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In Vitro Autoradiographic Determination of Cell Kinetic Parameters in Adenocarcinomas and Adjacent Healthy Mucosa of the Human Colon and Rectum[1]

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SUMMARY

Using *in vitro* double labeling with two different doses of [^{3}H]thymidine, we measured the S-phase duration and the labeling index in biopsy samples taken from human rectal and colonic adenocarcinomas. Samples from the adjacent healthy mucosa, taken at the same time as the tumor samples, were simultaneously subjected to the same measurements. The mean labeling index in tumors was higher than in the normal mucosa (32.5 and 17.0%, respectively). The mean S-phase value was also longer than in the normal tissue (19.4 *versus* 11.2 hr). These observations are discussed with reference to similar observations made in tumors of the human epidermis, especially considering the possible relationship of an increased S-phase duration with carcinogenesis. While no definitive conclusions may be reached, it seems prudent to consider an S-phase duration that is longer than normal to be a justification for attentive follow-up of the patient.

INTRODUCTION

Little data have been reported concerning the direct comparison of cell kinetic parameters in human solid tumors and in the adjacent normal tissue from which they originated (9, 15, 19). There is in fact a lack of data concerning normal human tissues; this is due evidently to the ethical problems linked to the methods available for studying human beings. For the purpose of measuring cell kinetic parameters in normal and malignant tissues of the gastrointestinal tract of humans, we used the double-labeling method (24, 27). This approach, which was recommended for *in vitro* measurements of the S-phase duration and the turnover time in human tissues several years ago (4, 10–12), has been applied in this laboratory to a wide diversity of human (2–5, 11, 12, 15–18) and animal (10, 32–34) tissue samples.

The present work compares S phase and LI[3] measured in tumors and in the healthy tissue adjacent to them in the human rectal and colonic mucosa.

MATERIALS AND METHODS

Patients. Samples were taken from 8 adult patients, 5 females and 3 males, presenting a rectal (4 cases) or a colonic tumor (4 cases). For each patient 1 tumor sample and 1 sample of the normal adjacent mucosa were taken simultaneously either through a rectoscope or in the operating room immediately following resection. In Case 6, the sampling missed the tumor, and the biopsy was classified as normal after histological examination. Colonic cancers were treated by surgery alone, whereas rectal cancers were treated preoperatively by radiotherapy as well. In these cases, biopsy samples were taken before the treatment was started. Case 5, a 77-year-old woman, was treated by radiotherapy alone (2 exposures of 3450 rads each).

Biopsy and Incubation. The tumor sample and the tissue fragment from the adjacent healthy mucosa, were submitted together to the *in vitro* procedure, involving double labeling with different levels of [^{3}H]thymidine doses (28 Ci/mmole). The samples were collected in ice-cold incubation medium. Sections about 1 mm thick were made under a binocular lens in order to maintain proper orientation of the tissue fragments. Tissue sections were then incubated in 25-ml flasks containing 2 ml of medium (Eagle's powder Medium BME, diploid, purchased from General Biochemicals, Inc., Chagrin Falls, Ohio) supplemented with 10% calf-inactivated serum (Wellcome Research Laboratories, Beckenham, Kent, England). The pH was adjusted from 7.2 to 7.4 by adding drops of 5% sodium bicarbonate solution.

Double-Labeling Method. The tissue specimens were first incubated for 15 min in a low concentration of [^{3}H]thymidine (0.5 μCi/ml) (I.R.E., Mol, Belgium). After repeated rinsing in Eagle's medium, some of the specimens were fixed in Bouin's fluid to control the effect of single labeling by the low dose. Remaining samples were incubated for an additional 60 min in unlabeled medium. They were then incubated for 15 min in the presence of a high concentration of [^{3}H]thymidine (40 μCi/ml). At the end of the incubation they were rinsed and fixed in Bouin's fluid, and autoradiographs were prepared from histological sections of the tissue.

Autoradiography. Sections (4 ± 1 μm) were covered with

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[3] The abbreviations used is: LI, labeling index (percentage of cells that are labeled by a "pulse-labeling" with [^{3}H]thymidine).

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nuclear emulsion Ilford K5 in gel form (Ilford Ltd., Ilford, Essex, Great Britain) by dipping the slides into the melted emulsion, diluted in an equal volume of distilled water. The autoradiographs were exposed for 20 days, developed in Dektol (Eastman Kodak Co., Rochester, N. Y.) for 2 min at room temperature, and fixed in Kodak Rapid-Fixer (Kodak). After being rinsed in tap water for 1 hr, the slides were kept overnight in 70% alcohol. The tissues were then stained with hematoxylin and eosin, dehydrated, cleared in xylol, and mounted in DPX mounting medium (Michrome, Buckinghamshire, Great Britain).

Interpretation of the Autoradiographs. As previously reported (3, 11, 12) [^{3}H]thymidine does not label the whole thickness of the tissue fragment. Therefore, we restricted our counts to the 1st 3 crypts located near the edges of the section or, in tumors in which the cryptal architecture was destroyed, to a depth of 4 cell diameters starting from the edge of the section. In this area, grain counts and labeling indices were reproducible and the latter did not depend on the dose of [^{3}H]thymidine used. The distribution histograms of the number of grains per cell, obtained with a single low or high dose of [^{3}H]thymidine, allowed us to determine the appropriate range of doses to be used for distinguishing between "weakly" and "heavily" labeled cells. Typical distributions are shown in Chart 1. With 0.5 and 40 μCi/ml, a grain count of 40 grains/nucleus was chosen as the upper limit for the weakly labeled population in double-labeled material. This produced very few misplaced weakly labeled cells (3% in the normal mucosa and 6% in tumor) and, at most, 4% of heavily labeled cells. In the latter instance, this number is most probably reduced when dealing with doubly labeled cells because, in most of the heavily labeled cells, the effects of both pulse labeling are additive. At least 300 labeled cells were scored and classified as weakly (N_W) or heavily (N_H) labeled. The S-phase duration (S) was then calculated by the following formula (30): $N_H/N_W = S/t$, where t is the time interval between the 2 administrations of [^{3}H]-thymidine, *i.e.*, 1 hr in these assays. The percentage of pulse-labeled cells, or LI, was measured in the same material by scoring as labeled the heavily labeled cells only. The ratio S/LI was calculated. This measures the turnover time, defined as the inverse of the rate of cell production. In steady-state populations, provided all cells are proliferating (*i.e.*, growth fraction = 1) and assuming homogeneous distribution of the cells in the cycle, this is equal to the cell cycle time. It is a rough approximation of the potential doubling time of the population, *i.e.*, the time it would take to double the number of cells it contains if there were no cell loss (28).

RESULTS

The results are summarized in Table 1. The mean S-phase duration in tumors is longer than in the normal adjacent tissue ($p \leq 0.001$).

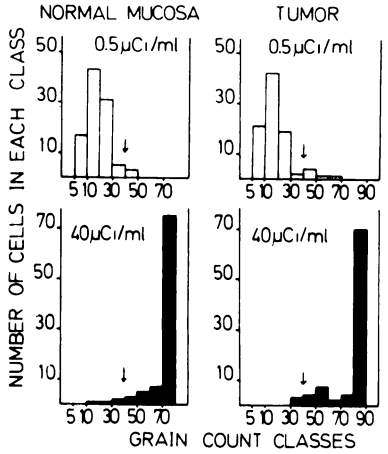

Chart 1. Frequency histograms of grain count over labeled nuclei in autoradiographs of tissue from adenocarcinoma and adjacent mucosa of the same patient, incubated for 15 min with 2 different doses of [^{3}H]thymidine. *Ordinate*, percentage of cells in each class; *abscissa*, class interval. Autoradiographs were exposed for 21 days. *Arrow* indicates, in double-labeled material, the upper limit chosen by us for the weakly labeled cell population.

Table 1

S-phase duration, LI, and turnover time in human rectal and colonic carcinoma and in the normal adjacent mucosa (double-labeling method, in vitro)

	S-phase duration (hr)		LI (%)		Estimated turnover time (S/LI)	
Case[a]	Tumor	Adjacent mucosa	Tumor	Adjacent mucosa	Tumor	Adjacent mucosa
1	21.5	11.8	26.5	16.6	81.1	71.0
2	21.6	9.6	31.3	24.6	69.0	39.0
3	22.0	14.5[b]	50.4	15.6	43.6	92.9
4	15.2	10.2	32.1	12.3	47.2	82.9
5	20.0	11.6	30.9	10.5	64.7	110.0
6		9.0		14.5		66.8
7	17.0	16.2[b]		24.1		67.2
8	18.8	9.5	24.1	25.0	70.5	38.0
		9.0	32.8	10.0	57.3	90.0
Mean ± S.E.	19.4 ± 0.98	11.2 ± 0.85	32.5 ± 3.21	17.0 ± 2.02	61.9 ± 5.06	73.0 ± 6
		≤0.001[c]		≤0.001[c]		Not significant

[a] Cases 1 to 4, colon; Cases 5 to 8, rectum.
[b] See text concerning these values.
[c] p level of the significance of the difference with normal values.

In Cases 3 and 6, the S phase was longer in the nonmalignant tissue than is usually observed. In both instances, the topographic distribution of labeled cells was abnormal; labeled nulcei appeared even at the top of the crypts. Counting of single-labeled material demonstrated that lightly labeled nuclei exhibited the expected grain count. Artifactual low grain count, resulting in "false negative" cells in the lightly labeled population, could give an overestimated S-phase duration. However, this can be excluded here, since counting of single-labeled material demonstrated that, in both cases, the lightly labeled nuclei exhibited the expected grain count. The LI is higher in tumors than in the normal mucosa. The calculated turnover time values are not systematically lower or higher in the tumor than in the normal counterpart. The mean value for all the tumors does not differ from that obtained for the normal tissue.

DISCUSSION

All our samples were taken from the periphery of the tumors, that is, from well-vascularized zones where proliferative activity is known to be more intense (20). This may explain why the LI values we obtained are higher than those that have been reported by others (1.7 to 23.1% in Refs. 31 and 35; 10.9% in Ref. 19).

The literature offers very scarce data concerning normal human tissue and even less concerning a direct comparison by a single method, between tumors and their normal counterpart. Using a double-labeling method, Fabrikant (9) observed that S phase was about twice as long, in epitheliomas of the larynx and bronchus, as it was in normal homologous tissue. Hoffman and Post (19) have proposed that S-phase duration could be longer in carcinomas from the human colon, rectum, and stomach than in the normal mucosa (19), but the evidence to support this view, namely, grain counts over labeled nuclei after [^{3}H]thymidine administration, was not decisive.

Comparing results obtained for tumors in a given laboratory with values reported by others for the normal tissue may be misleading because of the large variation in the results between laboratories (see, *e.g.*, Refs. 20–23, 29). This can be explained on the basis of discrepancies in the interpretation of the "labeled mitoses curve," as well as by lack of precision of the latter due to limitations in the sampling of tissues from humans. In this work, we avoided such bias by performing the comparison between normal and malignant tissue in parallel assays, using the same methodological process for both.

Our conclusion is that S phase is about 2 times longer in carcinomatous tissue than in normal rectal and colonic mucosa. A similar finding was made in this laboratory when comparing basal cell carcinoma with the normal human epidermis (15). Moreover, further work has shown that S phase remains unmodified in several benign diseases of the skin, whereas it is lengthened in premalignant tissues (18). If one considers the data of the literature (see review in Ref. 13), there is an indication of this in the fact that the reported S phase for several human cancers is generally longer than what is reported for normal human tissues.

Further work would be necessary before concluding whether a long S-phase duration may be taken as an indication of malignancy or premalignancy and, especially in the latter case, which length would be taken as critical. In this respect, our observations of 2 patients with a longer than normal S-phase duration associated with atypical localization of labeled (*i.e.*, proliferative) cells deserve attention. Atypical distribution of proliferating cells in the gastrointestinal tract has been observed in several pathological situations: villous and adenomatous polyps (2, 6–8), familial polyposis (3), and ulcerative colitis (4). High frequency of cancerization is associated with all these diseases (14, 25, 26). The only observed case of a very long S phase (22 hr) in an apparently normal rectal mucosa was associated also with the atypical location of the germinative compartment along crypt walls. This was in a 50-year-old woman who had developed 3 colonic cancers during the 10 previous years, and whose brother and sister both died from colonic cancer.

This matter remains open for further analysis. However, previous work from this laboratory on the human skin (15, 18), together with this investigation on the rectal and colonic mucosa, warrants the suggestion that it would be safe, from a clinical point of view, to consider any incidental observation of an increased S-phase duration as a reason for suspicion of developing cancer, at least justifying an attentive follow-up of the patient.

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