomycin D	0.010	0.8 ± 0.2
Bisantrene (ADAH)	0.50	7.1 ± 1.5
Doxorubicin	0.60	2.3 ± 0.3
L-Alanosine	50	3.5 ± 1.4
Acridinyl Anisidide (m-AMSA)	1.0	1.3 ± 0.2
Cytosine Arabinoside (ARA-C)	0.20	1.3 ± 0.5
Diaziquone (AZQ)	1.0	9.9 ± 1.2
Carmustine (BCNU)	2.0	12.5 ± 1.1
Bleomycin	2.0	2.6 ± 1.1
Cyclophosphamide	70	79.0 ± 9.9
Dibromodulcitol (DBD)	5.0	10.4 ± 1.4
Galactitol (DAG)	2.0	7.3 ± 1.0
5-Fluorouracil	10	0.8 ± 0.2
Mitoguazone (MGBG)	50	1.0 ± 0.2
Hydroxyurea (HUR)	60	5.1 ± 1.4
Methotrexate	1.0	0.8 ± 0.2
Mitomycin C	0.040	1.0 ± 0.1
N-Phosphonacetyl-L-aspartic acid (PALA)	200	1.7 ± 0.8
Cisplatin (CDDP)	1.5	14.1 ± 2.2
Procarbazine	5.0	78.5 ± 4.5
Triazinate (TZT)	40	1.3 ± 0.4
Vinblastine	0.050	3.3 ± 0.2
Teniposide (VM-26)	10	1.3 ± 0.2
Etoposide (VP-16)	10	0.4 ± 0.2
Sodium Azide	600	0.7 ± 0.3
Mercuric Chloride	100	0.05 ± 0.05

[1] Culture concentration of each chemotherapeutic agent was selected to approximate the mean plasma concentration present in patients one hour following administration of a maximum tolerated dose.

[2] Tabulated data are the mean ± 1 SEM for each group (n=6 vehicle control cultures; n=3 drug-treated cultures).

in higher frequencies of proliferation and chemosensitivity than were observed for the drug incorporation method in an earlier series of specimen cultures. While similar frequencies of drug sensitivity were observed for the two methodologies when data are expressed relative to the number of assays (A), higher frequencies were observed when data is normalized with respect to the number of cultures (C). These findings coupled with those depicted in Figure 1 suggest that in vitro tumor cell growth may be enhanced by minimizing mechanical manipulations of cells prior to culture inoculation.

Assessment of Tumor Cell Colony Formation Following Culture Surface Application of Multiple Drug Concentrations

In a subsequent series of 117 primary tumor cell cultures as well as secondary cultures derived from 4 xenograft-passaged human renal carcinomas, each drug was applied at three concentrations. As noted in previous testing of single concentrations, tumor cell cultures exhibited a wide range of sensitivities to a given agent. For example, colony formation by one renal carcinoma (Figure 2A)

Table II
Evaluability and Chemosensitivity of Primary Human Tumor Cultures Following Two Methods of Drug Application[1,2]

Criteria	Drug Incorporation	Drug Overlay
Consecutive evaluable specimen cultures (C):	195	145
Cultures exhibiting significant growth and successfully assayed (A):	82 (42% of C)	76 (52% of C)
Drug sensitive:	55 (67% of A; 28% of C)	55 (72% of A; 38% of C)

[1] Table entries indicate the number and normalized frequencies of specimen cultures meeting each criterion

[2] Sensitivity refers to > 70% inhibition of colony formation by one or more chemotherapeutic agents present at clinically relevant concentrations.

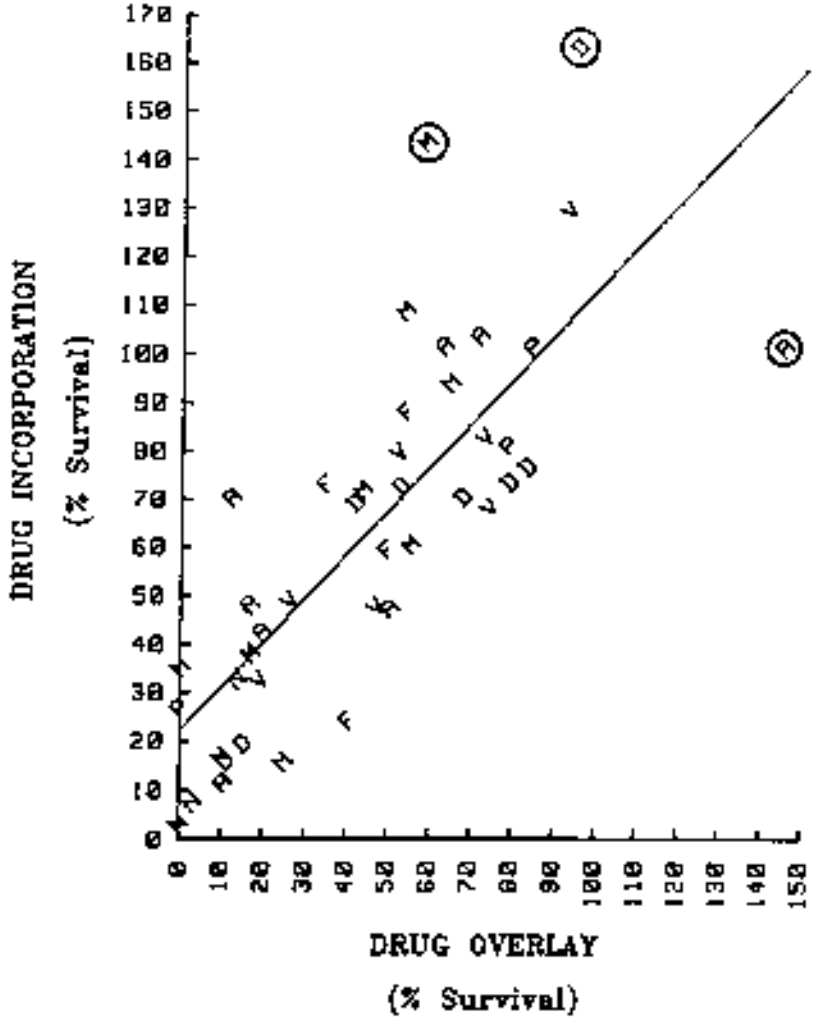

Figure 1. A Comparison of Drug Activities in Primary Human Tumor Cell Culture Following Two Methods of Drug Application. The graph depicts paired mean percent survival data gathered from nine individual specimen cultures (see text). N = sodium azide (600 µg/ml); F = 5-fluorouracil (10 µg/ml); M = mitomycin C (0.04 µg/ml); A = actinomycin D (0.01 µg/ml); D = doxorubicin (0.6 µg/ml); V = vinblastine (0.05 µg/ml); P = cisplatin (1.5 µg/ml).

was sensitive to all agents at therapeutically relevant concentrations (1x) except mitoguazone. By contrast, colony formation by another renal carcinoma (Figure 2B) was resistant to all agents at the same respective 1x concentrations following identical applications. At higher concentrations (10x and 100x) actinomycin D, mitoguazone and mitomycin C were "active", whereas L-alanosine, etoposide and vinblastine were inactive. Similar in vitro drug sensitivity profiles were observed in subsequent cultures; and, in fact, in vitro colony formation by the former specimen was markedly inhibited (<15% of control growth) by lesser concentrations (0.1x) of actinomycin D, mitomycin C, and vinblastine. Coefficients of variation for the colony count of primary, secondary as well as cell line cultures inoculated from a single, "bulk" cell suspension were generally small: less than 20% for colony counts exceeding 80/500 mm^2 (e.g., see Figure 2).

DISCUSSION

A standardized laboratory assay capable of identifying effective chemotherapeutic agents for individual tumor specimens would be a useful adjunct to the clinical management of cancer patients. While the "human tumor stem cell assay" was designed specifically for this purpose (Salmon et al, 1978), certain technical features of the original assay complicate its performance. For example, the conventional protocol requires that prior to setting up bilayer cultures, aliquots of cell suspension be transferred to separate tubes, each containing a different drug, the same drug at different concentrations, as well as respective drug vehicles (Alberts et al, 1980; Soehnlen et al, 1980). While few tubes may be required for small tumor specimens, larger specimens require many tubes. Not only is tube handling cumbersome, but it is our empirical judgement that excessive mechanical manipulation of cells suspended in culture medium at elevated temperatures may lead to diminished in vitro cell proliferation. Moreover, addition of a small volume of stock cell suspension (at high density) to each tube followed by thorough mixing and application of three, 1 ml aliquots to individual plates using a different pipet for each tube reduces the accuracy and precision of delivering the same number of cells to each dish of a given specimen culture.

As an alternate approach to culture and chemosensitivity testing in the present study, the potential utility of preparing all cultures from a single, "bulk" cell suspension

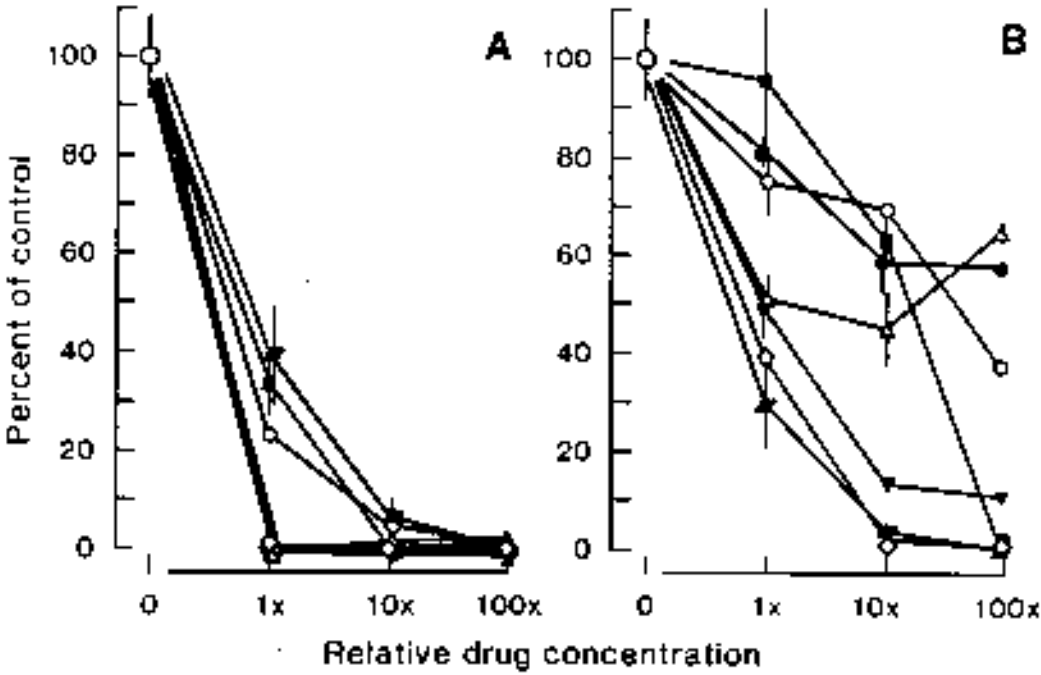

Figure 2. Colony Formation by Two Human Renal Carcinomas (Xenograft-passaged) Following Drug Application to Soft-agarose Culture Surfaces. Colony counts in drug-treated cultures (n=3) are expressed relative to that in vehicle control cultures (○, n=6) as percent. The mean ± 1 SEM for each group is depicted. The following agents were evaluated (1x concentration, μg/ml): ▼ actinomycin D (0.010); ● L-alanosine (5.0); ○ etoposide (1.0); ▰ mitoguazone (50); ◇ mitomycin C (0.04); ▲ vinblastine (0.050); and ■ mercuric chloride (1.0).

followed by culture surface drug application was assessed. Such a methodology was observed previously to be suitable in human tumor cell line cultures (Alley et al, 1982). In the present study surface application of a large battery of clinically useful drugs brought about excellent inhibition of tumor cell proliferation in cell line as well as primary and secondary cultures. The increased frequency of detecting chemosensitive cell cultures following surface drug application appeared to result from improved tumor cell growth (evaluability rate) afforded by fewer mechanical manipulations prior to culture. Thus, the method of drug application appears to be a subtle, but important factor

which influences the ease of performance and evaluability of human tumor cell cultures of this type.

SUMMARY

Previous methods of evaluating human tumor cell chemosensitivity using soft-agar colony formation assay have required that cell suspensions be aliquoted into multiple tubes, one for each drug concentration and each drug vehicle prior to culture inoculation. In the present study, the utility of an alternate method of drug exposure was investigated: Application of drug and/or drug vehicle to culture surfaces following cell inoculation. Culture surface drug application was observed 1) to provide a means to avoid mechanical manipulation of cells in the presence of drug and 2) to reduce the number of steps for chemosensitivity testing required by other methodologies. In addition, the preparation of all culture dishes from a single, "bulk" cell suspension for each specimen culture appeared 1) to improve the accuracy and precision of culture inoculation and 2) to facilitate growth in soft-agar colony formation assays.

ACKNOWLEDGEMENTS

The authors wish to acknowledge preparation of human solid tumor specimens by the pathology staff of St. Marys Hospital and Rochester Methodist Hospital, culture and chemosensitivity testing by Mary Adams, Linda Foster, Barbara Furlow, Sue Gossman, Sharon Guy, Dane Mathieson, Cindy Ghl, and Carol White, and manuscript preparation by Shelly Nicklay.

REFERENCES

Alberts, D.S., Chen, H.-S., and Salmon, S.E. In vitro drug assay: Pharmacologic considerations. In S.E. Salmon (ed.), Cloning of Human Tumor Stem Cells, Prog. Clin. Biol. Res., Vol. 48, New York: Alan R. Liss, 1980, 197-207.

Agrez, M.V., Kovach, J.S., Beart, R.W. Jr., Rubin, J., Moertel, C.G., and Lieber, M.M. Human colorectal carcinoma: Patterns of sensitivity to chemotherapeutic agents in the human tumor stem cell assay. J. Surg. Oncol., 1982a, 20:187-191.

Agrez, M.V., Kovach, J.S., and Lieber, M.M. Cell aggregates in the soft agar "human tumor stem-cell assay". Brit. J. Cancer, 1982b, 46:880-887.

Alley, M.C., Uhl, C.B., and Lieber, M.M. Improved detection of drug cytotoxicity in the soft-agar colony formation assay through use of a metabolizable tetrazolium salt. Life Sci., 1982, 31:3071-3078.

Alley, M.C. and Lieber, M.M. Improved optical density of colony enlargement and drug cytotoxicity in primary soft agar cultures of human solid tumour cells. Brit. J. Cancer, February, 1984, in press.

Alley, M.C., Powis, G., Appel, P.L., et al. Activation and inactivation of cancer chemotherapeutic agents by rat hepatocytes cocultured with human tumor cell lines. Cancer Res., February, 1984, in press.

Kressner, B.E., Morton, R.R.A, Martens, A.E., et al. Use of an image analysis system to count colonies in stem cell assays of human tumors. In S.E. Salmon (ed.), Cloning of Human Tumor Stem Cells, Prog. Clin. Biol. Res., Vol. 48, New York: Alan R. Liss, 1980, 179-193.

Lieber, M.M., Ames, M.M., Powis, G., and Kovach, J.S. Drug sensitivity testing in vitro with a liver microsome "activated" soft agar human stem cell colony assay. In I.J. Fidler and R.J. White (eds.), Design of Models for Testing Cancer Chemotherapeutic Agents, New York: Van Norstrand Reinhold, 1982, 12-18.

Salmon, S.E., Hamburger, A.W., Soehnlen, B., et al. Quantitaion of differential sensitivity of human tumor stem cells to anticancer agents. New Engl. J. Med., 1978, 298:1321-1327.

Soehnlen, B., Young, L., and Liu, R. Standard laboratory procedures for in vitro assay of human tumor stem cells. In S.E. Salmon (ed.), Cloning of Human Tumor Stem Cells, Prog. Clin. Biol. Res., Vol. 48, New York: Alan R. Liss, 1980, 331-338.