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Relation of In Vitro Colony Survival to Clinical Response in a Prospective Trial of Single-Agent Chemotherapy for Metastatic Melanoma

By Frank L. Meyskens, Jr, Lois Loescher, Thomas E. Moon, Bonnie Takasugi, and Sydney E. Salmon

We have used the effect of therapeutic agents on clonogenic growth in agar to discriminate between active and inactive agents for malignant melanoma. We report a prospective study of single-agent chemotherapy for metastatic melanoma. Forty-five separate in vitro/in vivo correlative trials were conducted in 34 patients. A number of agents were used in these evaluations, including actinomycin D, Amsacrine, bisantrene, mitoxantrone, BCNU, vinblastine, vindesine, 5-fluorouracil, MGBG, etoposide, interferon, tamoxifen, and 13-cis-retinoic acid. At the "cut-off" concentration, a colony survival $< 30\%$ was designated as "sensitivity" and $> 30\%$ as "resistance." Clinical sensitivity was designated to include complete, partial, and mixed responses and was predicted in eight of 18 trials (44%). Clinical resistance (nonresponse) was predicted correctly in 24 of 27 cases (89%). Using Fisher's exact test the association of in vitro and in vivo results was significant ($P = .05$). These results offer further support for the concept that clonogenic assays may help select useful agents for clinical trials in metastatic melanoma.

AN IN VITRO ASSAY that could accurately predict clinical response to anticancer therapy would be of use for selecting therapeutic compounds for further development or for individual patients.[1-3] In vivo studies of animal tumors have documented that a small subpopulation of cells (tumor stem cells) is responsible for the growth and regrowth of tumors after subcurative therapy.[4] Studies of human tumor stem cells cannot be performed in situ; however, closely related clonogenic tumor cells have been studied in assays developed to estimate the frequency and properties of human tumor stem cell populations.[5-7]

Using a bilayer agar human tumor colony assay (HTCA), we have extensively studied the biology and chemosensitivity of clonogenic cells obtained from biopsies of human melanomas.[8-18] We have demonstrated that these cells are malignant melanocytes as analyzed by light and transmission electron microscopy[8,11] and found that the number of colonies formed was linearly related to the number of cells plated.[8,12] A classic survival curve to ionizing radiation with D_0's between 2.0 and 3.5 Grey can also be demonstrated.[14] Additionally, we have shown that cells in human melanoma colonies can self-renew, as demonstrated both by replating of primary colony cells in agar[12] and by injection of colonies into nude mice with the subsequent development of human melanoma tumors.[17] Other experiments have shown that the self-renewing cells are not limited to a few colonies but are widely distributed,[18] suggesting that primary melanoma colony growth in this clonogenic assay is representative of the stem cell compartment of this tumor. Overall, these studies have provided a sound biological basis for the use of this HTCA to study the in vitro chemosensitivity of human melanoma.

In a prior correlative clinical study, we carried out a retrospective analysis of 48 in vitro/in vivo comparisons and included patients receiving multidrug therapy.[9] Using the same response criteria as in our current prospective trial, the assay was 86% accurate (25 of 29 trials) in identifying drugs to which the tumor was resistant in vivo and 63% accurate (12 of 19 comparisons) in selecting drugs to which the tumor was clinically sensitive. Similar retrospective observations have been recorded by two other investigators using different culture conditions for identification of clonogenic cells.[7,19] These results with malignant melanoma and favorable prediction

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rates in our pilot study, which evaluated m-AMSA against human malignancies using a clonogenic assay in a prospective design,[20] prompted us to undertake a prospective trial in patients with metastatic malignant melanoma. Correlative in vitro/in vivo accuracy was 44% (eight of 18 trials) for sensitivity and 89% (24 of 27 comparisons) for resistance.

PATIENTS AND METHODS

Patient Studies

All patients had metastatic malignant melanoma. Biopsy specimens from subcutaneous or skin sites in 51 consecutive patients with metastatic melanoma were cultured and growth adequate for evaluation of chemosensitivity in vitro was obtained in 34. Forty-five clinical trials of single agents in these 34 patients formed the basis for the in vitro/in vivo correlations. In vitro studies were performed either before chemotherapy or at least four weeks after the last course of chemotherapy. In 40 of 45 cases, patients had been treated with prior chemotherapy before entering this study. Only patients whose clinical trials were evaluable for response were included in the in vitro/in vivo correlation studies. Clinical evaluation of response to treatment was assessed with standard Southwest Oncology Group criteria. Complete response (CR) represented objective disappearance of all evidence of melanoma; partial response (PR), > 50% regression of all measurable disease; mixed response (MR), > 50% regression of one or more evaluable disease sites but not all sites. Improvement (IMP) represented 25% to 50% tumor regression. Lesions were measured in at least two dimensions and the product of maximal distances used to determine size. As the clinical situation did not always allow waiting for the in vitro result, correlations between in vitro and clinical responses reported in this paper include a combination of prospective correlative trials (results of biopsy not known before therapeutic agent was started, ten trials) and decision-aiding trials (selection of therapeutic agent based on in vitro results, 35 trials).[1] The data from these two types of trials were combined, as the prospective correlative group did not contain a sufficient number of trials for separate analysis and their inclusion did not affect the overall correlative accuracy.

Preparation of Specimens

Stock solutions of intravenous formulations of 1,3-bis-chloro-(2-chloroethyl)-1 nitrosourea (BCNU), cisplatin (DDP), melphalan (PAM), actinomycin D (AD), vindesine (VDE), vinblastine (VB), amsacrine (AMSA), methotrexate (MTX), hydroxyurea (HU), 5-fluorouracil (5-FU), bisantrene (BIS), mitoxantrone (MITOX), methylglyoxalbisguanyl hydrazone (MGBG), tamoxifen (TAM), medroxyprogesterone acetate (MP), and etoposide (VP-16) were prepared in sterile buffered saline or water and stored at −70 °C in aliquots sufficient for individual assays; 13-cis-retinoic acid (13cRA) was stored in 100% dimethylsulphoxide (DMSO) in light-protected vials at −70 °C as a stock solution (10^{-3}mol/L) and diluted with medium just before use. The compound was used only in dim light conditions as previously described.[15] Recombinant human leukocyte interferon (IFN-αA) was handled as previously described.[21]

Tumor cell suspensions were transferred to tubes and adjusted to a final concentration of 10^6/mL in the appropriate drug or control medium. Each drug was tested at concentrations that we calculated from pharmacokinetic data as pharmacologically achievable in vivo. Final concentration ranges (in μg/mL) were 0.001 to 0.100 for AD, 0.05 to 0.50 for BIS, 0.1 to 1.0 for AMSA, 0.01 to 0.10 for VB, 0.01 to 0.10 for MITOX, 0.01 to 0.20 for BCNU, 0.01 to 0.10 for VDE, 0.05 to 5.00 for 5-FU, 0.01 to 0.10 for VP16, 0.01 to 1.00 for DDP, 0.005 to 0.050 for MGBG, 0.01 to 1.00 for PAM, and 1 to 10 for HU. Final concentration ranges for other drugs were 13cRA (5×10^{-5} to 5×10^{-7}mol/L), TAM and MP (10^{-5} to 10^{-8}mol/L), and IFN-αA (0.4 to 10.0 ng/mL). A minimum of two and frequently three concentrations of drugs was used.

Preparation of Specimens and Culture Assay

Single-cell suspensions were prepared from tumor biopsy by mechanical techniques described previously.[9] Cells were incubated with or without drugs for one hour at 37 °C in medium with 10% heat-activated fetal calf serum (HIFCS). The cells were then centrifuged at 150 g for ten minutes, washed twice with serum-free medium, and prepared for culture. Loss of cells during this procedure was < 5% in both the control and drug-treated samples. IFN-αA, 13cRA, MP, and TAM were tested by continuous rather than one-hour exposure and incorporated into the agar gel matrix.

The culture system of Hamburger and Salmon has been extensively described.[5,9] In brief, 5×10^5 cells were suspended in a 1-mL volume of 0.3% agar containing 10% horse serum in enriched Connaught Medical Research Laboratories (Ontario, Canada) Medium 1066 and plated over a 1-mL nutrient feeder layer of McCoy's 5A medium with 10% HIFCS in 0.5% agar in 35 mm² plastic Petri dishes. Conditioned medium was not required in the feeder layer. Plates were routinely monitored after plating for single-cell dispersion and all control and drug assays were in triplicate. Plates were examined on day 1 for aggregates. Those containing more than ten aggregates were discarded, but this is rare in melanoma, as good cell dispersion is usual. Plates were incubated at 37 °C in a humidified atmosphere containing 6% CO_2 for ten to 21 days. Melanoma tumor colony-forming unit (TCFU) colonies > 60 μm in diameter were counted with the Omnicron (Bausch and Lomb, Rochester, NY) image analysis system. The mean plating efficiency was 0.05% (median, 0.01%; range, 0.006% to 0.400%). At least 30 colonies per control plate were required to assess the results of chemosensitivity assays, and this occurred in 34 of 51 samples tested. A linear relationship between the number of cells plated and the number of colonies produced in control cultures has previously been demonstrated.[8,12] Morphology of the neoplastic melanoma cell colonies was further defined with a dried-slide technique and a combination of Papanicolaou and melanin staining.[8,22]

Statistical Analysis

All assay data were stored on a Wang 2200 C laboratory computer disc file. Plating efficiencies were calculated from the total number of cells plated and were not corrected for the proportion of nontumor cells in the sample. Standard error of the mean for individual data points (mean of triplicate plates) aver-

aged 5%. The mean of triplicate observations of the survival of TCFU for each patient was plotted against drug concentration. Because of practical limits on the number of cultures that can be set up on fresh biopsies, higher plating concentrations were not routinely used to define drug dose effects at TCFU levels < 5% of control.

Previous experience with the assay has suggested that survival of TCFU at one-tenth the calculated pharmacologically achievable concentration × time (one hour) (cut-off concentration) provides a useful parameter to relate to clinical response for drugs with a short half-life and one-tenth the steady state concentration for drugs with long half-lives.[15,23,24] The cut-off concentrations specified for concentration × time (one hour) (in µg/mL) were AD (0.01), VB (0.05), VDS (0.05), MGBG (0.05), MITO (0.05), AMSA (0.10), VP16 (0.10), DPP (0.50), MEL (0.10), 5-FU (0.50), and BIS (0.50). The cut-off concentrations used for agents tested by continuous exposure were 4 ng/mL for IFN-αA, 10^{-6} mol/L for TAM and MP, and 5 · 10^{-6} mol/L for 13-cRA. These concentrations were selected on the basis of an extensive compilation of the literature[24] and our own pharmacokinetic studies.[21,24,25] The doses necessarily represent an approximation to drug concentrations that could be found in the plasma of any individual patient. On the basis of training sets of in vitro/in vivo correlations in experiments with ovarian cancer,[26,27] multiple myeloma,[26,28] and malignant melanoma,[9] patients with melanoma were classified as sensitive if survival of TCFU was ≤ 30% at the cut-off concentration and resistant if > 30%. In our initial study with melanoma, 38% colony survival allowed the best retrospective "fit" for true positive and true negative correlations, although 30% colony survival gave similar results.[9] In the present trial we have selected 30% colony survival as the boundary, as this has become the standard for this assay at numerous institutions. This simple approach to classifying in vitro response has developed from a careful analysis of the more complex "area under the curve"[23] and should be viewed as an operational method of determining in vitro chemosensitivity and relating TCFU survival to clinical response.

RESULTS

Relationship of In Vitro Colony Survival and In Vivo Clinical Response

A median of ten drugs per tumor specimen (range, one to 19) and a total of 444 drug tests were performed in vitro. The results served as the basis for these trials. Overall, in 63 tests (14%) survival of TCFU was decreased to < 30%. Twelve different drugs were used in the correlative trials and include AD (ten trials), BIS (eight trials), interferon (seven trials), AMSA (five trials), TAM (four trials), VB (three trials), MITOX (two trials), BCNU (two trials), VDE (one trial), 5-FU (one trial), VP16 (one trial), and 13cRA (one trial).

The relationship of colony survival at the cut-off concentration of the drug to clinical effect is depicted in Fig 1. The details of the clinical re-

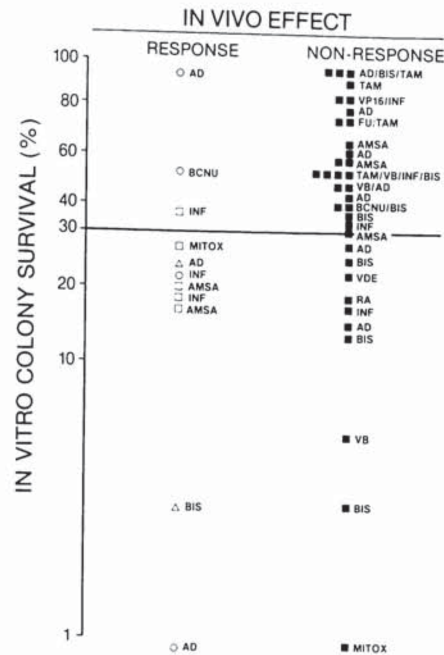

Fig 1. Relationship of in vitro colony survival and in vivo clinical effect. Symbols indicate clinical effect: △, CR; ○, PR; □, MR; ■, stable or progression (nonresponse).

sponses of all patients are summarized in Table 1. In 27 cases, colony survival was > 30%, and in 24 of the trials, no clinical response occurred. In 18 cases, colony survival was < 30%, and in eight of the trials a clinical response occurred. Among the eight patients whose colony survival was < 30%, two CRs, two PRs, and four MRs were recorded. The overall in vitro/in vivo correlations for this prospective trial are summarized in Table 2. Correlative in vitro/in vivo accuracy was 44% for true positive and 89% for true-negative. Using Fisher's exact test, the association between the in vitro and in vivo outcomes was significant (P = .05).

The overall clinical response rate was 24% (11 of 45 patients) for all patients entered on this study. For the 17 patients whose cells did not grow sufficiently for drug evaluation, four (24%) responded to single agents. These response percentages were compared with the correlative true-positive accuracy (47%) for the assay and both were significantly different (P = .05, Fisher's exact test).

DISCUSSION

We have performed a prospective single-agent correlative trial for metastatic malignant melano-

Table 1. Description of Clinical Responses of All Patients in Prospective Trial

Patient No.	Drug	In Vitro Colony Survival* (%)	Clinical Response Type	Duration (mo)	Description
1A	AD	10	PR	4	Multiple lymph nodes
2B	BIS	30	CR	6	4 × 2 cm lymph node
3	AMSA	19	MR	2	50% decrease in liver, stable nasal mass
2A	IFN-αA	21	MR	6	Disappearance of lymph nodes; some subcutaneous nodules
1B	AMSA	22	MR	3	Disappearance of some subcutaneous nodules
4	IFN-αA	24	PR	6	Subcutaneous lung nodules and liver
5	AD	28	CR	5	Multiple lymph nodes
6	DHAD	28	MR	2	Disappearance of some subcutaneous nodules
7	IFN-αA	36	MR	3	Disappearance of some subcutaneous nodules
8	BCNU	49	PR	4	Large pelvic mass
9	AD	94	PR	4	Lymph nodes

NOTE. Type of response: CR = complete, PR = partial, MR = mixed, DHAD = dihydroxyanthracenedione.

*Percentage of survival at cut-off concentration of drug as defined in Patients and Methods.

ma relating colony survival to clinical response. Using as an operational definition 30% colony survival at a predesignated cut-off concentration, the predictive accuracy for true-negatives was 89%. These results are similar to our findings in a multidrug retrospective trial in melanoma in which the agar assay was 86% accurate in identifying drugs to which the patient was resistant in vivo and 63% accurate in identifying drugs to which the tumor was clinically sensitive. The results for predicting true-negatives are similar to those obtained for other tumor types[27-30] and is comparable to the accuracy for predicting clinical resistance reported by Tveit et al[19] and Kirkwood and Marsh[7] using different methodologies to grow clonogenic human melanoma cells.

Table 2. Prospective In Vitro/In Vivo Correlations for Metastatic Melanoma*

		In Vivo Response CR	PR	MR	Nonresponse† Other	Total
In Vitro Survival*	S	2	2	4	10	18
	R	1	0	2	24	27

NOTE. In vitro survival: S ≤ 30% colony survival at cut-off concentration; R > 30% colony survival at cut-off concentration.

*Using Fisher's exact test, the association between the in vitro (< or > 30% colony survival) and in vivo (response or nonresponse) outcome was significant at $P = .05$.

†Nonresponse includes stable and progression.

Both our retrospective (63% accurate) and prospective (44% accurate) studies have demonstrated less accuracy in predicting true positives and is comparable to results obtained by others using different clonogenic bioassays.[7-19] Kirkwood and Marsh, using agar diffusion chambers to grow clonogenic melanoma cells, predicted clinical sensitivity accurately in five of ten cases when marked sensitivity was used as the in vitro criterion and in nine of ten cases when intermediate sensitivity was also included.[7] Tveit et al were able to show 91% correlative accuracy (ten of 11 cases) using the Courtenay and Mills procedure to assess the chemosensitivity of clonogenic melanoma cells.[19] However, clinical sensitivity criteria were more lenient in these two studies and included stable disease as responsive and therefore cannot be directly compared with our present experience, wherein stable disease is included in the nonresponse category.

The overall response rate in the present study is comparable to that reported by others when mixed responses are included as responses.[31,32] However, because most of the patients had biopsy of subcutaneous or skin metastatic sites (and therefore possibly less aggressive disease), growth of melanoma cells in agar could be select-

ing more responsive patients. This was not the case in this study, as the overall clinical response was 24% for patients whose biopsies grew as well as for those 17 patients whose biopsies did not grow. The response rate of 24% was significantly less ($P = .05$) than the 44% response rate seen in patients who were treated with drugs that had produced TCFU survival of < 30% in vitro.

Our work has provided a basis for the experimental study of clonogenic human melanoma cells. Our previous data, the current study, and the work of others using different clonogenic bioassays support the proposition that the response of clonogenic melanoma cells to chemotherapeutic agents can be accurately used to predict cellular and tumor resistance. The results of HTCA in melanoma are similar to those observed in other tumor types except for the lower true-positive rate.[1] However, such tumors as ovarian cancers show clinical response to a variety of agents.

The role of this bioassay in predicting clinical responsiveness remains to be clarified. The major limitation at present in the treatment of melanoma is the lack of effective agents for treatment. In lieu of effective drugs, no predictive assay is likely to play a major role in managing clinical therapy. Furthermore, 40 of 45 patients in this study had received prior therapy. Conceivably, in vitro assay may be of greater value in patients who have not had prior therapy, as previously treated patients with metastatic melanoma in relapse or with progressive disease rarely achieve a good remission with any therapeutic agents. Although sensitivity of clonogenic melanoma cells in vitro to chemotherapeutic drugs cannot be translated to clinical response with high accuracy, our results and those of others suggest that sensitivity in vitro may be useful as a screening modality. In the effort to identify new agents, overprediction of activity is clearly more desirable for screening than underprediction.

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