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Proliferative Activity of Human Tumors: Assessment Using Bromodeoxyuridine and Flow Cytometry

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Cell kinetics of human carcinoma xenografts and human solid tumors were evaluated by means of two-color flow cytometry using single sampling at appropriate time intervals after bromodeoxyuridine (BrdU) labeling. The tumors were resected several hours after the administration of BrdU, and flow cytometry was used to measure the DNA content and BrdU incorporation. Once the BrdU labeling index (LI) and DNA synthesis time (Ts) were obtained, the potential doubling time (Tpot) was calculated from these values. In 11 xenografts, the cell kinetic data were compared with the actual tumor doubling time (Td), and a good correlation between Tpot and Td was obtained ($r=0.91$, $P<0.005$). In a clinical study, 33 patients with gastric cancer, colorectal cancer, lung cancer, or other solid tumors were analyzed. Aneuploid tumors had a significantly higher LI value ($P<0.01$) and a shorter Tpot than diploid tumors. The cell loss rate of human tumors ranged from 40% to 80%. These cell kinetic parameters therefore accurately indicated the level of proliferative activity of human tumors.

Key words: Proliferative activity — Flow cytometry — Bromodeoxyuridine — Potential doubling time — Tumor doubling time

Many cell kinetic studies of human malignancies have been performed by the ^{3}H-thymidine labeling method.[1–3] However, this requires multiple sampling and laborious autographic techniques. In addition, administration of ^{3}H-thymidine to humans raises some ethical problems. Bromodeoxyuridine (BrdU)[2] is a pyrimidine analogue which is incorporated into DNA-synthesizing nuclei and has been widely used as a clinical radiosensitizer since the 1960s.[4–6]

Gratzner developed the method of detecting BrdU-labeled nuclei by using an anti-BrdU monoclonal antibody.[7] Subsequently, Dolbeare *et al.* developed a method to visualize BrdU-incorporating cells, while performing simultaneous analysis of the DNA content and the amount of incorporated BrdU using flow cytometry,[8] and since then BrdU has been used for many cell kinetic studies both *in vitro* and *in vivo.*

The DNA synthesis time (Ts) can be calculated by a single sampling procedure performed at appropriate time intervals after BrdU labeling,[9] and other kinetic parameters, such as the potential doubling time (Tpot) and the production rate (PR), can also be calculated to determine the proliferative activity of tumors.[10–12]

In this study, we applied these cell kinetic techniques to human carcinoma xenografts and compared the parameters with the actual tumor doubling time (Td). In a clinical study, we also investigated the proliferative activity of human solid tumors with these techniques.

MATERIALS AND METHODS

BrdU administration and tumor collection (1) Xenografts: Eleven human tumor xenografts were used for the experiments. They consisted of six gastric carcinomas (Shimizu, Kitakon, Matsubara, Ishigaki, Yoshikawa-P, Yoshikawa-M), one colonic carcinoma (Tamura), one cholangiocellular carcinoma (Namura), one maxillar carcinoma (Inoue), one bladder carcinoma (BT-8) and one osteosarcoma (Tanaka). BT-8 was kindly provided by Dr. K. Okada, from the Department of Urology of Fukui Medical School. All of these xenografts were serially transplanted into nude mice (BALBc/*nu*/*nu*) at our institution. BrdU was purchased from Sigma Chemical Co. (St. Louis, Mo.). BrdU (100 mg/g) dissolved in saline was administered intraperitoneally to tumor-bearing nude mice, and 4 to 6 h later the mice were killed. After resection, the tumors were minced into pieces using scissors and immediately fixed with cold ethanol.

(2) Human tumors: Thirty-three patients with gastric cancer, colorectal cancer, lung cancer, and other solid tumors underwent surgery at Fukui Medical School Hospital. All patients gave informed consent to receive BrdU before surgery. Commercially available BrdU (Radibut) was purchased from Takeda Chemical Industries Ltd. (Osaka). One gram of BrdU dissolved in 100 ml of saline was given to the patients intravenously before surgery.

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[2] Abbreviations used in this paper: BrdU, bromodeoxyuridine; DI, DNA index; LI, labeling index; RM, relative movement; Ts, DNA synthesis time; Tpot, potential doubling time; Td, tumor doubling time; FS, fluorescence of S-phase cells; FG_1, fluorescence of G_1-phase cells; FG_2, fluorescence of G_2-phase cells.

During surgery, a few pieces of tumor about 5 mm in diameter were obtained from the primary or the metastatic lesions, and these tissue samples were treated as described above.

Preparation After fixation with ethanol, tumor fragments were disaggregated with 0.5% pepsin solution (pH 1.5) using a magnetic stirrer and filtered through a 40-μm pore nylon mesh to remove the remaining cell aggregates. The cells were washed with PBS containing 0.5% Tween-20 and preserved after resuspension in ethanol.

Staining A modification of the method of Dolbeare *et al.*[8] was used. Ethanol-fixed cells were washed with PBS containing 0.5% Tween-20 and incubated in 2 *N* HCl for 20 min to denature double-stranded DNA. After neutralization with 0.1 *N* $Na_2B_4O_7$ and washing with PBS containing 0.5% Tween-20, the cells were next incubated in 20 μl/ml of anti-BrdU antibody (Becton Dickinson, Calif.) for 60 min at 37°C. The anti-BrdU antibody preparation contained PBS, 0.5% Tween-20, and 0.5% bovine serum albumin. The cells were washed and incubated in 20 μl/ml of FITC-conjugated anti-mouse goat IgG for 60 min at 37°C. Cells were then washed again, incubated in RNase solution for 30 min at 37°C, washed one more time, and suspended in propidium iodine solution (50 μl/ml). This suspension was finally filtered through a 40-μm pore nylon mesh.

Flow cytometry The two-color distribution of BrdU (green) versus DNA content (red) was analyzed by using a flow cytometer (CS-20, Showa Denko K.K., Tokyo). A total of 40,000 cells were measured each time and all data were stored on a disc. DNA histograms were constructed using the same tumor tissue sample. As a normal diploid standard, a normal tissue sample was used for both mice and humans. The tumor was defined as diploid when the histogram showed one G_1G_0 peak, and defined as aneuploid when the histogram showed more than two G_1G_0 peaks.

(1) Labeling index (LI): The BrdU LI was determined as the percentage of green-fluorescent cells to all cells in the window. If some BrdU-labeled cells were shown to have recycled to G_1G_0 in the cytogram, the number of labeled cells was determined as the number of cells in the S-phase and G_2M plus half the number of labeled cells in the G_1G_0 area (Fig. 1).

(2) Relative movement (RM) and DNA Ts: At time 0, the mean position of the BrdU-labeled cells was midway between the G_1G_0 and G_2M peaks in the cytogram. If it is assumed that the rate of movement of S-phase cells is constant, at the time of tumor sampling (t) the BrdU-labeled cells would have shifted towards the G_2M peak and some of them would already have recycled to G_1G_0. RM was calculated by using the following formula: $RM = FS - FG_1/FG_2 - FG_1$. Next, Ts was calculated by using the following formula: $Ts = 0.5/(RM - 0.5) \times t$. The corrected formula $Ts' = 0.4/(RM - 0.6) \times t$ was used in this study[9-12] (Fig. 1).

(3) Tpot: If it is assumed that no cells are lost, then once the LI and Ts have been obtained, Tpot can be calculated by using the formula: $Tpot = Ts \times 100/LI/24$[10-12] (Fig. 1).

Td (1) Xenografts: Three- to five-week-old female BALBc/*nu*/*nu* nude mice were bred and maintained under specific pathogen-free conditions. The length, width, and height of the tumor were measured with calipers twice a week and the estimated volume was then calculated as follows: (Volume) = 1/2 × (length) × (width) × (height). Td was calculated in terms of number of days after transplantation (X) and the natural logarithmic value (Y) of estimated tumor volume. A correlation factor and sample regression equation ($Y = a + bX$) were set up and Td was obtained by using the formula: $Td = \ln 2/b$.

(2) Human tumors: The estimated tumor volume was determined from X-ray films of computed tomographic films, and Td was calculated as described above.

RESULTS

Xenografts Fig. 1 shows serial cytograms of xenografts (BT-8) after the intraperitoneal administration of BrdU. Fig. 1A shows the cytogram 20 min after administration. The mean DNA content was situated midway between the G_1G_0 and G_2M peaks. Fig. 2B shows the cytogram

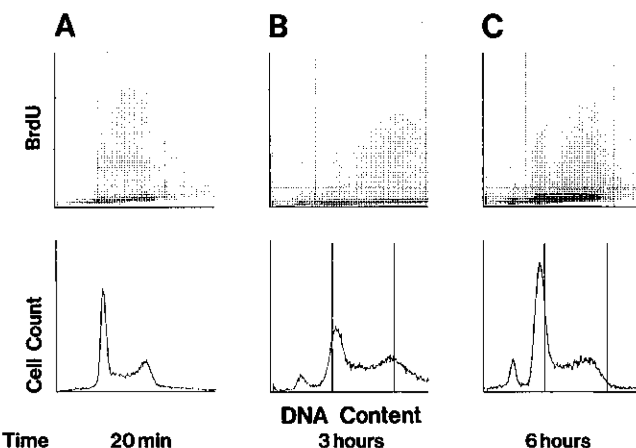

Fig. 1. Serial cytograms of two-color flow cytometry of xenografts (BT-8). Horizontal broken lines indicate the cut-off level of BrdU labeling and vertical broken lines indicate the window. After 20 min, BrdU-labeled cells lay between the G_1G_0 and G_2M peaks, while after 3 h the BrdU-labeled cells had shifted towards G_2M. After 6 h, many cells had reached G_2M and recycled to G_1G_0.

obtained 3 h after administration, when the labeled cells have shifted to the right. Fig. 1C shows the cytogram obtained 6 h after administration, by which time many cells had recycled to G_1G_0.

Fig. 2 shows the correlation between Td and Tpot. The regression line was Y=0.17+0.94X and a significant correlation was found between the two parameters (r=0.91, P<0.005).

Human tumors Adverse reactions attributable to BrdU were observed in 5 of the 33 patients. Nausea occurred in 3 patients, conjunctival congestion in one patient, and numbness of the tongue in one patient. All these reactions were transient and required no specific therapy. Representative DNA histograms and two-color cytograms are shown in Fig. 3. Fig. 3A shows a diploid pattern, while Figs. 3B and 3C show aneuploid patterns. The LI value was obtained in 91% of the patients and Ts and Tpot values were obtained in 67%.

Table I summarizes the cell kinetic data for the human tumors. No significant differences were observed among gastric, colorectal, and lung cancer. Table II shows the kinetic data for primary and metastatic lesions; again no differences were observed. Table III shows the results in relation to DNA ploidy. Aneuploid tumors had a significantly higher LI (P<0.01), a longer Ts, and a shorter Tpot than the diploid tumors. Actual Td values were observed in 3 patients with lung and colon cancer.

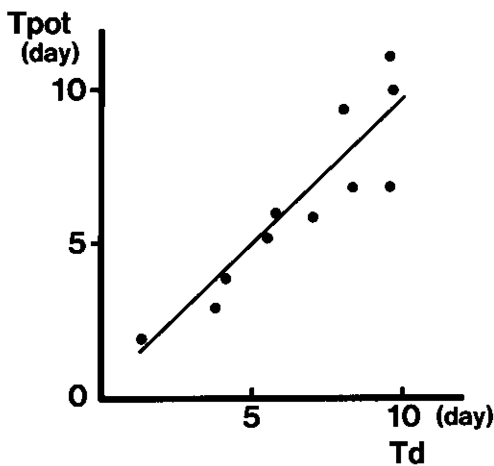

Fig. 2. Correlation between the Tpot and the actual Td. Y=0.173+0.944X; r=0.915, P<0.005.

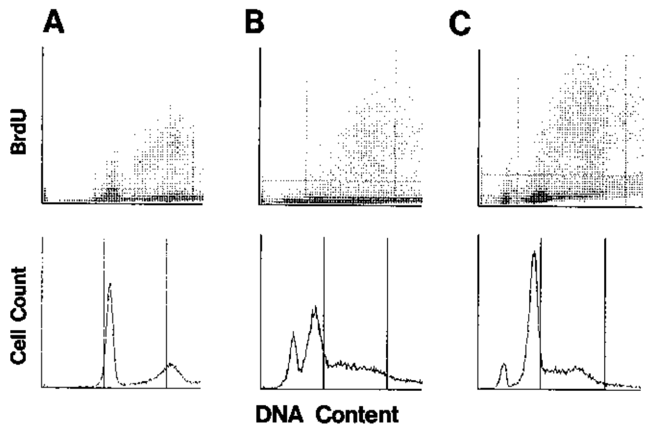

Fig. 3. Cytograms of two-color flow cytometry and DNA histograms of fresh human tumors. A: Colonic cancer (DI=1.0; LI=17.8%; Ts=12.4 h; Tpot=3.0 days). B: Breast cancer (DI=1.6; LI=8.5%; Ts=14.7 h; Tpot=7.2 days). C: Gastric cancer (DI=2.2; LI=13.0%; Ts=17.8 h; Tpot=5.1 days).

Table I. Kinetic Data for Solid Tumors from 33 Patients

Diagnosis		No. of patients	LI (%)	Ts (h)	Tpot (days)
Gastric cancer	(Primary)	8	9.6±6.0	17.4±2.6	9.0±8.3
	(Metastasis)	5	12.8±6.4	23.3±8.7	6.1±0.7
Colorectal cancer	(Primary)	9	16.5±5.7	18.1±7.3	5.1±2.1
	(Metastasis)	3	9.9±4.3	16.2±1.6	9.5±6.2
Lung cancer	(Primary)	6	7.8±3.9	16.1±6.5	7.1±3.2
Breast cancer	(Metastasis)	1	8.5	14.7	7.2
Penile cancer	(Primary)	1	12.9	13.3	4.3
Pancreas cancer	(Metastasis)	1	6.1	ND	ND
Cholangiocellular C.	(Primary)	1	4.7	ND	ND
Mature teratoma	(Primary)	1	2.3	ND	ND

LI=labeling index; Ts=DNA synthesis time; Tpot=potential doubling time; ND=not determined.

Table IV shows the Tpot, Td, and cell loss data for these tumors.

DISCUSSION

A number of parameters are used clinically to assess the proliferative potential of human tumors. Previous reports have stated that the DNA ploidy pattern gives a good indication of the prognosis of human malignancies.[13, 14] One of the parameters commonly used to determine the degree of proliferative activity is the S-phase fraction,[15] although the fraction obtained from a univariate DNA histogram is not always accurate. Since an antibody specific for BrdU was developed by Gratzner,[7] this compound has been adopted for cell kinetic studies. Immunohistochemical staining has been used to visualize DNA-synthesizing cells with the anti-BrdU antibody. This method has recently been used instead of ^{3}H-thymidine autoradiography.[16, 17] Autoradiography takes longer to perform and is relatively ineffective in the discrimination of weakly labeled cells from unlabeled cells. In contrast, BrdU-labeled nuclei are more distinctly visualized.

Table II. Kinetic Data in Primary and Metastatic Lesions

	No. of patients	LI (%)	Ts (h)	Tpot (days)
Primary	26	11.3±6.7	17.5±5.9	6.5±5.0
Metastasis	9	10.1±5.6	19.0±6.1	7.7±4.3

LI=labeling index; Ts=DNA synthesis time; Tpot=potential doubling time.

Table III. Relationship between Kinetic Data and DNA Ploidy Pattern

Ploidy	No. of patients	LI (%)	Ts (h)	Tpot (days)
Diploid	14	6.7±5.6	16.1±3.6	10.4±8.4
Aneuploid	21	14.0±5.3	19.0±7.3	5.8±2.2

LI=labeling index; Ts=DNA synthesis time; Tpot=potential doubling time.

In 1983, Dolbeare *et al.*[8] developed a method to visualize BrdU-incorporating cells and allow the simultaneous analysis of DNA content and BrdU incorporation by flow cytometry. Since the anti-BrdU monoclonal antibody became available, two-color flow cytometry coupled with BrdU labeling has been widely used to analyze the cell cycle.[18, 19]

There are both *in vivo* and *in vitro* labeling methods. The incubation of cell suspensions or small tissue blocks at 3 atm,[20] and the perfusion of organs resected surgically with perfluoro compounds[21] have been used for *in vitro* labeling. However, in all of these methods BrdU is not always incorporated by the tumor, and it is also possible for cell viability to be decreased by the disruption of the tumor tissue. Administration of ^{3}H-thymidine to patients is a problem because this radioactive material is hazardous to humans. Cytocidal and teratogenic effects have also been reported to result from the prolonged administration of high-dose BrdU.[22, 23] However, the daily intravenous infusion of BrdU (600 mg/sq) for several weeks can be tolerated without serious adverse effects. The dose used in this study was far below the dose used therapeutically and none of the patients experienced adverse effects that required specific treatment.

We studied the cell kinetics of human carcinoma xenografts and human solid tumors using two-color flow cytometry to analyze single samples taken at appropriate intervals after *in vivo* BrdU labeling. In the xenografts, a close relationship between Tpot and Td (r=0.91, P< 0.005) was observed and the regression line Y=0.17+0.94X was obtained. These results show that this method can evaluate the proliferative activity of tumors.

In the clinical study we found no kinetic differences between primary and metastatic lesions, but aneuploid tumors had a significantly higher LI and shorter Tpot than diploid tumors, indicating that aneuploid tumors have a higher proliferative activity and grow more rapidly than diploid tumors. However, the aneuploid tumor had a longer Ts than the diploid tumors. It is known that

Table IV. Potential Doubling Time, Tumor Doubling Time, and Cell Loss in Clinical Cases

Patient	Diagnosis	Tpot (days)	Td (days)	Cell loss (%)
1	Small cell carcinoma of lung	11.5	41.5	72.3
2	Squamous cell carcinoma of lung	6.2	33.2	81.3
3	Metastatic lesion of colon cancer	13.5	22.1	38.9

Tpot=potential doubling time; Td=tumor doubling time.

the cycle time is longer in malignant cells than in normal cells.[24] Our results suggest that the cell cycle time might be even longer in aneuploid tumors than in diploid tumors, although the doubling time was shorter in the former than in the latter. It might be mainly due to higher LI that the aneuploid tumors had a shorter doubling time.

The doubling time of most human tumors has been reported to be over 20 days. The Tpot values for the human tumors determined in this study were smaller than the actual Td values, although the Tpot values for the xenografts were consistent with the actual Td values. In exponential growth of xenografts the cell loss is negligible, but the growth of human tumors is affected by many factors. We found that the cell loss rate of human tumors ranged from 40% to 80%, in accord with previous reports.[25, 26]

In conclusion, in the xenografts the Tpot values corresponded to the tumor proliferative activity. The clinical study showed that aneuploid tumors had a shorter Tpot than diploid tumors, but the Tpot values were smaller than the actual Td values. The differences between them were considered to be due to cell loss, and the parameters obtained with this method were considered to represent adequately the tumor proliferative activity. The method should be able to provide valuable information for deciding on the appropriate treatment of human tumors.

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