Growth Kinetics of Human Colorectal Carcinoma1

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ABSTRACT

In this study, we investigated the influence of some of the variables of the thymidine labeling index (TLI) in human colorectal carcinoma. These variables were: cell suspensions versus tissue fragments; incubation with 5-fluoro-2'-deoxyuridine; method of tissue procurement; location in the large bowel; and TLI distribution in different areas of the tumor. Mean TLI values for cell suspensions and tissue fragments were 3.4 (range, 0.1 to 7) and 1 (0.1 to 2.6), respectively. Incubation with 5-fluoro-2'-deoxyuridine significantly reduced tritiated thymidine incorporation. There were no differences in TLI values between biopsy and surgical samples and in different areas of the large intestine. Median TLI of cell suspensions in 47 tumors was 2.25 (0.1 to 10.1). These results show that the TLI of colorectal carcinoma is low and correlates with its slow growth. Cell suspension provides a more representative and unbiased sample than tissue fragments in cell kinetics studies.

INTRODUCTION

Human colorectal carcinoma is a slow-growing solid tumor. Welin et al. (14) estimated that the median doubling time of 20 colorectal carcinomas measured by serial roentgenographic studies was 620 days, a value that would indicate that tumor cell growth rates are low. However, initial studies using [3H]dThd3 label in vivo in patients with primary colorectal carcinoma demonstrated that 16 to 23% of the tumor cell population was in S-phase (4,13). Techniques were later developed to measure cell kinetic parameters in vitro by incubating fragments of colorectal carcinoma with the radiolabeled precursor. In 5 patients, Lieb and Lisco (6) reported TLI of 16 in carcinoma fragments incubated with [3H]dThd before microsections were prepared. Using similar tissue preparations, Bleiberg and Galand (1) reported TLI of 33 in carcinoma fragments from 6 tumors, and Meyer and Prioleau (7) confirmed high TLI of 18 in 90 patients.

Such high TLI in tumors that are relatively slow growing suggest a bias in the methodology to determine the percentage of cells in S-phase. Indeed, all of the reports intimated that slow diffusion of [3H]dThd into whole tissue specimens may limit the accuracy of the TLI determination. Because the label did not uniformly incorporate into the tissue fragments, only areas where adequate labeling could be readily seen were counted. To circumvent this problem, Lesher et al. (5) used mechanical disruption of tissue fragments to obtain a single cell suspension and reported a mean TLI of 7 and a GF of 30 in 17 colorectal carcinoma specimens. The GF was determined by the primer-available DNA-dependent α-DNA polymerase technique which measures the presence of α-DNA polymerase in proliferating cells (11).

The purpose of this study was to evaluate various methods to determine cell kinetic parameters of colorectal carcinoma and to define cytokinetic values as a function of location of the carcinoma in the colon and rectum in a large number of patients.

MATERIALS AND METHODS

Tumor. Tumor tissue was obtained from patients with histologically proven adenocarcinoma of the colon or rectum. Tissue was obtained either by proctoscopic biopsy before operative resection or from surgically resected specimens. In either case, at least 0.5 cm of tissue was obtained from the growing edge of the intraluminal portion of the tumor mass, placed in ice, 0.9% NaCl, and processed within 1 h. For proctoscopic specimens, adjacent biopsies were obtained for histological confirmation. For surgical specimens, tissue adjacent to the surgical pathology was used.

Preparation of Tumor Cell Suspension. Cell suspensions were made by slicing tumor tissue into 1-mm fragments with a scalpel blade in a Petri dish placed on ice. Care was taken to avoid crushing or tearing the tissue with forceps. Fragments were then exposed to digesting medium (12 ml) for 45 min at 37°C in a trypsinizing flask. The digesting medium consisted of RPMI Medium 1640 (Grand Island Biological Co., Great Neck, NY), 10% fetal calf serum, 0.1% DNAse Type I (450 Kunitz units/ml; Sigma Chemical Co., St. Louis, MO) and 0.14% collagenase type I (1.5 to 2.0 units/ml; Sigma). After incubation, fragments were allowed to settle to the bottom of the flask in an ice bath. The cell suspension was then chilled in ice for 4 min to inhibit further uptake of label and was centrifuged at 500 x g for 5 min. The pellet was resuspended in 0.5 ml of this mixture, and the cells were incubated at 37°C for 30 min in a 5% CO2 water bath with continuous agitation (2). The cell suspension was then chilled in ice for 4 min to inhibit further uptake of label and was centrifuged at 500 x g for 5 min. The pellet was resuspended in 1.5 ml of cold 0.9% NaCl and cytocentrifuged onto acid-cleaned slides. Slides were immediately fixed in absolute methanol containing 10% (v/v) acetic acid for 15 to 30 min and then air dried.

Primer-available DNA-dependent DNA Polymerase Assay. The PDPI assay was performed according to the method of Schiffer et al. (11). The cell suspension was cytocentrifuged onto acid-cleaned slides and air dried. Slides were dipped in a 0.25% agar solution at 42°C and air dried. A glass ring with paraffin was placed over the slide to surround the cell cluster and formed a well to contain the required reagents. Three ml of a mixture of deoxy-5'-triphosphates of adenosine (83 mm), guanosine (83 mm), and cytidine (83 mm), 5 mm MgCl2 and 12.8 mm KCl in 0.083 M Tris-HCl (pH 8.0) were mixed with 1.2 g of Ficoll, 0.03 ml of [3H]TTP (2.5 mCi/ml; specific activity, 40 to 80 Ci/mmole; New England Nuclear), and 0.03 ml of 0.78% o-mercaptoethanol-Tris buffer. Each well received 0.5 ml of this mixture, and the cells were incubated at 37°C for 30 min in a 5% CO2 incubator. The reaction was terminated by placing slides in cold 0.9% NaCl for 10 min, and the cells were fixed in 3 changes

1 Supported by Grant CA 34465 from the National Cancer Institute.
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3 The abbreviations used are: [3H]dThd, tritiated thymidine; TLI, thymidine labeling index; GF, growth fraction; PDPI, primer-available DNA dependent α-DNA polymerase index; FdUrd, 5-fluoro-2'-deoxyuridine.

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of acidic formaldehyde for 10 min each. Slides were then rinsed in tap water for 30 min before processing for autoradiography.

Autoradiography. Autoradiographs were prepared by using Kodak NTB-2 liquid photographic emulsion. Autoradiographs were dried at room temperature for 1 h and exposed in light-tight boxes at 4°C for 7 d for TLI assays and for 14 d for the PDPI assay. Cells were then stained with hematoxylin-eosin. Both indices were determined by counting 1000 cells/slide on 4 slides for each tumor sample.

Effect of FdUrd on the TLI. Changes in the TLI induced by FdUrd were determined using single-cell suspensions. A cell suspension was prepared by enzyme digestion and split into 2 parts. One aliquot was prepared for TLI as described above. The other half of the cell suspension was incubated with 10⁻⁴ M FdUrd for 30 min at 37°C in a 5% CO₂ incubator under normal atmospheric pressure. The cells were washed 3 times with 0.9% NaCl solution and then incubated with [³H]dThd before processing for autoradiography.

Cell Suspension versus Tissue Fragments. The TLI of cell suspensions was compared to the TLI of tissue fragments. Tumor specimens were cut into 1-mm fragments and split into 2 equal parts. A cell suspension was prepared from half of the fragments for the TLI. The second half of fragments was incubated with [³H]dThd for 30 min at 37°C and then exposed to enzyme digestion to obtain a cell suspension for TLI determination. Histological slides were prepared from the residual 1-mm fragments after enzyme digestion. These slides were then prepared for autoradiographic analysis to determine the efficiency of enzyme digestion to selectively remove labeled cells from the tissue fragments.

RESULTS

Enzyme digestion of 1-mm tumor fragments on the average yielded suspensions with 78% single cells, 9% doublets, 4% triplets, 2% quadruplets, and about 7% clumps of 5 to 10 cells. Viability of all cells, as determined by trypan blue exclusion, was 80%.

Incubation of the cell suspensions with FdUrd inhibited the incorporation of the radioactive label into cells (Table 1). Cell suspensions from 5 different tumors incubated with FdUrd showed a marked decrease in the TLI.

The next experiments determined the differences in TLI between cell suspensions and 1-mm tissue fragments of 4 colorectal carcinomas. Table 2 shows that the mean TLI was 4.4 in cell suspensions, while the mean index for the tissue fragments was 1.1. The efficiency of enzyme digestion to remove labeled cells from the tissue fragments was documented with autoradiography of histological preparations of the residual tissue fragments. No label was observed in these tissue fragments after enzyme digestion. The effect of the procurement method of tumor tissue specimens, either by proctoscopic biopsy techniques or from surgically resected specimens, on the TLI was also evaluated.

Tumor tissue was obtained from surgically resected specimens immediately after they were removed from the patient. As shown in Table 3, the median TLI was 2.0 for tumor tissue obtained by proctoscopy (n = 41 tumors) and 3.1 for operative specimens (n = 6 tumors).

The TLI was then determined in carcinomas from different areas of the large intestine. Tumors were obtained from the right, left, or rectosigmoid regions of the large bowel. The majority of tumor specimens (75%) were from the rectosigmoid region, a frequency consistent with the distribution of adenocarcinoma in the large bowel. There were no significant differences in TLI of tumors located in different areas of the large intestine (Table 4).
To assess intratumor TLI reproducibility, the TLI was determined in different regions of 9 tumors (Table 5). For each tumor, the TLI was determined in 2 or 3 separate areas of the intraluminal growing edge, identified macroscopically as viable. Five such tumors showed minimal differences of less than 50%. Four other tumors showed TLI differences of 2.0-fold or greater. The PDPI was measured in conjunction with the TLI in the multiple biopsy specimens of the 9 tumors. The TLI was consistently lower than the corresponding mean PDPI for each tumor. The GF, as determined by the PDPI, ranged from 4.4 to 26.6 with a median value of 11.8.

One tumor specimen consisted of a large, broad-based villous adenoma adjacent to an ulcerating colon carcinoma. Tumor tissue was available from both tumors for TLI and PDPI measurements. The TLI and PDPI for the carcinoma were 3.4 and 11.2, respectively. The TLI and PDPI for the villous adenoma were 1.0 and 4.7, respectively.

**DISCUSSION**

**In vivo** administration of [³H]dThd and **in vitro** labeling of tumor cells have been used to determine cell kinetic parameters of colorectal cancer cells, resulting in a wide discrepancy of kinetic data. **In vivo** labeling procedures with infusion of labeled substrates such as [³H]dThd may be hazardous and are ethically difficult to justify. Therefore, Braunschweiger et al. (2) have emphasized the need to develop **in vitro** procedures for obtaining kinetic data in solid tumors. To this end, **in vitro** methods have been developed that use the label and random counting of labeled cells. In these reports there were, however, many areas where incorporation was limited, and these labeled cells is not possible. Instead, they focus on areas where incorporation of [³H]dThd into tissue fragments is limited, and investigators have reported that random counting of labeled cells is not possible. Instead, they focus on areas where incorporation of [³H]dThd into tissue fragments is possible. These values, however, are not consistent with a median tumor doubling time of 620 days (14).

Labeling of tissue fragments may be associated with procedural problems that contribute to such high indices. Diffusion of nutrients and radioactive precursors into tissue fragments is limited, and investigators have reported that random counting of labeled cells is not possible. Instead, they focus on areas where incorporation of [³H]dThd into tissue fragments is possible. These values, however, are not consistent with a median tumor doubling time of 620 days (14).

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The use of cell suspensions allows uniform incorporation of label and random counting of labeled cells. Braunschweiger et al. (2) described a mechanical method to obtain cell suspensions from tumor tissue fragments. Using cell preparation, Lesher et al. (5) calculated a mean TLI of 7 for human colon tumors with a GF estimate of 25. By using gentle enzyme digestion of similar tumors, we have been able to obtain an 80% single cell suspension with 80% cell viability by trypan blue exclusion. This cell suspension yielded a median TLI of 2.25 in 47 tumor specimens and a median GF of 11.8 in 9 tumor specimens.

Obviously, enzyme digestion or mechanical disruption of a solid mass of tumor cells into single cell suspensions has its own inherent difficulties. Disruption, either mechanical or enzymatic, could reduce cell viability and selectively remove non-S-phase cells from the tissue, accounting for a lower estimate of S-phase cells. At the present time, there is no analytical method to refute this possibility.

Another limiting factor of solid tumor cell kinetic measurements is the inability to recognize growth parameters of various clones. It is possible that in a suspension of cells derived from a solid tumor, the TLI and PDPI may differ among the heterogeneous cell population. Currently, there are no methods to identify and separate different clones of cells before cell kinetic parameters are measured. It is assumed that in the preparation of a cell suspension, various cell populations in the solid tumor fragments contribute randomly to the cell suspension.

A more important factor contributing to the low TLI and growth fraction, based on the PDPI, is the limited availability of nutrients within a solid tumor. Based on the concepts proposed by Tannock (12), the growth potential of individual cells is determined by proximity of cells to the adjacent feeding capillary. Cells adjacent to a capillary have adequate nutrients, while those furthest from the capillary are relatively anoxic and deficient of essential nutrients. Presumably, a heterogeneous population of cells is equally affected by the availability of nutrients determined by proximity to the feeding capillary.

The present study has clarified other details regarding the TLI. Meyer and Connor (8) proposed that incubation of label with FdUrd enhanced uptake of dThd label by blocking endogenous thymidylate synthetase. However, our observations showed that preincubation of cells with FdUrd did not enhance incorporation of label but rather, reduced it. Our results also showed that the TLI was not significantly affected by the method of tissue procurement or location in large intestine or in different areas within a tumor. The observed variation in TLI and PDPI can be readily accounted for by the random error of a binomial distribution of labeled cells in a sample size of 1000 cells (10).

Using the median value of 2.25 for the TLI and a median value of 11.8 for the GF (PDPI), we estimated theoretical values for the growth kinetics of human colon carcinoma (Table 6). Assuming a Tₐ of 24 h and based on median tumor volume doubling time of 620 d (14), we calculated a cell cycle time of 4.6 d and a potential doubling time of 39 d. From these values, the calculated cell loss rate was 94%. This rate is compatible with the slow growth of colorectal carcinoma and is consistent with the 98.7% cell loss rate reported by Campinejohn et al. (3). Thus, it appears that our technique provides data that reflect the actual growth dynamics of human colorectal tumors more closely.

**Table 6**

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<th>Parameter</th>
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<tr>
<td>Cell loss</td>
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*Based on the data of Welin et al. (14).
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REFERENCES


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