

Correlation of Drug Response in Human Tumors Histocultured *in Vitro* with an Image-Analysis MTT End Point and *in Vivo* Xenografted in Nude Mice

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Abstract. We have in this study used the 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl 2-H-tetrazolium bromide (MTT) end point in our histoculture drug-response assay. We have previously demonstrated that the formazan crystals formed by MTT reduction by mitochondrial succinate dehydrogenase reflect polarized light and can be measured by pixel analysis in intact tissue. The results described here indicate a total specificity of 93.8% and a total accuracy of 74.6% of the MTT end point for drug response in histoculture correlating with nine different human xenograft tumors grown in nude mice with respect to the *in vivo* drug response data. This *in vitro* system allows prediction of positive and negative responses to drugs, with a rate of 70% and 71.8%, respectively. The system described here has potential for clinical use because of the possibility of simultaneous description of the MTT values and heterogeneous response to drugs within individual tumors.

A large effort has been made over the past few decades to develop *in vitro* methods for precise prediction of *in vivo* chemosensitivity of human tumors. See Hoffman for reviews of the development of chemosensitivity assays [1-3]. The human tumor clonogenic assay by Hamburger and Salmon [4] has achieved 91% predictability for clinical chemoresistance and 69% for chemosensitivity [5-7]. However, only 30% of

Abbreviations: MTT, 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl 2-H-tetrazolium bromide; MMC, mitomycin C; 5-FU, 5-fluorouracil; DDP, cisplatin; Melph, Melphalan; DOX, Doxorubicin; RW, relative mean tumor weight; ip, intraperitoneally; iv, intravenously.

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tumors attempted to be cultured in the clonogenic assay have been evaluable (6).

Kern and Weisenthal, with agar-based and suspension culture systems, were able to accurately predict chemoresistance but were not able to accurately predict chemoresponsiveness [19]. One of the major problems was in the use of single-cell suspensions derived from disaggregated tumor tissue, thereby removing the tumor cells from their native histology.

Because the present efficacy of any single agent still remains at 10-25%, resulting in a high negative rate in most *in vitro* chemosensitivity assays it is important to develop methods to accurately determine the relatively infrequent positive responses to chemotherapy.

The gel-supported three-dimensional histoculture assay introduced by Hoffman *et al* [9-12] and based on earlier work by Leighton (21) allows the culture of fresh surgical specimens while maintaining their cell-to-cell contact in a three-dimensional native tissue architecture. The histoculture assay possesses the theoretical potential for accurate prediction of chemosensitivity based on tissue architecture, tumor-stromal interaction and differentiated function [1-3,9-12]. Using the end point of [³H]thymidine incorporation measured by histological autoradiography, it was shown that *in vivo* chemoresistance was correlated at an 86% rate comparing the drug response of human tumor xenografts grown *in vitro* and in nude mice. Overall *in vitro-in vivo* correlation of drug response varied depending on the drug, from a 53% rate for 5-fluorouracil to a 78% rate for doxorubicin [11].

Using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) end point (17, 18) on cells in suspension, relatively-high *in vitro-in vivo* correlations were observed [8]. The MTT end point was applied to the histoculture assay in a recent study (23) and was found to increase the overall *in vitro-in vivo* correlative ability of the assay. However, formazan crystal formation from MTT due to succinate

Table I. Summary of drug response comparison of 9 human xenografts grown *in vivo* in nude mice and *in vitro* in three-dimensional sponge-gel supported histoculture. Calculations were made by doing the following:

Percent accuracy of the MTT assay = [(no. true positives + no. true negatives)/no. total]x100

Percent specificity = [no. true negatives/(no. true negatives + no. false positives)]x100

Predictive value positive = [no. true positives/(no. true positives + no. false positives)]x100

Predictive value negative = [no. true negatives/(no. true negatives + no. false negatives)]x100

True positive = number of sensitive cases *in vitro* and *in vivo*

True negative = number of resistant cases *in vitro* and *in vivo*

False positive = number of sensitive cases *in vitro* and resistant *in vivo*

False negative = number of resistant cases *in vitro* and sensitive *in vivo*

	Mitomycin C	Doxorubicin	5-Fu	Cisplatinum	Melphalan
True positive	3	0	2	1	1
True negative	2	6	6	5	5
False positive	0	1	0	1	0
False negative	4	1	1	2	1
Accuracy	0.56	0.75	0.89	0.67	0.86
Specificity	1	0.86	1	0.83	1
Predict. pos. val.	1	0	1	0.50	1
Predict. neg. val.	0.33	0.85	0.86	0.71	0.83
Total Accuracy of the Assay = 74.6%	Total Predict. pos. val. = 70.0%				
Total Specificity of the Assay = 93.8%	Total Predict. neg. val. = 71.8%				

dehydrogenase activity was measured by spectrophotometric methods after extraction which not only does not allow simultaneous histological observation but is labor intensive.

As Phillips *et al* (20) have pointed out, using an animal model to compare *in vivo* and *in vitro* drug response is a much more controlled experiment than a clinical trial with its myriad of uncontrolled variables. Utilizing this approach with nude mice, with all its advantages of a constant and reproducible cohort of human tumor xenografts for the *in vivo* arm, we have in this study used our previously developed image analysis technique to measure the MTT end point in the histoculture assay by the ability of the formazan crystals to reflect polarized light in intact tissue (22) to obtain more accurate *in vitro-in vivo* correlations than were obtained with the use of the [³H]thymidine end point.

Materials and Methods

BALB/c nu/nu mice originating from the Central Institute for Experimental Animals (Kawasaki) were obtained from CLEA Japan Inc., Tokyo Japan. Six-to-eight-week-old mice weighing 20-22g were used.

Human tumor xenografts. Tumors evaluated include the gastric cancers St-4, St-15, St-40, H-111, and SC-2-JCK; the colon cancers Co-3, Co-4, Co-6 and Co-8; the breast cancers MX-1 and MCF-7; the small-cell lung cancers Lu-24, Lu-130 and H-69; hepatoma Li7; and neuroblastoma CR-NB9. H-111 was kindly provided by Dr. M. Fujita, Osaka University. SC-2-JCK was established at the Central Institute for Experimental Animals, Kawasaki and provided by Dr. K. Maruo. MX-1 was provided by Dr. K. Inoue, Cancer Chemotherapy Center Tokyo. The other strains were established in the Pathology Division, the National Cancer Research Institute and at Keio University School of Medicine, Tokyo. All these tumors were maintained in the Keio University School of Medicine by serial transplantation into nude mice [13-15].

Drugs. Drugs used were mitomycin (MMC), doxorubicin (DOX), 5-fluorouracil (5FU), cisplatin (DDP) and melphalan (Melph). The drugs were obtained from Sigma Chemical Co, and dissolved in physiological saline, except for melphalan which was dissolved in ethanol. Drug concentrations used were the following: MMC 100 ng/ml; DOX 29 ng/ml; 5FU 4.0 μg/ml; DDP 1.5 μg/ml; Melph 1.0 μg/ml. These concentrations are believed to represent clinical concentrations *in vitro*.

Histoculture. Tumors were explanted using the sponge-gel-supported three-dimensional *in vitro* histoculture system developed by Hoffman *et al* [9-12] based on the earlier work of Leighton (21). Specialized collagen gels manufactured from pigskin were purchased from Health Design Incorporated, Rochester, NY. Collagen gels were removed from their sterile packages and cut with scissors into cm² pieces and placed in 6-well plates.

Tumors in the exponential growth phase in nude mice were resected aseptically, necrotic tissues were cut away and the remaining healthy tumor tissues were minced with scissors into 2-mm diameter bits in Hanks' balanced salt solution and further minced into 4-to-10 1-mm diameter bits, which were placed on the same collagen surface.

Two ml/well of Eagle's minimum essential medium containing 10% serum were added into 6-well plates, at which volume the upper part of the gels were reached but not covered. All drug concentrations were tested in triplicate wells.

The histocultures were incubated for a total of 2 days at 37°C in a humidified atmosphere containing 95% air /5% CO₂.

MTT assay. The activity of living cells in and on the collagen gels was evaluated using the MTT end point [8,22]. 100-μl of MTT solution, prepared by dissolving the powder at 8mg/ml in phosphate-buffered saline and filtering through a 0.45 μm membrane filter (Millipore, Bedford, MA), was added to each well and the plates were further incubated for 1-3 hours. At this point frozen sections of 4 μ were made. The resulting slides were dipped in 1.25 μg/ml propidium iodide (PI) for 2 minutes.

For image analysis as previously described (22), a Nikon Optiphot microscope with an RCA video camera was used. Slides were viewed under both bright-field light as well as polarizing light without bright-field light. The cells containing formazan crystals due to MTT reduction brightly reflect the polarized light generated by a mercury lamp. The

slides were also analysed for PI fluorescence whereby the dye was activated using a DM-580 G2A filter. The number of bright pixels from PI fluorescence was calculated also by using the Fas-Com program (11, 12). Cell morphology could be observed due to PI fluorescence whereby cancer cells could be distinguished from stromal cells. The ratio of formazan bright pixels to PI bright pixels was calculated for each drug concentration tested and compared to the control value in order to obtain the amount of drug-induced inhibition. The image was digitized by a digitizer board and the area of brightness corresponding to the number of labeled or bright cells was calculated as the area of enhanced pixels by the Fas-Com version of the P-See program (The Microworks, Del Mar, CA) run on an IBM PC XT clone. The area of enhanced pixels is proportional to the number of labeled cells.

In vivo chemosensitivity testing. Tumor fragments were inoculated into the subcutaneous tissue of either side of the backs of nude mice. The length and width of the tumors were measured with sliding calipers three times a week by the same person. The tumor weight was calculated using the formula: Tumor weight (mg) = length (mm) x [width (mm)]²/2. When tumors reached 100-300mg, usually 2-3 weeks after the tumor inoculations, tumor-bearing mice were randomized into control and treated groups, consisting of at least four mice each, and treatment was initiated. All the drugs were dissolved in 0.2ml of 0.9% NaCl per 20 g of body weight. The drugs were administered bolus ip for cyclophosphamide, MMC, and DDP, iv for DOX, whereas 5-FU was given ip at a schedule of q4dx3. The doses administered were 6, 9, 8, 60 mg/kg, and 80 mg/kg for MMC, DDP, DOX, 5-FU, and cyclophosphamide respectively, which were determined as the maximum tolerated doses for nude mice in our previous reports [14-16]. Mice and tumors were observed 3 times a week for 3 weeks after the initial treatment and the relative mean tumor weight (RW) was calculated as W_i/W_o where W_i is the mean tumor weight at the initiation of treatment and W_o is the tumor weight at the time of measurement. The anticancer effects of drugs were evaluated in terms of the lowest Trw/Crw during the experiment, where Trw is the relative mean tumor weight of the treated group and Crw is that of the control group at the same time. The antitumor activity was evaluated as positive when the lowest Trw/Crw during the experiment was less than 42%, which is equivalent to reflecting a 25% reduction of each diameter [14-16].

Results and Discussion

Table I shows a summary of the drug-response results obtained from the comparison between histoculture with the non-colorimetric MTT end point measured by pixel analysis and the *in vivo* results. The results described here indicate a total specificity of 93.8% and a total accuracy of 74.6% of the non-colorimetric MTT end point in histoculture with nine different human xenograft tumors grown in nude mice with respect to the *in vivo* drug-response data. This system allows prediction of positive and negative responses to drugs, with a rate of 70% and 71.8% respectively. Although the overall correlative value of histoculture with the MTT end point measured by pixel analysis was in some cases less than the same system with the MTT end point measured by extraction and optical density measurement (23), the system described here has good potential because of the possibility of simultaneous description of the MTT values and heterogeneous response to drugs of individual tumors.

The histoculture method with the MTT end point measured by pixel analysis allows the simultaneous observation of

the histology of human tumor specimens *in vitro* and gives a relatively high correlation with *in vivo* drug response and resistance. Data are available in as little as two days after the culture of the specimen is initiated which gives the possibility of wide-spread application of this method for cancer patients. Clinical usefulness is expected from such an accurate, safe, simple and convenient chemosensitivity test.

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