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Regional heterogeneity and pharmacodynamics in human solid tumor histoculture

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Abstract *Purpose*: Human solid tumor histocultures represent a clinically relevant experimental system for pharmacodynamic study. The evaluation of the drug-induced antiproliferative effect in histocultures is usually performed by manual microscopic scoring of individual cells. This procedure, because of its labor intensive nature, is performed on a single microscopic field, i.e. the field with the highest proliferative activity. Because regional heterogeneity in a solid tumor may result in different drug sensitivities in different parts of a tumor, there is the question as to whether the pharmacodynamic data determined in the most proliferative field is representative of those in the whole tumor. This question was addressed in the present study. *Methods*: A recently developed automated image analysis method was used to measure the labeling index of tumor cells. The drug-induced inhibition of DNA precursor incorporation into nuclei of cells in the region with the highest proliferative activity was compared to the inhibition in cells in the entire histoculture. This study was performed in human bladder tumor histocultures treated with several drugs (doxorubicin, mitomycin C, paclitaxel and 5-fluorouridine). A total of 724 pairs of data obtained from untreated and drug-treated histocultures (each data point representing the average of 1 to 6 tumor histocultures) were analyzed. *Results*: The absolute value of the labeling index in the most proliferative region (LI_{one}) was significantly higher than the absolute value of the labeling index in the whole tumor (LI_{all}), in both untreated and drug treated samples (mean difference of 18%, range 1–27%). However, when the absolute LI values in drug-treated samples were normalized to the values in untreated controls and expressed as a percentage of control, and used to construct the concentration-response curves, the two curves obtained using LI_{one} and LI_{all} yielded comparable pharmacodynamic parameters, i.e. curve shape parameters and drug concentrations that produce 30, 50, and 70% inhibition. *Conclusion*: These results indicate comparable pharmacodynamics in the most proliferative region and the whole tumor, and confirm the validity of using the most proliferative field for evaluating chemosensitivity in solid tumor histocultures.

Key words Tumor heterogeneity · Solid tumor histocultures · Automated image analysis · Pharmacodynamics

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Introduction

The most common experimental model for studying drug effect is continuous human cancer cell lines maintained as monolayer cultures, mainly because of the availability of cell lines and the relative ease of the experimental techniques and data interpretation associated with this system. The major shortcoming of the monolayer system is that the cells, usually used for studies while they are in the exponential growth phase, are relatively homogeneous with respect to cell cycle kinetics, growth rate, access to oxygen and nutrients, and drug uptake. To better simulate the microenvironments in human solid tumors, three-dimensional systems such as spheroids [19] and tumor histocultures [20] have been used. The major distinction between the spheroid and histoculture systems is that the former is homogeneous with respect to cell types, does not include fibroblasts or stromal tissues, and does not reflect the intersubject heterogeneity seen in patients. Histocultures are derived from surgical specimens of solid tumors from individual patients and contain both tumor and stromal tissues. Hence, histocultures offer the advantages of coexistence of tumor and stromal cells, maintenance of tissue architecture, cell–cell interaction, and inter- and intratu-

moral heterogeneity. The maintenance of cell–cell interaction in the histoculture system offers the opportunity to study the response of tumor cells without excluding possible environmental effects.

The histoculture system was first introduced in the 1950s [12] but lost its popularity after the introduction of continuous cell lines. In the early 1980s, Hoffman and coworkers reintroduced the use of patient tumor histocultures to predict patient response to chemotherapy [20]. A limited number of laboratories, including our own have since adopted the system for studying the pharmacodynamics of anticancer drugs (see, for example, references [5, 8, 9, 15, 16, 24]). In general, the response of tumor histocultures to drug treatment is several orders of magnitude lower than the response of monolayer cultures of continuous human cancer cell lines [26]. The clinical relevance of the human tumor histoculture system has been demonstrated in retrospective and semiprospective preclinical and clinical studies showing that drug response in patient tumors correlates with the sensitivity and resistance of cancer patients to chemotherapy as well as with patient survival [7, 11, 20].

The use of human solid tumor histocultures for pharmacodynamic studies poses several challenges. First, the success rate of growing histocultures is usually less than 100%, in part because different tumor types have different growth requirements and in part because of suboptimal specimen quality (e.g. low ratio of tumor-to-stromal cell density, contamination of tumors by microbes, damage due to electrocaulterization during surgery). Second, the procedures used for growing the histoculture and for evaluating drug effects are labor-intensive because they require dissecting the tumors into 1 mm^3 pieces, preparing a slide for each tumor piece, and evaluating drug response by microscopic examination of individual cells. The third and the most challenging problem is the intratumoral heterogeneity, i.e. different regions in an individual histoculture have different biological properties which can result in different drug responses. For example, a common pharmacodynamic endpoint is the inhibition of DNA synthesis, measured by the incorporation of DNA precursors such as bromodeoxyuridine (BrdUrd) or ^{3}H-thymidine into DNA [2, 14, 17, 24]. The DNA precursor labeling indices (LI) in different regions of the histoculture can vary by severalfold [21]. Ideally, drug effect should be determined by evaluating all cells in the histoculture. However, because the traditional method for measuring drug effect in histocultures is by manual microscopic scoring of individual cells, the counting of large numbers of cells will be highly labor intensive and impractical. The approach used in the previous studies is to analyze a small region of the specimen, typically containing about 200 to 500 cells, and to use a large number of replicates so that the results are representative of the entire tumor specimen [3, 10, 13, 25, 27]. To standardize field selection in individual histocultures and to minimize selection bias, the customary method is to select the most proliferative field in each sample for evaluation [8, 9, 15, 16, 18, 20, 21, 24], as proposed by Hoffman and coworkers [20]. The rationale is that drug effect measured in this region is likely to be a conservative measurement rather than an overestimate.

Because regional heterogeneity in a solid tumor may affect the chemosensitivity of tumor cells in different parts of a tumor, there is the uncertainty that the pharmacodynamics determined in one region of the tumor may not represent the pharmacodynamics in the entire tumor histoculture. To address this question, we have developed image analysis methodologies to analyze drug effect on large numbers of tumor cells [22, 23]. These methods use several parameters (i.e. cell size, grey level, hue value) to identify and count tumor cells, and determine their labeling status with DNA precursors. We have shown that the image analysis method is a valid alternative to visual scoring of individual cells [23]. Furthermore, by incorporating a motorized microscope stage into the system, the analysis can be fully automated, requiring no investigator supervision after the initial setup. These methods eliminate limitations due to human fatigue and reduce the time requirement of the evaluation process, thereby enabling evaluation of entire tissue sections.

In the present study, we used our newly developed image analysis methods to compare the drug effect in the most proliferative region and in the entire cross-section of tumor histocultures. We evaluated the effect of four anticancer agents, i.e. mitomycin C, doxorubicin, paclitaxel and 5-fluorouridine, in histocultures of bladder tumors excised from patients. Inhibition of BrdUrd incorporation was used as the pharmacodynamic endpoint.

Materials and methods

Chemicals and supplies

Paclitaxel and mitomycin C were gifts from Bristol-Myers Squibb (Princeton, N.J.). Doxorubicin and 5-fluorouridine were purchased from Sigma Co. (St. Louis, Mo.), cefotaxime sodium was purchased from Hoechst-Roussel (Somerville, N.J.), gentamicin from Solo Pak Laboratories (Franklin Park, Ill.), Minimal essential medium (MEM), fetal bovine serum, glutamine, and nonessential amino acids from GIBCO Laboratories (Grand Island, N.Y.), sterile pigskin collagen (Spongostan standard) from Health Designs Industries (Rochester, N.Y.), and BrdUrd from Sigma Co. (St. Louis, Mo.). Antibody against BrdUrd and the 3,3′-diaminobenzidine kit were obtained from BioGenex (San Ramon, Calif.), and the linked streptavidin-biotin immunoperoxidase kit from Dako (Carpinteria, Calif.). All chemicals and reagents were used as received.

Tumor histoculture

Primary human bladder tumors were obtained via transurethral resection or cystectomy and provided by the Tumor Procurement Service at The Ohio State University Comprehensive Cancer Center. Tumor specimens were placed in Hank's medium 10 to 30 min after surgery, and maintained at 4 °C until use. Histocul-

ture of the tumors was performed as previously described [8, 9]. Briefly, specimens were dissected into 1 mm^3 pieces. Four to six tumor fragments were placed on each 1 cm^2 piece of prehydrated collagen gel, and cultured in six-well plates in a humidified atmosphere of 95% air and 5% CO_2. The culture medium consisted of MEM fortified with 9% heat-inactivated fetal bovine serum, 0.1 m*M* nonessential amino acids, 100 μg/ml gentamicin, and 95 μg/ml cefotaxime. The pH of the medium was 7.4.

Pharmacodynamic studies

Ten specimens of human transitional cell bladder carcinoma were cultured and used in the studies. After culturing for 3 to 4 days, tumor histocultures were treated for 2 h with drugs. This is the treatment duration used in intravesical therapy of bladder cancer [15]. We used four drugs with different action mechanisms, including three drugs that have demonstrated activity in bladder cancer patients, i.e. doxorubicin, mitomycin C, and paclitaxel [7, 8, 15], and one drug, 5-fluorouridine, which has demonstrated activity in human bladder tumor histocultures [18]. Six tumors were treated with doxorubicin (0.01 to 100 μ*M*), three with mitomycin C (0.3 to 150 μ*M*), and one with paclitaxel (0.01 to 10 μ*M*), 5-fluorouridine (0.04 to 400 μ*M*), or one of four combinations of paclitaxel and 5-fluorouridine in concentration ratios of 10:90, 40:60, 60:40, and 90:10. The selected drug concentrations are clinically achievable during intravesical therapy where the dose is dispersed in a relatively small dosing volume of 20 to 40 ml [8, 9, 16]. The total number of pharmacodynamic experiments was 15. After drug treatment, tumor pieces were washed three times with 5 ml drug-free medium and then cultured in the presence of 40 μ*M* BrdUrd for 48 h. Next, tumor histocultures and the supporting collagen gels were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections (5 μm) of the paraffin-embedded tissues were mounted on slides, processed for immunohistochemical staining of BrdUrd (see below) and counterstained with hematoxylin. All nuclei were stained blue by hematoxylin whereas the BrdUrd-labeled nuclei were stained brown. Control tissues were treated similarly but were not treated with drugs.

Drug effect was measured by the inhibition of DNA synthesis, i.e. a reduction in BrdUrd labeling. The BrdUrd LI of each tumor piece was determined in two regions: (1) the 400× magnification field which showed the highest percentage of BrdUrd-labeled cells (LI_{one}), and (2) the entire tissue section (LI_{all}). The concentration-effect curves of doxorubicin, mitomycin C and 5-fluorouridine were analyzed using Eq. 1 to determine the values for IC_{30}, IC_{50}, and IC_{70}, i.e. concentrations needed to produce 30, 50, and 70% inhibition of DNA synthesis, respectively.

$$E = E_0\left(1 - \frac{C^n}{K^n + C^n}\right) \quad (1)$$

where E is the LI of drug-treated tissues and is expressed as a percentage of the LI of control tissues, E_0 is the baseline LI in the absence of drug (i.e. 100%), C is the drug concentration, K is the concentration at one-half E_0, and *n* is a curve shape parameter.

Determination of BrdUrd labeling

Tissue sections were deparaffinized and hydrated. The BrdUrd antigen was unmasked by boiling the slides in 700 ml citrate buffer (10 m*M* citric acid, pH 6.0) for 15 min. Following antigen retrieval, the slides were cooled for 15 min and rinsed in phosphate-buffered saline (PBS). Immunohistochemical detection of BrdUrd was performed using the linked streptavidin-biotin immunoperoxidase kit. Briefly, nonimmune goat serum was applied to the tissues for 10 min to block nonspecific binding sites. After excess goat serum had been removed, the anti-BrdUrd antibody, diluted 1:250 in bovine serum albumin (5 mg/ml), was applied to the tissue at room temperature in a humidified chamber for 30 min. After rinsing in PBS, biotinylated linker antibody and streptavidin-peroxidase were applied sequentially for 20 min each, followed by rinses with PBS. Peroxidase activity was detected with 3,3′-diaminobenzidine after incubation for 5–7 min. The slides were rinsed in water, counterstained with hematoxylin, dehydrated and coverslipped.

Image analysis

The fraction of BrdUrd-labeled nuclei was determined by image analysis using a SAMBA 4000 image analysis system (Imaging Products International, Chantilly, Va.) equipped with a motorized stage. Specimens were viewed through an Axioskop microscope at 400× magnification (Zeiss, Thornwood, N.J.) and images were captured with a CCD color video camera mounted on top of the microscope. The analogue images were transformed with the image processor into 640 × 480 digital images having 256 grey levels and 8-bit density resolution. Prior to analysis, the three color channels of the camera were balanced by adjusting the light intensity of the microscope and the gain and offset of the camera using a program provided with the image analysis system.

The image analysis program used for evaluation of BrdUrd staining was as described previously [22, 23]. This method evaluates the labeling in individual cells in four steps. First, a grey level threshold (GLT) was used to distinguish the nuclei stained by hematoxylin (blue color) and/or diaminobenzidine (brown color) from the unstained background objects. The GLT defined the upper grey level limit for selecting pixels for analysis; darkly stained pixels that had grey values below the GLT were selected for analysis whereas brighter pixels were excluded. A second parameter, size range, was used to reject irrelevant objects such as cellular debris and collagen that did not meet the size range criteria. Third, the BrdUrd-labeled nuclei (brown color) were distinguished from the unlabeled nuclei (blue color) using hue thresholds (HT). The brown color is composed of magenta, yellow and red hues, whereas the blue color is recognized as blue hues. The values of the blue hues (i.e. between 50 to 100) are between the magenta hues (<35 to 50) and the yellow and red hues (>200). Two HT values were used to distinguish the brown and blue colors HT2 which separates the blue hues from the yellow and red hues was fixed at 120, and HT1 which separates the blue hues from the magenta hues was selected automatically by the computer [23]. Four, to eliminate background immunostaining, only nuclei that showed more than the minimum percent of brown (MPB) staining were identified as BrdUrd-labeled. The values of HT1 and GLT were defined automatically by the computer for each field (unless drug-induced morphological changes required adjustments in GLT and HT1 selection, see below), the value of HT2 was set to 120, and the size range and the MPB value were defined by the investigator for each tumor. These procedures were adequate to analyze 65% of the samples. Analysis of the remaining 35% of the samples required modification of the image analysis algorithm, as follows.

About 25% of the samples showed very intense, dark-brown BrdUrd staining and light-blue hematoxylin staining. In these specimens, the contrast between the two stains exceeded the contrast between blue nuclei and background, and thereby resulted in computer selection of an erroneously low GLT value which in turn caused erroneous classification of blue staining as background staining. Consequently, the cell number was underestimated, resulting in an overestimation of LI. For these tumors, it was necessary to define a minimum GLT value (GLT_{min}) based on visual inspection of the sample. By this method, the excessively low computer-selected GLT values were automatically reset to GLT_{min} to ensure the identification of the light-blue nuclei.

In the remaining 10% of the samples, we observed that treatment with high concentrations of doxorubicin or mitomycin C resulted in condensed nuclei that showed a dark-blue hematoxylin staining. This interfered with the computer's selection of the HT1 value and resulted in a lower HT1 value (i.e. < 50) than is normally associated with the blue color. The lower hue value overlapped with the magenta hues. Therefore, the condensed dark-blue nuclei were mistaken as brown nuclei. To solve this problem, we used only one hue value, i.e. HT2, to separate the blue color from the brown color in tumors treated with doxorubicin or mitomycin C. Omis-

sion of HT1 and therefore exclusion of the magenta hues had minimal effect on the recognition of brown color, because the magenta hues typically represented a small percentage, i.e. <30%, of all brown staining. This modification was not required for the analysis of tumors treated with paclitaxel and 5-fluorouridine, alone or in combination, because these tumors did not show condensed, darkly staining nuclei.

To verify that the changes in GLT and HT selection were appropriate and allowed the image analysis routines to correctly identify blue and brown nuclei, we compared, in more than 20 fields per tumor, the labeled and unlabeled nuclei identified by image analysis with those identified by visual evaluation, using the Red Dot routine described previously [23]. The results showed that the computer-determined LI agreed with those estimated by visual inspection of the field.

For a typical pharmacodynamic experiment which used 60 tumor pieces, determination of all LI_{one} values required 30 to 40 min and determination of LI_{all} required about 18 h.

Determination of LI_{one}

For the determination of LI_{one}, the tissue pieces were scanned at low power, i.e. 100× or 200× magnification, to preselect the region with the highest percentage of BrdUrd-labeled cells. The selected regions were then analyzed by image analysis. Because one 400× camera field was approximately one-half the size of a 400× ocular field, two adjacent images were scored to determine LI_{one}. A minimum of 50 cells were evaluated per tumor piece. In histocultures with low cell densities, multiple adjacent fields were evaluated until at least 50 cells had been analyzed.

Determination of LI_{all}

Determination of LI_{all} was a two-step procedure that involved first describing the location of each tumor piece and its boundaries and then scoring the labeled cells to determine the BrdUrd LI_{all}. Definition of tissue location and boundaries was performed using a computer program. Briefly, a low power, 25× magnification image of each tumor piece was displayed on the monitor where its perimeter was outlined with the computer mouse, and the specific location of the histoculture on the microscope stage was recorded. Next, the analysis parameters, i.e. size range and MPB, were defined, and the determination of BrdUrd LI_{all} proceeded with no further investigator assistance. The computer directed movement of the microscope from field to field and from histoculture to histoculture. The focus was adjusted automatically approximately every four fields to compensate for the slight variation in the focal planes in which cells were located. Histocultures that exceeded the size of the screen were divided into several sections and the number of BrdUrd-labeled and unlabeled nuclei from each section were combined to determine the LI_{all} of the entire histoculture.

Data analysis

Six histocultures were placed on each collagen sponge. However, some histocultures were not located on the same plane as others on the same collagen sponge and therefore did not appear on the same slide. Hence, each microscopic section contained between one and six histocultures. Likewise, because of the disorderly positioning of the multiple histocultures on the collagen gel and because LI_{one} and LI_{all} were determined on separate occasions, it was difficult to correctly match and compare LI_{one} and LI_{all} of the same histoculture. To overcome this uncertainty, we elected to compare the average values of LI_{one} and LI_{all} of all histocultures on each histologic section. Hence, each LI value represents the average of results obtained from one to six histocultures. We evaluated 244 sections on three different days to determine the extent of interday variation in pharmacodynamics. Of the 732 (3 * 244) total possible pairs of LI_{one} and LI_{all}, 8 pairs yielded inconsistent data due to difficulty in defining the image analysis parameters because of the small size and poor quality of the tissue sections. We compared the remaining 724 pairs of LI_{one} and LI_{all} values in two ways. The first was to compare their absolute values to determine the extent of differences between the two parameters. The second was to compare their values normalized by the values of untreated controls. These normalized LI represented drug effect, or E in Eq. 1.

Differences in the pharmacodynamic parameters determined using LI_{one} and LI_{all} (i.e. n, K, IC_{30}, IC_{50}, and IC_{70}) were compared using the two-tailed Student's paired t-test. The statistical significance of the frequency of experiments where the two LI measurements yielded different pharmacodynamic parameters was analyzed using Fisher's Exact test. Differences at the 5% level of significance were considered significant.

Results

Characteristics of bladder tumor histocultures

The untreated tumor histocultures varied widely in size, proliferative activity, and cell density. The number of fields per tumor histoculture, an indication of size, was 88 ± 46 (mean ± SD, range 36–201). The absolute values of LI_{one} and LI_{all} were 46.0 ± 17.3% (range 2.1–94.3%) and 23.6 ± 12.7% (range 1.6–51.4%), respectively. The cell density was 60 ± 10 cells per field for the untreated controls. For the drug-treated samples, the cell density per field ranged from 0 (i.e. where the drug effect approached 100%) to approximately the same level as in the controls.

Comparison of absolute values of LI_{one} and LI_{all}

We did not find consistent differences in the LI values for tumors treated with the four drugs. Hence, the data were pooled for analysis.

The relationship between the absolute values of LI_{one} and LI_{all} is illustrated in Fig. 1. Of the 724 pairs of observations, 650 pairs showed higher values of LI_{one} (median of differences of 18.6%, range 0.01–83.9%) whereas 74 pairs showed equal or higher values of LI_{all} (median of differences of 2.4%, range 0.0–32.6%) than LI_{one}. Interestingly, the latter 74 pairs were derived from five of the six tumors treated with doxorubicin and three of the three tumors treated with mitomycin C. As discussed in the Methods, these tumors displayed condensed, intensely staining nuclei which could have led to errors in the image analysis results.

We evaluated the relationship between the extent of the drug effect and the differences between LI_{one} and LI_{all}. Five levels of drug effects, i.e. no effect (controls), minimal (1–20% inhibition of LI), low (21–50%), moderate (51–80%) and high (81–100%), were compared. The results, summarized in Fig. 1, showed significant correlations between the absolute values of LI_{one} and LI_{all}, at all levels of drug effect. In all cases, LI_{one} was significantly greater than LI_{all} ($P < 0.001$). The difference between the two values decreased from 27.0% to 1.2% when the drug effect was increased from minimal to high (Table 1).

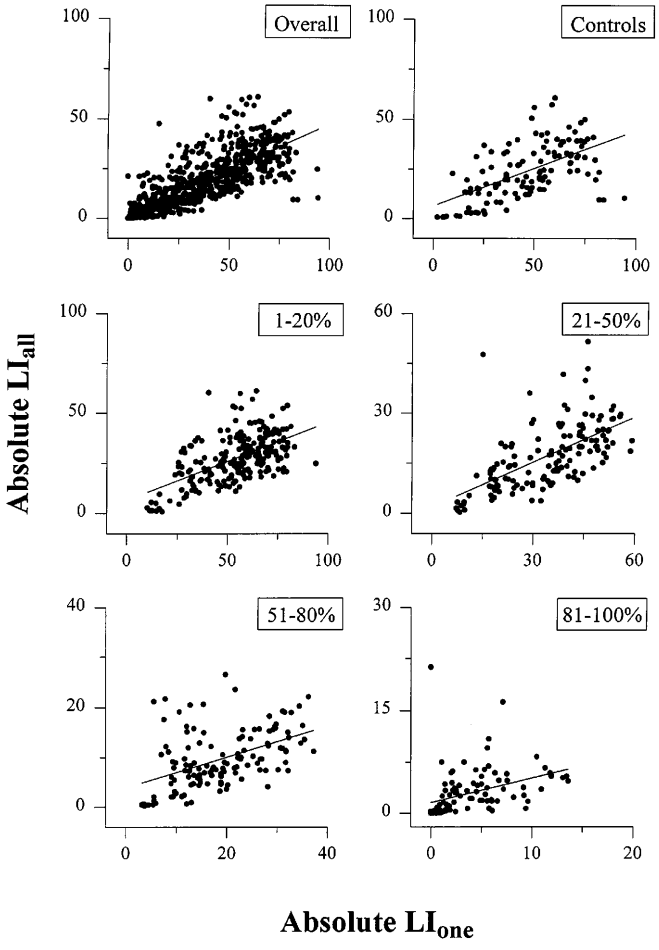

Fig. 1 Relationship between the absolute values of LI_{one} and LI_{all}. The results of linear regression are shown. For all data, $n = 724$, $r^2 = 0.613$, $P < 0.0001$. For untreated controls, $n = 121$, $r^2 = 0.324$, $P < 0.0001$. For samples that showed 1–20% drug-induced inhibition, $n = 235$, $r^2 = 0.327$, $P < 0.0001$. For samples that showed 21–50% drug-induced inhibition, $n = 142$, $r^2 = 0.374$, $P < 0.0001$. For samples that showed 51–80% drug-induced inhibition, $n = 127$, $r^2 = 0.223$, $P < 0.0001$. For samples that showed 81–100% inhibition, $n = 99$, $r^2 = 0.148$, $P < 0.0001$

Comparison of normalized values of LI_{one} and LI_{all}

When the LI values of the drug-treated samples were normalized by the values of the control samples and expressed as a percentage of control, the normalized LI_{one} and LI_{all} were not different when the drug effect was ≤50% whereas LI_{all} was significantly higher than LI_{one} when the drug effect was >50% (Table 1). Except for the highest drug effect, the normalized LI_{one} was significantly correlated with the normalized LI_{all} (Fig. 2). In all cases, the correlations between the normalized values of LI_{one} and LI_{all} were lower than the correlation between the absolute values LI_{one} and LI_{all}, as indicated by the lower coefficients of determination (Fig. 2).

Comparison of pharmacodynamic parameters using LI_{one} or LI_{all}

Figure 3 shows the concentration-response curves obtained using LI_{one} or LI_{all}. In agreement with our previous data [8, 9, 17], treatment with doxorubicin, mitomycin and 5-fluorouridine resulted in sigmoidal, concentration-dependent inhibition of BrdUrd incorporation, whereas paclitaxel treatment, either alone or in combination with 5-fluorouridine, resulted in partial inhibition in some tumors and had no effect in other tumors.

Pharmacodynamic parameters were obtained by analyzing the concentration-response curves using Eq. 1. Of the 15 experiments, one did not show a measurable drug response. Samples obtained from each experiment were analyzed for LI_{one} and LI_{all} on three separate days. The triplicate results were used to determine whether the pharmacodynamics determined using LI_{one} were different from the pharmacodynamics determined using LI_{all}, for each experiment. Results of the comparison are shown in Table 2. Of the 14 experiments which showed a measurable response, two showed different IC values obtained with LI_{one} and LI_{all}, but there was no relationship between the differences and the LI used. For example, one experiment showed higher IC values of doxorubicin for pharmacodynamic analysis using LI_{one}, whereas the second experiment showed higher IC values of paclitaxel and 5-fluorouridine for analysis using LI_{all}. Likewise, there was no consistent pattern in the differences of the other pharmacodynamic parameters determined using either method. The overall results indicated

Table 1 Comparison of LI_{one} and LI_{all}. LI_{one} is the LI in the most proliferative region of the histoculture and LI_{all} is the LI of the entire tumor histoculture. The differences between the absolute values of LI_{one} and LI_{all} and between the normalized values of LI_{one} and LI_{all} were compared using the two-tailed Student's paired *t*-test. The normalized LI values were obtained by dividing the absolute LI values of the drug-treated samples with the absolute LI values of the untreated controls

Drug effect	Absolute LI			Normalized LI		
	n	$LI_{one} - LI_{all}$ (mean ± SD)	*P*-value	*n*	$LI_{one} - LI_{all}$ (mean ± SD)	*P*-value
0% (control)	121	22.4 ± 17.4	<0.0001	121	3.3 ± 31.8	NS
1–20% inhibition	235	27.0 ± 14.1	<0.0001	235	4.0 ± 35.3	NS
21–50% inhibition	142	17.4 ± 9.9	<0.0001	142	−3.1 ± 28.4	NS
51–80% inhibition	127	9.1 ± 8.0	<0.0001	127	−9.3 ± 32.1	<0.005
81–100% inhibition	99	1.2 ± 3.2	<0.0005	99	−8.5 ± 16.6	<0.0001
Overall	724	17.6 ± 15.3	<0.0001	724	−2.2 ± 32.7	NS

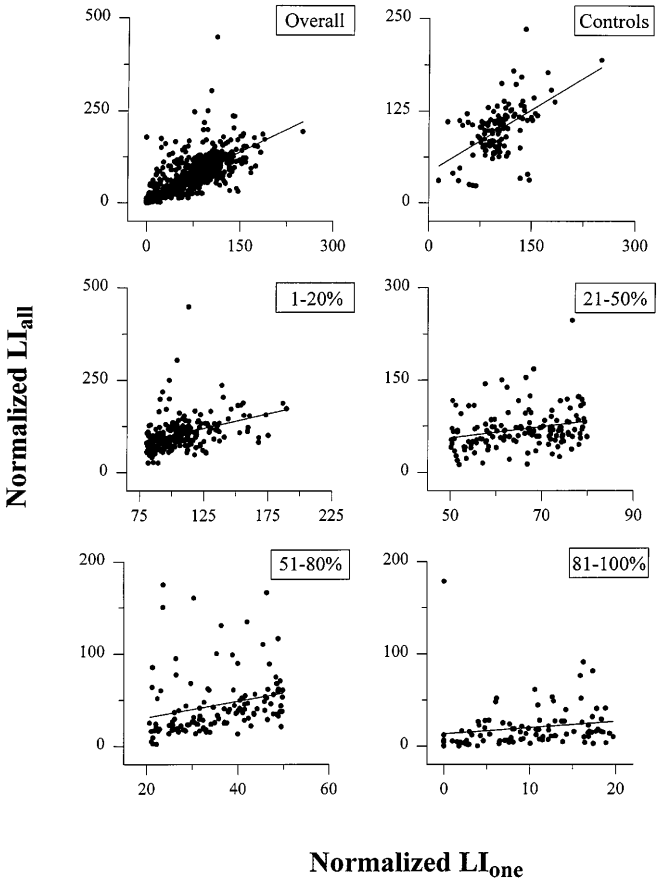

Fig. 2 Relationship between the normalized values of LI_{one} and LI_{all}. The results of linear regression are shown. For all data, $n = 724$, $r^2 = 0.489$, $P < 0.0001$. For untreated controls, $n = 121$, $r^2 = 0.284$, $P < 0.0001$. For samples that showed 1–20% drug-induced inhibition, $n = 235$, $r^2 = 0.142$, $P < 0.0001$. For samples that showed 21–50% drug-induced inhibition, $n = 142$, $r^2 = 0.062$, $P < 0.01$. For samples that showed 51–80% inhibition, $n = 127$, $r^2 = 0.061$, $P < 0.01$. For samples that showed 81–100% drug-induced inhibition, $n = 99$, $r^2 = 0.028$, $P = 0.097$

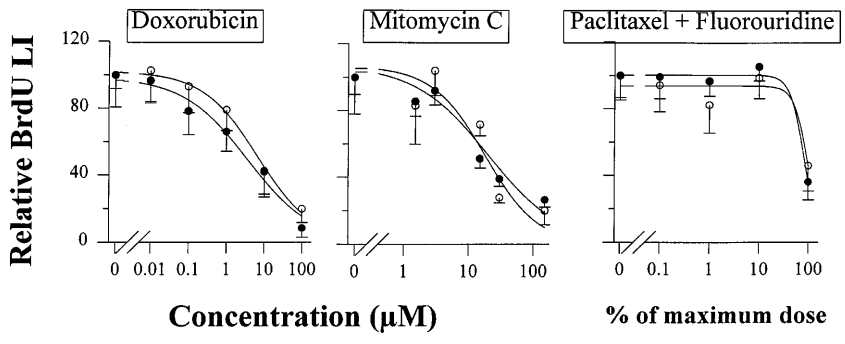

Fig. 3 Drug concentration-response relationship. Inhibition of BrdUrd LI by a 2-h treatment with doxorubicin, mitomycin C, and paclitaxel plus 5-fluorouridine. For the latter, the drug concentrations are expressed as the percentage of the maximum dose (100% is 10 μ*M* paclitaxel and 6.7 μ*M* 5-fluorouridine). Paclitaxel alone produced less than 50% inhibition (data not shown). (○ mean LI_{one}, ● mean LI_{all}; *bars* one standard deviation; *lines* computer-fitted lines using Eq. 1)

Table 2 Comparison of pharmacodynamic data determined in the most proliferative field with the data determined in the entire histoculture. To determine whether the pharmacodynamic data obtained in the most proliferative region differed from the data obtained from the entire histoculture, we compared the pharmacodynamics obtained in the 14 experiments in which tumors showed a response to drug treatment. The results of one experiment in which the tumor did not respond to paclitaxel treatment are not included. For paclitaxel, the maximum effect was <50%. Hence, only the IC_{30} is provided. Samples obtained from each experiment were analyzed for LI_{one} and LI_{all} on three separate days. The triplicate results were analyzed by the two-tailed paired Student's *t*-test to determine whether the pharmacodynamic parameters determined using LI_{one} were significantly different from the parameters determined using LI_{all}. The results are presented as the frequency of the experiments that showed no significant differences in the pharmacodynamic parameters. Fisher's Exact test was used to determine whether there were differences in the pharmacodynamic parameters determined using either the normalized LI_{one} or the normalized LI_{all}; the *P*-values are indicated

Drug	Frequency of experiments in which no differences in pharmacodynamic data were detected				
	n	K	IC_{30}	IC_{50}	IC_{70}
Doxorubicin	3/5	4/5	4/5	4/5	4/4
Mitomycin C	3/3	3/3	3/3	3/3	2/3
Paclitaxel	1/1	1/1	1/1	0/0	0/0
5-Fluorouridine	1/1	1/1	1/1	1/1	1/1
Paclitaxel plus 5-Fluorouridine	4/4	3/4	3/4	3/4	1/1
All	12/14	12/14	12/14	11/13	8/9
P-values from Fisher analysis	0.48	0.48	0.48	0.48	1.0

that the pharmacodynamic parameters, i.e. *n*, K, IC_{30}, IC_{50}, and IC_{70}, determined using LI_{one} were comparable to the corresponding parameters estimated using LI_{all}.

Interday variation in LI_{one}, LI_{all} and pharmacodynamic parameters

To determine whether pharmacodynamic measurements using LI_{one} were more variable than those using LI_{all}, we evaluated the interday variation in LI_{one}, LI_{all}, and the estimated pharmacodynamic parameters. The pharmacodynamic experiments were each evaluated in triplicate using image analysis parameters defined independently

Table 3 Interday variation in LI and pharmacodynamic parameter estimates. The LI data were obtained from the results of 15 experiments. One experiment did not show a response to drug treatment. Hence, the pharmacodynamic parameters were obtained from the results of 14 experiments (*CV* coefficient of variation)

Parameter	Analysis of one field		Analysis of all fields	
	n	CV Median (range)	*n*	CV Median (range)
Absolute LI	240	12.3 (0–126)	240	12.1 (2–173)
Normalized LI	240	11.5 (0–129)	240	10.7 (0–173)
n	15	17.5 (0–78.6)	15	15.4 (0–84.5)
K	15	22.1 (2.3–59.3)	15	35.4 (3.3–75.2)
IC_{30}	15	33.4 (6.9–67.1)	15	39.8 (4.2–138.2)
IC_{50}	14	19.6 (2.5–62.3)	13	25.9 (3.6–90.6)
IC_{70}	10	24.2 (7.7–103.7)	10	24.1 (9.1–118.8)

on three different days. The coefficients of variation (CV) of the absolute and normalized values of LI_{one} and LI_{all}, and the corresponding pharmacodynamic parameters are summarized in Table 3. The comparable CV for each of the parameters indicates comparable interday variation for the two methods.

Discussion

Among the 724 pairs of absolute values of LI_{one} and LI_{all}, 90% showed significantly higher LI_{one}. Although this finding is as expected because we selected the most proliferative region to determine the LI_{one}, it confirms the heterogeneous proliferative activity within a solid tumor. A comparison of the absolute and normalized values of LI_{one} and LI_{all}, as a function of the drug effect, reveals the differences in the chemosensitivity of cells in the most proliferative regions and cells in the whole tumor histoculture. For example, the diminishing differences between the normalized LI_{one} and LI_{all} values when the drug effect was >50% (Table 1) suggests a greater reduction in LI_{one} and therefore a greater chemosensitivity of cells in the most proliferative region at higher drug concentrations. Collectively, these data confirm that heterogeneities in proliferative activity and drug response are retained in tumor histocultures, which supports the application of this experimental model for studying regional heterogeneity in solid tumors.

In the drug-treated samples, the different values of normalized LI_{one} compared to normalized LI_{all} (Table 1) did not result in differences in pharmacodynamic parameters. This is in part due to the relatively large interday variation in the results, i.e. coefficient of variation of 20–50% for all parameters, compared to the 9% difference in the normalized LI_{one} and normalized LI_{all}. The interday variation is comparable to those commonly observed in pharmacodynamic studies [1, 4, 6], but is significantly lower than the up to >120-fold variation in the chemosensitivity of tumors from individual patients [2, 8, 9, 15, 17]. The variation in the LI values observed in the present study may have resulted from the interday variation in (1) the selection of image analysis parameters [22, 23], (2) the selection of the most proliferative fields, (3) the investigator-defined tissue boundaries for LI_{all}, and (4) the number of histocultures evaluated per drug concentration for LI_{one} and LI_{all}. It is noted that the variation in the image analysis results is comparable to the variation in manual visual analysis, as we have shown previously [23].

To our knowledge, this is the first study which compared the LI in one region of a solid tumor histoculture with the entire tumor histoculture. Our results demonstrate that the pharmacodynamics measured by the inhibition of DNA synthesis in the most proliferative fields are indistinguishable from the pharmacodynamics measured in the entire tumor histocultures. In addition, because the LI_{one} method required only 3% of the time required for the LI_{all} method, our data affirm the traditional and more time-efficient method of studying only the most proliferative region.

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References

1. Andreotti PE, Cree IA, Kurbacher CM, Hartmann DM, Linder D, Harel G, Gleiberman I, Caruso PA, Ricks SH, Untch M, Sartori C, Bruckner HW (1995) Chemosensitivity testing of human tumors using a microplate adenosine triphosphate luminescence assay: clinical correlation for cisplatin resistance of ovarian carcinoma. Cancer Res 55: 5276–5282
2. Au JL-S, Wientjes MG, Rosol TJ, Koolemans-Beynen A, Goebel EA, Schuller DE (1993) Histocultures of patient head and neck tumors for pharmacodynamics studies. Pharm Res 10: 1493–1499
3. Botticelli AR, Criscuolo M, Martinelli AM, Botticelli L, Filoni A, Migaldi M (1993) Proliferating cell nuclear antigen/cyclin in incidental carcinoma of the prostate. Virchows Arch A: Pathol Anat Histopathol 423: 365–368
4. Bressolle F, Bologna C, Edno L, Bernard JC, Gomeni R, Sany J, Combe B (1996) A limited sampling method to estimate methotrexate pharmacokinetics in patients with rheumatoid arthritis using a Bayesian approach and the population data modeling program P-PHARM. Eur J Clin Pharmacol 49: 285–292
5. Chang S-G, Slocum HK, Perrapato SD, Tóth K, Huben RP, Rustum YM (1992) Glucose consumption end point in primary histoculture indicates recovery of human tumors from drug treatment. In Vitro Cell Dev Biol 28A: 585–587
6. Dutta S, Matsumoto Y, Ebling WF (1996) Is it possible to estimate the parameters of the sigmoid Emax model with truncated data typical of clinical studies? J Pharm Sci 85: 232–239
7. Furukawa T, Kubota T, Hoffman RM (1995) Clinical applications of the histoculture drug response assay. Clin Cancer Res 1: 305–311
8. Gan Y, Wientjes MG, Badalament RA, Au JL-S (1996) Pharmacodynamics of doxorubicin in human bladder tumors. Clin Cancer Res 2: 1275–1283
9. Gan Y, Wientjes MG, Schuller DE, Au JL-S (1996) Pharmacodynamics of taxol in human head and neck tumors. Cancer Res 56: 2086–2093
10. Kalns JE, Millenbaugh NJ, Wientjes MG, Au JL-S (1995) Design and analysis of in vitro antitumor pharmacodynamic studies. Cancer Res 55: 5315–5322

11. Kubota T, Sasano N, Abe O, Nakao I, Kawamura E, Saito T, Endo M, Kimura K, Demura H, Sasano H, Nagura H, Ogawa N, Hoffman RM (1995) Potential of the histoculture drug-response assay to contribute to cancer patient survival. Clin Cancer Res 1: 1537–1543
12. Leighton J (1954) The growth patterns of some transplantable animal tumors in sponge matrix tissue culture. J Natl Cancer Inst 15: 275–293
13. Lynch DA, Clarke AM, Jackson P, Axon AT, Dixon MF, Quirke P (1994) Comparison of labeling by bromodeoxyuridine, MIB-1, and proliferating cell nuclear antigen in gastric mucosal biopsy specimens. J Clin Pathol 47: 122–125
14. Robbins KT, Connors KM, Storniolo AM, Hanchett C, Hoffman RM (1994) Sponge-gel-supported histoculture drug-response assay for head and neck cancer. Correlations with clinical response to cisplatin. Arch Otolaryngol Head Neck Surg 120: 288–292
15. Schmittgen TD, Wientjes MG, Badalament RA, Au JL-S (1991) Pharmacodynamics of mitomycin C in cultured human bladder tumors. Cancer Res 51: 3849–3856
16. Schmittgen TD, Au JL-S, Wientjes MG, Badalament RA, Drago JR (1991) Cultured human bladder tumors for pharmacodynamic studies. J Urol 145: 203–207
17. Schmittgen TD, Weaver JM, Badalament RA, Wientjes MG, Klein EA, Young DC, Au JL-S (1994) Correlation of human bladder tumor histoculture proliferation and sensitivity to mitomycin C with tumor pathobiology. J Urol 152: 1632–1636
18. Song D, Wientjes MG, Gan Y, Au JL-S (1997) Bladder tissue pharmacokinetics and antitumor effect of intravesical 5-fluorouridine. Clin Cancer Res 3: 901–90
19. Sutherland RM (1988) Cell and environment interactions in tumor microregions: the multicell spheroid model. Science 240: 177–180
20. Vescio RA, Redfern CH, Nelson TJ, Ugoretz S, Stern PH, Hoffman RM (1987) In vivo-like drug responses of human tumors growing in three-dimensional gel-supported primary culture. Proc Natl Acad Sci USA 84: 5029–5033
21. Vescio RA, Connors KM, Youngkin T, Bordin GM, Robb JA, Umbreit JN, Hoffman RM (1990) Cancer biology for individualized therapy: correlation of growth fraction index in native-state histoculture with tumor grade and stage. Proc Natl Acad Sci USA 87: 691–695
22. Weaver JR, Au JL-S (1997) Comparative image and visual analysis of proliferation markers in human solid tumors. Cytometry 27: 189–199
23. Weaver JR, Au JL-S (1997) Application of automatic thresholding in image analysis scoring of cells in human solid tumors labeled for proliferation markers. Cytometry 29: 128–135
24. Wientjes MG, Pretlow TG, Badalament RA, Burgers JK, Au JL-S (1995) Histocultures of human prostate tissues for pharmacologic evaluation. J Urol 153: 1299–1302
25. Wijsman JH, Van Dierendonck JH, Keijzer R, Velde CJ van de, Cornelisse CJ (1992) Immunoreactivity of proliferating cell nuclear antigen compared with bromodeoxyuridine incorporation in normal and neoplastic rat tissue. J Pathol 168: 75–83
26. Yen WC, Schmittgen TD, Au JL-S (1996) Different pH dependency of mitomycin C activity in monolayer and three-dimensional cultures. Pharm Res 13: 1887–1891
27. Yu CC, Hall PA, Fletcher CD, Camplejohn RS, Waseem NH, Lane DP, Levision DA (1991) Haemangiopericytomas: the prognostic value of immunohistochemical staining with a monoclonal antibody to proliferating cell nuclear antigen (PCNA). Histopathology 19: 29–33