Tumor Biology: Use of Tiled Images in Conjunction with Measurements of Cellular Proliferation and Death in Response to Drug Treatments

Eric J. Wexler,¹ Ellen M. Gravallese, Philip M. Czerniak, James J. Devenny, Janina Longtine, Michael K. K. Wong, Andrew M. Slee, and Janet S. Kerr

General Pharmacology, DuPont Pharmaceuticals Co., Wilmington, Delaware 19880-0400 [E. J. W.]; P. M. C., J. J. D., A. M. S., J. S. K.]; New England Baptist Bone and Joint Institute and Department of Medicine, Beth Israel Deaconess Medical Center, Boston, Massachusetts 02115 [E. M. G.]; Department of Pathology, Brigham and Women’s Hospital, Boston, Massachusetts 02115 [J. L.]; and Department of Medicine, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania 15213 [M. K. K. W.]

ABSTRACT

Tumor growth is dependent on the balance between cell proliferation and cell death, and these events occur heterogeneously within an individual tumor. We present a methodology that provides integrative information about cell kinetics, cell death, and cell growth within individual tumors in animals treated with cytotoxic chemotherapeutic agents. Using HCT-116 and NCI-H460 cells, human colonic adenocarcinoma and non-small cell lung cells, respectively, traditional xenograft studies were performed. The tumor-bearing animals were treated with cyclophosphamide (Cytoxan), gemcitabine (Gemzar), or mitomycin C, and extensive analysis of the tumors was studied. Cell kinetics were evaluated by measuring the apoptotic and proliferation indices. The ability to image an entire tumor section using “tiling” by creating a large montage from many high-resolution images makes it possible to identify regional differences within areas of tumor and to demonstrate differences in these tumor regions after treatment with selected chemotherapeutic agents. Two specific areas within tumors have been identified: (a) areas of viable cells within the cell cycle, determined by bromodeoxyuridine and/or morphological characteristics determined by hematoxylin staining; and (b) areas of necrosis determined by the absence of bromodeoxyuridine and proliferating cell antigen–labeled cells coupled with morphological changes. By standardizing the tumor size to 100 mm², different patterns of tumor responses to chemotherapeutic agents were determined. By creating such tiled images and by quantitating cell cycle kinetics, it is possible to gain a more complete understanding of tumor growth and response to treatment, leading to the development of more reliable methods for assessing the clinical behavior of anticancer drugs.

INTRODUCTION

The efficacy of cancer treatment is usually defined as the ability of agents to elicit tumor shrinkage, termed “tumor response.” Although there is an understanding of the effects of individual agents on tumor cells, much less is known about their global effect on the tumor as a whole. It is becoming increasingly clear that tumor growth is a balance between cell death and cell growth and that these events may occur heterogeneously within an individual tumor. Understanding tumor response is critical, because decisions about the activity of antitumor agents are made on the basis of this response. We present a methodology that provides integrative information on cell kinetics, cell death, and cell growth within individual tumors in animals treated with cytotoxic chemotherapeutic agents. We are using these chemotherapeutic agents as tools and the dosing and time points as examples of this methodology.

The human tumor xenograft model has been used as a preclinical model for the discovery of anticancer drugs. Evaluation of tumor growth has relied on the overall size and weight changes of the tumors. Because tumor growth is regulated through the balance of cell proliferation and cell death, measurement of these cellular processes is critical in assessing the kinetics of tumor growth. One, therefore, needs methods to quantitate cellular proliferation and cell death (both apoptosis and necrosis) within the tumors. The ability to create large montages from many high-resolution images makes the investigation of entire tumor tissue sections possible and characterization of areas of distinct tumor morphology feasible. One purpose of the present study was to develop a method using “tiling” to identify regional differences within the entire tumor sections. In addition, we sought to identify changes in regional morphology and kinetics of tumor growth after treatment with selected chemotherapeutic agents. The ability to image entire tumor sections requires more time than conventional analyses but allows visualization and quantitation of distinct areas of tumor morphology.

We have focused on two human cell lines grown in a traditional xenograft model: colon adenocarcinoma cells (HCT-116) and non-small cell carcinoma cells (NCI-H460). Three chemotherapeutic agents were dosed in the model: cyclophosphamide (Cytoxan), gemcitabine (Gemzar), and mitomycin C. Using standard immunohistochemical methods, we have assessed cell proliferation and apoptosis to evaluate parameters of cell kinetics. Use of entire tumor sections, coupled with information gained from this analysis, permit a more complete understanding.

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¹ To whom requests for reprints should be addressed, at General Pharmacology, DuPont Pharmaceuticals Co., Route 141 and Henry Clay Road, P.O. Box 80400, Wilmington, DE 19880-0400.
MATERIALS AND METHODS

Reagents and Therapeutic Agents. All reagents and chemotherapeutic agents were chemical grade and purchased from Sigma Chemical Co. (St. Louis, MO) or through VWR Scientific (Bridgeport, NJ). For the proliferation indices BrdUrd2 and PCNA kits and the corresponding mouse isotype control antibody were purchased from Zymed Laboratories (San Diego, CA). To access cellular apoptosis, Invitrogen Apoptag plus kit was purchased from Invitrogen (Purchase, NY). The chemotherapeutic agents used were: Cytotoxan (Bristol-Myers Squibb, Princeton, NJ), Gemzar (Eli Lilly & Co., Indianapolis, IN), and mitomycin C (Sigma Chemical Co.).

Cell Lines and Cell Culture. HCT-116, a human colon adenocarcinoma cell line, and NCI-H460, a human non-small cell lung carcinoma cell line, were purchased from American Type Culture Collection (Manassas, VA). HCT-116 cells were maintained in culture in McCoy’s heat-inactivated fetal bovine serum, 2% L-glutamine, and 2% penicillin/streptomycin. NCI-H460 cells were maintained in culture in DMEM supplemented with 10% FCS, 2% L-glutamine, and 2% penicillin/streptomycin. Both cell lines were maintained at 37°C with 5% CO2 . Subconfluent cultures were trypsinized using 0.25% trypsin-EDTA, pelleted using centrifugation, counted with a hemocytometer, resuspended in complete media, and diluted to appropriate concentrations.

Animal Care. All animal studies were conducted in a facility accredited by the American Association for the Accreditation of Laboratory Animal Care. Female athymic Swiss nu/nu mice at 6–8 weeks of age were used for the xenograft studies (Taconic Farms, Germantown, NY). They were housed five to a cage in sterile, polycarbonate filter-capped microisolator cages. All animals were kept in temperature-controlled rooms, maintained in a barrier facility on 12-h light/dark cycles, and provided with food and water ad libitum.

Human Cell Implantation and Xenograft Studies. Either HCT-116 cells or NCI-H460 cells were implanted s.c. into the inguinal area at 1 × 107 cells (0.1 ml) per mouse. At the end of 7–10 days postinjection, tumor volumes were determined by measuring the tumors in two dimensions, and the volume was calculated using the formula for a prolate ellipsoid: length (mm) × width (mm)/2 = mm3. Because the tumors were not removed from the animals, tumor volume was converted to weight by assuming unit density (i.e., 1.0 cm3 = 1.0 g; Ref. 2). Animals were placed in groups so the tumor weights were not statistically different among the groups prior to treatment. Selected chemotherapeutic drugs (cyclophosphamide, Cytotoxan; gemcitabine, Gemzar; and mitomycin C) were administered daily i.p. over 14–15 days. At the end of treatment, tumors were removed and weights were determined and expressed as mean ± SE. TGI was determined as the ratio of the percent tumor weight from treated animals divided by tumor weights from control animals for a given dose of drug.

2 The abbreviations used are: BrdUrd, bromodeoxyuridine; PCNA, proliferating cell nuclear antigen; TGI, tumor growth inhibition; LI, labeling index.

To begin to characterize the interplay between the processes of tumor growth and tumor cell death, a time course study was performed, dosing 120 mg/kg Q3Dx3 gemcitabine and evaluating tumors after drug treatment. The BrdUrd-LI and apoptotic index at 1, 2, 3, and 4 days after completion of the third drug dose were determined, as described below.

Immunohistochemistry: BrdUrd Incorporation. BrdUrd (0.2 ml/mouse) was injected i.p. 2 h before mice were sacrificed. This allowed time for BrdUrd incorporation into proliferating cells. To ensure BrdUrd was consistently incorporated, additional tissues from the small intestine were collected from all animals injected with BrdUrd. Tumor and small intestine sections were excised and trimmed of fat, and serial sections no thicker than 0.5 cm were processed for paraffin embedding by fixing in 10% buffered formalin for 4 h and dehydrated with graded alcohols: 80%, 95% twice, and 100% three times for 60 min per bath. The samples were cleared with three baths of xylene and then infused with paraflin via two additional baths. Using a microtome (Olympus America Inc., Melville, NY), sections were sectioned at 4 μm and placed onto silane-coated slides and air-dried. To ensure consistency of the tissue sections and to maximize differences among treatments, the largest cross-sectional diameter of each tumor was selected. The slides were stored at room temperature.

The Zymed BrdUrd staining kit was used for visualization of incorporated BrdUrd. BrdUrd labeling identifies cells in the S phase of the cell cycle. The slides were deparaffinized, hydrated in PBS, and processed through 0.3% hydrogen peroxide to quench endogenous peroxides. Tissues were then treated with 0.125% trypsin and denatured. Sections were then blocked using the kit blocking reagent, incubated with biotinylated mouse anti-BrdUrd for 60 min at room temperature, followed by streptavidin-peroxidase incubation for 10 min, and 2–5 min with the diaminobenzidine chromagen solution. The sections were counterstained with hematoxylin and coverslipped with a permanent mounting medium. The three negative controls used were: an isotype control, a non-BrdUrd injected tumor, and a no primary antibody section; BrdUrd-injected mouse intestine was used as the positive control. Five areas of the same size (0.05 mm) and at least four tumors per group were analyzed.

PCNA Staining. The Zymed PCNA staining kit was used as a marker for cell proliferation, because PCNA levels are elevated in actively proliferating cells during the S, G2, and M phases of the cell cycle. The tissue preparation was performed as outlined above for BrdUrd, and the staining and visualization were done according to the manufacturer’s specifications.

Apoptosis. Invitrogen Apoptag plus kit was used to visualize and quantify the number of cells undergoing apoptosis. Tissues were fixed in 10% neutral buffered formalin, paraffin embedded, and sectioned at 4 μm, and the kit protocol was followed. This kit uses the method of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling for in situ labeling of fragmented DNA. The slides were counter-stained with methyl green. The negative control tissue section was the primary antibody solution with the dUTP substrate but without dUTP, and the positive control tissue was tissue from the mouse.
mammary gland. Five areas of the same tumor and at least four tumors per group were analyzed.

**Image Analysis and Ratio of Tumor Areas.** Image analysis was performed with the M2 MCID Image Analysis System (Imaging Research, Toronto, Canada), coupled with the Olympus AX70 microscope.

**BrdUrd-LI.** To calculate the LI, viable cells (BrdUrd-positive cells plus unstained cells) were counted from five same-sized areas (0.05 mm²), containing no edges, artifacts, or necrotic portions, and from at least four separate tumors per group. Using 20 individual cells, the average nuclear size was calculated to determine the number of cells if clumps of cells were counted. Automated counting was done using hue, intensity, and saturation as criteria for separating labeled from unlabeled cells. The ratio of BrdUrd-labeled cells to the total number of cells counted (both labeled and unlabeled cells in histologi-

![Fig 1 A. composite image (tiled) of an HCT-116 tumor section at ×10. B, tiled image of a serial section of the same HCT-116 tumor showing BrdUrd-positive cells. Cells within central area of the tumor are not in S phase. C and D, insets (×40) of a portion of sections shown in A and B, respectively. H&E counterstain (A and C) or hematoxylin (B and D). The arrow indicates a necrotic area.](image-url)
cally viable areas of tumor determined by hematoxylin staining) was determined, and this ratio was multiplied by 100. This is defined as the BrdUrd-LI. For both the proliferating cells and the apoptotic cells the data are expressed as percentages rather than total number of positive cells per field. We reasoned that sampling errors were due to the heterogeneity of the tumors and, therefore, the selected fields would be minimized.

**Percentage of Apoptotic Cells.** The percentage of labeled cells was determined using the same methodology as for the BrdUrd-LI: percentage of labeled cells = positive labeled cells/(positive labeled cells + unstained cells) × 100.

**Characterization of Tumor Areas.** Tiled images of tumor sections were created, which resulted in resolution of the entire tumor section at ×10 (16–100 individual frames captured and stitched together). The different regions of the tiled tumor were traced manually. Because the tumors were variable in size, each tumor section was standardized to 100 mm² and the percentage of necrotic and viable areas per section were calculated based on 100 mm². The tiled tumor section was divided into two distinct areas: (a) the area containing BrdUrd-stained cells plus the area containing histologically viable but unstained cells; and (b) the necrotic area determined by morphological changes via hematoxylin staining. To confirm that many of the unstained cells within the tumor were still within the cell cycle, additional studies were performed to determine whether the cells not labeled with BrdUrd would label with PCNA.

Using standardized tumor areas, two percentages were derived: viable tumor (areas containing both unlabeled but histologically viable cells plus BrdUrd-labeled cells) and necrotic tumor determined by H&E. The percentage of viable tumor area was calculated as the ratio of viable BrdUrd-labeled cells plus unlabeled cells to the total tumor area, including necrotic portions of the tumor, × 100. The percentage of necrotic tumor area was calculated as the ratio of the necrotic area to the total tumor area × 100.

**Statistical Methods.** Statistical differences were determined using the Student’s t test and the nonparametric Mann-Whitney test. A P < 0.05 was considered significant.

**RESULTS**

Figs. 1 and 2 show tiled images of HCT-116 and NCI-H460 untreated tumors with serial sections of H&E staining or hematoxylin staining and BrdUrd labeling. These sections clearly show the heterogeneity within the HCT-116 tumor (Fig. 1, A and B). The HCT-116 tumor grew in organized sheets composed of areas of poorly differentiated cells with a high nuclear:cytoplasmic ratio and prominent nucleoli. There was some inflammatory response. Central areas of the tumor contained frankly necrotic cells. Viable tumor areas were predominately located around the tumor periphery. BrdUrd-stained cells were concentrated in the central portions of the viable areas.

The untreated NCI-H460 tumors (Fig. 2, A and B) had viable tumor cells present in the peripheral areas of the specimen. Tumor growth was in fronds, and occasional mitotic figures were present. Tumor cells contained large nuclei with...
prominent nucleoli. Deep to the viable tumor was an admixture of areas containing clearly necrotic cells and areas containing cells that, by routine histology, appeared viable but smaller. BrdUrd labeling was seen again, predominantly in central areas of clearly viable tumor fronds. The BrdUrd-labeled HCT-116 and NIH-H460 tumors under 300 oil demonstrate the distribution of nuclei, nucleoli, and mitotic figures within these representative tumor sections (Fig. 3, A and B). The BrdUrd-labeled intestine clearly demonstrates positively stained cells in intestinal crypts (Fig. 4B).

Fig. 5 shows serial sections of an untreated HCT-116 tumor with PCNA-labeled cells (A) and with BrdUrd-labeled cells (B). Serial sections show that many tumor cells not labeled with BrdUrd did label with PCNA and, therefore, were within the cell cycle, confirming cells not labeled with BrdUrd were viable. We used this criterion in addition to cellular morphology with hematoxylin staining to delineate the viable portions of tumor sections.

Table 1 shows xenograft results from mitomycin C-, cyclophosphamide (Cytoxan)-, and gemcitabine (Gemzar)-treated mice with colonic adenocarcinoma (HCT-116) or non-small cell lung (NCI-H460) tumors. Mitomycin C (1 mg/kg/day for 14 days) consistently reduced tumor growth compared with controls in animals with both HCT-116 and NCI-H460 tumors.

Cyclophosphamide was considered inactive against the HCT-116 tumors at 50 mg/kg/day and the NCI-H460 tumors at 80 mg/kg/day for 14 days, because the TGI values were 45% and 36%, respectively. Gemcitabine was active against the

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Fig. 3  A, section of HCT-116 tumor from a mouse injected with BrdUrd 2 h before removal, demonstrating BrdUrd-stained cells, a mitotic figure, nuclei, and nucleoli. 100 × under oil, hematoxylin counterstain. B, Section of NCI-H460 tumor from a mouse injected with BrdUrd 2 h prior to removal, demonstrating BrdUrd-stained cells, nuclei and nucleoli (×100 under oil, hematoxylin counterstain).

Fig. 4  A, section of intestine of a non-BrdUrd injected mouse. B, section from intestine from a mouse injected with BrdUrd 2 h before removal (×40, hematoxylin counterstain).
HCT-116-inhibiting tumor growth by >90% and inactive against the non-small cell lung tumor with 32% TGI.

Because we determined the LI only at one time point, we have compared control and treated groups within the same experiment (Table 1, A and B). In the HCT-116 tumors cyclophosphamide at 50 mg/kg/day reduced the LI by 30%, from 28% in the control group to 20% (P < 0.05, Table 1). Gemcitabine dosed at 120 mg/kg Q3Dx5 lowered LI to 21%, a 26% decrease. Treatment with mitomycin C dosed daily did not significantly change the LI. In contrast, in the NCI-H460 tumor group mitomycin C treatment reduced the LI from 22% to 10%, a 55% decrease in proliferation (P < 0.05), whereas gemcitabine and cyclophosphamide treatments did not significantly affect this index. Therefore, the LI does not always correlate with the change in tumor weight.

With both cyclophosphamide and gemcitabine, the percentage of apoptotic cells (apoptotic index) significantly increased in the HCT-116 tumors, suggesting this process significantly contributes to the reduction in tumor growth. (Table 1B). The apoptotic indices were not significantly different in the mitomycin C-treated animals with either tumor, although the mitomycin C-treated NCI-H460 tumors were significantly smaller than untreated controls (Table 1A), demonstrating the apoptotic index does not always correlate with the change in tumor weight.

Morphology of the drug-treated tumors differs from that of

Table 1  TGI, BrdUrd-LI, and apoptotic cell index

<table>
<thead>
<tr>
<th>Treatment (dose)</th>
<th>Tumor weight, g (TGI)</th>
<th>BrdUrd-LI</th>
<th>Apoptotic cell index (%)</th>
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<tr>
<td></td>
<td>HCT-116 (%)</td>
<td>NCI-H460 (%)</td>
<td>HCT-116 (%)</td>
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<tr>
<td>A.</td>
<td></td>
<td></td>
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<tr>
<td>Vehicle control</td>
<td>1069 ± 107</td>
<td>1275 ± 81</td>
<td>29.9 ± 1.9</td>
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<tr>
<td>Mitomycin C</td>
<td>455 ± 40^d</td>
<td>145 ± 60^d</td>
<td>21.6 ± 3.0</td>
</tr>
<tr>
<td>(1 mg/kg/day; Q1Dx14)</td>
<td>(58)</td>
<td>(80)</td>
<td></td>
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<td>B.</td>
<td></td>
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<tr>
<td>Vehicle control</td>
<td>974 ± 145</td>
<td>1585 ± 162</td>
<td>28.1 ± 1.3</td>
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<tr>
<td>Cyclophosphamide</td>
<td>540 ± 69</td>
<td>NT</td>
<td>35.7 ± 0.6</td>
</tr>
<tr>
<td>(50 mg/kg/day; Q1Dx14)</td>
<td>(45)</td>
<td></td>
<td></td>
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<tr>
<td>Cyclophosphamide</td>
<td>NT</td>
<td>1015 ± 6.5</td>
<td>NT</td>
</tr>
<tr>
<td>(80 mg/kg/day; Q1Dx14)</td>
<td></td>
<td>(36)</td>
<td></td>
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<tr>
<td>Gemcitabine</td>
<td>78 ± 28^d</td>
<td>1196 ± 138</td>
<td>20.8 ± 3.5</td>
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<tr>
<td>(120 mg/kg; Q3Dx5)</td>
<td>(92)</td>
<td>(32)</td>
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Results are mean ± SE.  
^a TGI is defined as the ratio of the percent tumor weight from treated animals divided by the tumor weights from control animals × 100.  
^b LI is defined as the ratio of the number of BrdUrd labeled cells divided by the total number of cells counted from five proliferating fields × 100.  
^c Apoptotic cell index is defined as the ratio of labeled cells divided by the total number of cells counted from five proliferating fields × 100.  
^d P < 0.05 compared with vehicle controls.  
^e NT, not tested.

Fig. 5  Serial sections of a HCT-116 tumor stained with PCNA (A) and with BrdUrd (B; ×40, hematoxylin counterstain).

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<th>Tumor Tiling and Tumor Biology</th>
<th>3366</th>
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the controls. To ensure consistency of the tissue sections and to maximize differences among treatments, the largest cross-sectional diameter of each tumor was selected. The tiled sections maximize differences among treatments, the largest cross-

distribution of the tumor with BrdUrd-positive cells. Abine-treated mouse (120 mg/kg Q3D x5) demonstrating the uniform

, composite image (tiled) of HCT-116 tumor from a gemcit-

abine and a mitomycin C-treated HCT-116 tumors; cyclophosphamide and the mitomycin C-treated NCI-H460 tumors. 

such analysis demonstrates apoptosis and cell proliferation at multiple time points. Additionally, through the use of “tiling,” we quantitated the percentage of the tumor that was viable, with and without drug treatment. Taken together, these three approaches allow for a more thorough understanding of cellular kinetics of tumor growth and response to therapy.

To the best of our knowledge, this is the first report of complete tumor sections that have been both visualized and analyzed with such high resolution, allowing appreciation of the complexity of the tumor. All of the drugs used in these studies have been or are being used in the clinic against colon and non-small lung tumors. Cyclophosphamide inhibits DNA cross-linking and synthesis (3); mitomycin C inhibits DNA cross-linking and slows the rate of cellular proliferation (4), whereas gemcitabine is an antimetabolite that inhibits cellular proliferation in S phase (5). These drugs had different effects on the inhibition of tumor growth as well as cellular proliferation (the BrdUrd-LI) and the percentage of apoptotic cells (apoptotic index). Although cyclophosphamide was not effective in inhibiting growth against either solid tumor (Table 1), it did inhibit the proliferative index in the HCT-116 tumor cells and increased the apoptotic index in the same cells. Cyclophosphamide is primarily used clinically against leukemias and lymphomas (6). Gemcitabine, an effective drug against a broad base of human tumors in xenografts (7), was efficacious against colon but not non-small cell lung tumor growth dosed at 120 mg/kg Q3Dx5. Although cell proliferation was decreased in both tumors with gemcitabine treatment, these reductions were not significant. The percentage of apoptotic cells increased significantly in the HCT-116 tumors, suggesting that cell death was the primary contributor to the reduction in tumor size with this dosing regimen. This drug is used against aggressive tumors such as adenocarcinomas of the pancreas in single and combination therapies (8). Mitomycin C reduced both colon and lung tumor growth, but significantly reduced cellular proliferation only in the NCI-H460 lung tumors.

These data demonstrate that assessing TGI by simply measuring changes in tumor weight of untreated animals compared with controls does not accurately assess the kinetics of tumor growth and response to treatment. Apoptosis is an important end point because more anticancer drugs have been reported to induce this process in tumor cells (9). We quantified apoptosis, coupled with cellular morphological changes (10), to
detect the percentage of apoptotic cells in a mixed cell population at the end of 14–15 days of daily dosing. We counted the total number of cells in each field (≈2000) and expressed the terminal deoxynucleotidyl transferase-mediated dUTP-nick end labeling-positive cells as a percentage of the total. We reasoned that counting total cells within each selected field was more consistent than counting only the positive cells per field, because the tumors were heterogeneous. The percentage of apoptotic cells in the tumors from untreated animals was higher but in the range reported for other tumors (11). The percentage of apoptotic cells within the tumor cell population of the HCT-116 tumors treated with cyclophosphamide or gemcitabine was significantly increased compared with controls, which was not seen with mitomycin C-treated tumors, suggesting that this mechanism may be important for the response of these tumor cells to those drugs. It is possible that the apoptotic cell fragments from the mitomycin C-treated cells may be more efficiently phagocytosed by neighboring cells (12), so that changes in the apoptotic cell numbers were not observed. Because the percentage of apoptotic cells was determined at only one time point, such a possibility cannot be ruled out.

The BrdUrd-LI has been used to investigate the proliferative characteristics of tumor cell populations to evaluate tumor growth (13). The percentage of proliferating cells in the HCT-116 and NCI-H460 tumors ranged from 22–35%, which is higher than reported in human melanoma (11). However, the majority of cells (65–78%) were not labeled with BrdUrd; a pulse exposure was used, so only those cells rapidly synthesizing DNA used the BrdUrd. A significant reduction in cellular proliferation does not consistently lead to a significant decrease in tumor weight as shown by the cyclophosphamide-treated HCT-116 tumors. On the other hand, a significant inhibition of tumor growth was demonstrated in the gemcitabine-treated HCT-116 tumors, and this was not accompanied by a statistically significant decrease in cellular proliferation. These data, again, suggest that analyzing only a single parameter does not accurately reflect overall kinetics of tumor growth.

Through the use of tiled images, a more complete picture of tumor morphology can be gained. The tiled images demonstrate differences in the patterns of proliferation and cell death within the tumors, from the extremes of the HCT-116-gemcitabine-treated and NCI-H460 mitomycin C-treated tumors that were...
essentially uniformly labeled with BrdUrd (Figs. 6 and 7) to the large control tumors with necrotic centers (Figs. 1 and 2). Standardizing these tissues to 100 mm² reflects these morphological observations (Table 2). HCT-116 tumor growth was inhibited by 90% after treatment with gemcitabine (Table 1), coupled with a 26% reduction in the percentage of proliferating cells and a significant increase in the percentage of apoptotic cells, which contribute to the reduction in tumor growth (14). The percentage of viable tumor after gemcitabine treatment was 85%, indicating that most of the remaining tumor was viable after drug treatment. Similar findings were present in the NCI-H460 mitomycin C-treated tumors.

The other drug-treated tumors did not exhibit any significant changes in percent area of tumor viability or necrosis, as shown in Table 2. Gemcitabine was clearly inactive against the NCI-H460 tumors in all parameters measured. Mitomycin C reduced the NCI-H460 tumor necrotic area. Clearly, the 50-mg/kg/day dose of cyclophosphamide had activity against the HCT-116 tumors, with a significant reduction in the proliferative index and a significant increase in the apoptotic index. However, when the tissue areas were normalized, the areas of tumor necrosis and viability in cyclophosphamide-treated tumors were not different from controls, suggesting that the overall tissue morphology remained constant. Although there is no direct evidence that these specific measurements, including standardizing the tumor tissues for viable and necrotic areas, lead to a more predictable assessment of the effects of chemotherapeutic agents on tumors, additional information does permit a more comprehensive assessment of the relationship between tumor morphology and treatment outcome.

The observation that the NCI-H460-mitomycin C-treated and HCT-116 gemcitabine-treated tumors were the smallest in size, with the least cellular proliferation, increased number of apoptotic cells, and virtually no necrotic areas within the tumor, is interesting. The relevance of these observations is unclear, but one could speculate that the treated tumors were not hypoxic or acidotic and had adequate

Table 2  Characterization of areas of tumor normalized to 100 mm²

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<tr>
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<th>Area of tumor viability (%)</th>
<th>Area of necrotic tumor (%)</th>
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<tr>
<td></td>
<td>HCT-116</td>
<td>NCI-H460</td>
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<tr>
<td></td>
<td>HCT-116</td>
<td>NCI-H460</td>
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<tr>
<td>A.</td>
<td></td>
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<tr>
<td>Vehicle control</td>
<td>77.5 ± 6.8</td>
<td>25 ± 5</td>
</tr>
<tr>
<td>Mitomycin C (1 mg/kg/day)</td>
<td>78.6 ± 10.0</td>
<td>96 ± 2.5c</td>
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<td>B.</td>
<td></td>
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<tr>
<td>Vehicle control</td>
<td>63.2 ± 5.4</td>
<td>41 ± 8.4</td>
</tr>
<tr>
<td>Cyclophosphamide (50 mg/kg/day)</td>
<td>61.3 ± 6.0</td>
<td>NT</td>
</tr>
<tr>
<td>Cyclophosphamide (80 mg/kg/day)</td>
<td>NT</td>
<td>46 ± 7.0</td>
</tr>
<tr>
<td>GEMCITABINE (120 mg/kg Q3Dx5)</td>
<td>85.8 ± 3.3c</td>
<td>61 ± 2.0</td>
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<tr>
<td></td>
<td>36.8 ± 5.4</td>
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<td>38.7 ± 6.0</td>
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<td>53.7 ± 7.3</td>
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<td></td>
<td>11.8 ± 7.1c</td>
<td>39.0 ± 1.9</td>
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Mean ± SE.

* The ratio of area of viable tumor including both BrdUrd-labeled plus viable unlabeled cells to the total tumor area normalized to 100 mm² × 100.

* The ratio of area of the tumor that is necrotic to the total tumor area × 100.

* P < 0.05 compared with vehicle controls.

* NT, not tested.
blood supply; they remain viable tumors. What happens in the tumors with continued treatment with these chemotherapeutic agents remains to be determined.

Evaluation of tumors at several time points after drug treatment, shown in the gemcitabine time course study, clearly generates additional information about the effect of the drug on the tumor. More of the tumor cells in the treated animals undergo apoptosis, while fewer cells are proliferating. By day 4 after the termination of gemcitabine treatment, the proliferative index is not significantly different from this index after 15 days of drug treatment, but the apoptotic index is significantly elevated.

In summary, we have described a new approach for investigating tumor responsiveness to chemotherapeutic agents. Through the use of tiling, we have demonstrated the heterogeneity within the tumors. Because of this heterogeneity, we have used the largest cross-sectional diameter of each tumor, reasoning that this section should be morphologically the most variable. We have identified two distinctly different areas within each tumor, an area containing proliferating and viable cells, and a necrotic area. As a result of treatment with the chemotherapeutic agents, the percentage of these areas within each tumor sections changed when the tumors were standardized to 100 mm². No areas of necrosis were found in the HCT-116 gemcitabine-treated and mitomycin C-treated NCI-H460 tumors. Tumors remained viable. Certainly, different drugs, dosing regimens, time points, and tumors will lead to different results, as shown by the two different dosing regimens with gemcitabine. Comparisons among groups at a single time point give valuable information. We used standard therapeutic agents, dosing regimens, and selected time points as tools to demonstrate the broad applicability of the methodology.

This methodology also may be applied in the clinical analysis of human tumors. For example, an analysis of tumors treated with chemotherapy before and after surgical removal, as in neoadjuvant or induction protocols, would provide information about the efficacy of treatment. This approach could also help answer questions related to the dose and fractionation of radiation treatments by providing insight into relative intratumor growth and death postradiation. Lastly, emerging concepts about the action of anticancer agents such as antiangiogenesis drugs suggest their effect may be to induce a state of tumor dormancy or stability through the balance of intratumor cell death and proliferation. The approaches outlined could be applied to these new agents and lead to a more rational design of treatment.

REFERENCES